CENTER for PRODUCE SAFETY

CPS 2015 RFP
FINAL PROJECT REPORT

Project Title
Evaluation of the efficacy of antimicrobial agents to prevent the transfer of *Listeria monocytogenes* from existing biofilms to produce or processing surfaces

Project Period
January 1, 2016 – December 31, 2017

Principal Investigator
Rolf Joerger
University of Delaware
Department of Animal and Food Sciences
018 Townsend Hall
Newark, DE 19716
T: 302-831-6605
E: rjoerger@udel.edu

Co-Principal Investigator
Gordon C. Johnson
University of Delaware
Department of Plant and Soil Sciences
Carvel Research and Education Center
16483 County Seat Highway
Georgetown, DE 19947
E: gcjohn@udel.edu

Objectives
1. *In laboratory experiments, determine the concentrations of hypochlorite, hydrogen peroxide, peracetic acid and other permissible antimicrobials that are required to prevent the transfer of Lm from an existing biofilm to an uncontaminated surface when tap water or water with different organic loads is the transfer medium.*
2. *Determine if Lm released from a biofilm is able to take refuge on a new surface coated with organic materials and bacteria.*
3. *Validate the results by monitoring the transfer of Lm existing as biofilms on leafy greens and fruits and vegetables to uncontaminated produce or equipment surfaces in the presence of antimicrobials. Also monitor the transfer from abiotic surfaces to leaves or produce surfaces.*

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research

FINAL REPORT

Abstract

Several laboratory studies have established that, like other bacteria, *Listeria monocytogenes* (*Lm*) attached to or growing on surfaces is less susceptible to antimicrobial agents than *Lm* growing in a planktonic state. Some limited evidence has also suggested that the presence of other bacteria along with *Lm* on surfaces can further reduce the susceptibility of *Lm* to antimicrobials. Therefore it was of interest to study the susceptibility of *Lm* in mixed-species biofilms to antimicrobials. Since bacteria can be released from surfaces on which they grow, especially under conditions of movement of the surrounding liquid, it was important to determine if *Lm* cells released from such surfaces or from mixed-species biofilms take some of the protective properties with them as they enter the surrounding liquid medium.

Pure *Lm* biofilms were established with a cocktail of six *Lm* isolates, five of which were from produce-related sources. Mixed-species biofilms grown at room temperature employed the *Lm* cocktail and four non-*Listeria* strains isolated from surfaces of a produce processing plant operating at ambient temperature. Mixed-species biofilms grown at refrigeration temperature included the *Lm* cocktail plus two non-*Listeria* isolated from a produce processing facility operating at refrigeration temperatures. Studies were conducted with cells attached to the surfaces of microtiter plate wells, pegs of the Calgary Biofilm Device (CBD), or coupons of different materials. The antimicrobials, sodium hypochlorite (NaOCl), peracetic acid (PAA), quaternary amines and alkaline oxychloride were used. For most assays, the concentration or exposure time that was capable of completely inactivating *Lm* in these systems was determined. In some cases, counts of *Lm* surviving treatments were obtained. Transfer of *Lm* from biofilms to other surfaces in the presence of antimicrobials was studied with the CBD system or in agitated beakers or microtiter plates, with blueberries or mung beans as the objects to which *Lm* could be transferred.

The data obtained showed that, while NaOCl and PAA at initial concentrations of 100 ppm inactivated planktonic *Lm* within seconds in water without an organic load, at least several minutes of exposure were required for inactivation of surface-attached *Lm*. Association of *Lm* with other bacteria isolated from produce processing environments further decreased susceptibility to antimicrobials, as did establishing the biofilms in high-nutrient conditions. Surface-associated *Lm* and *Lm* in mixed-species biofilms were easily released into surrounding medium, but the released cells were inactivated by antimicrobial concentrations and exposure times close or equal to those required for inactivation of planktonic cells, rather than those required for surface-associated cells. As a result, the antimicrobial agents studied could prevent transfer of *Lm* from surfaces it grew on to other surfaces, including blueberries, at commonly used concentrations in wash water; however, *Lm* associated with soil particles was transferred in a microtiter plate system to mung beans even in the presence of 250 ppm of NaOCl or PAA.

Background

2017 saw over 30 recalls of produce and produced-based items due to (possible) contamination with *Listeria monocytogenes* (*Lm*). The recalled items included apples and apple slices, various vegetable and salad mixes, corn, broccoli, beans, peas, edamame, soybean sprouts, spinach, cashews and macadamia nuts (FDA, 2018). Although no official data are yet available as to whether anyone became ill from these items, the economic losses due to a recall alone can be substantial. Avoiding such recalls is therefore an obvious goal by farmers, produce processors and retailers.

Lm in soil and irrigation water can contaminate produce in the field, and harvested produce, containers and machinery can carry the bacterium into processing environments. Once in the processing environment, opportunities exist for the spread of the bacterium from contaminated produce or soil to produce and equipment previously free of the pathogen. Although the presence of *Lm* in biofilms in produce processing plants has apparently not been demonstrated, it is assumed that, as in cheese, meat and fish processing plants, *Lm* can become part of the biofilm communities in these facilities. Such surface-attached bacterial communities are of concern to food safety because they are potential reservoirs from which pathogens can be released over time, and because such communities, once established, are difficult to eliminate with sanitizers.

Even though it can be debated whether *Lm* by itself can actually form structures that conform to the accepted definition of biofilms (Ferreira et al., 2014), multiple studies have shown that concentrations of antimicrobials able to kill planktonic *Lm* are insufficient to inactivate *Lm* that has been allowed to grow on a surface. For example, in a study comparing the efficacy of ozone, chlorine and hydrogen peroxide in eliminating sessile *Lm*, a concentration of these antimicrobials that was 16 times higher than that required for inactivation of planktonic cells was necessary (Robbins et al., 2005). Studies have routinely shown that it is difficult or impossible to achieve 5-log reductions of surface-grown *Lm* with various antimicrobials, including sodium hypochlorite, chlorine dioxide, electrolyzed water, peracetic acid, ozone, quaternary ammonium compounds, organic acids and trisodium phosphate (e.g., Hoelzer et al., 2014; Kostaki et al., 2012; Olmez and Temur, 2010; Vaid et al., 2010). Olszewska et al. (2016) tested commercially available antimicrobial preparations at concentrations recommended for use on food contact surfaces or for produce washes and found that the chlorine-based (Formula™) and QAC-based (Zep-amine™) sanitizers and levulinic acid/SDS-based (Fit-L) produce wash reduced *Lm* populations established on surfaces of the microtiter plates wells at most by 1.5 log CFU/ml after a 60-min exposure. Phenolic-based (Vesphene®) and QAC-based (Micronex®) sanitizers were more effective, as they reduced cell counts by 6.9 and 3.7 log CFU/ml, respectively, but even those reductions were insufficient to completely eliminate *Lm*.

While it is possible to completely inactivate sessile *Lm* on food contact surfaces (e.g., Aarnisalo et al., 2000; Carballo and Araujo, 2012; Cruz and Fletcher, 2012; Fatemi and Frank, 1999; Robbins et al., 2005; Rodrigues et al., 2011; Srey et al., 2014) by using high concentrations of antimicrobials, the use of such concentrations may be illegal or simply undesirable when in direct contact with food, due to health concerns or damage to food quality or equipment.

The elimination of surface-attached *Lm* in produce processing environments, as in other food processing facilities, is further complicated by the high probability that *Lm* is part of biofilm communities consisting of multiple species of microorganisms. To examine the fate of *Lm* in such communities, Fagerlund et al. (2017) co-cultured seven *Lm* strains and 16 strains belonging to various other genera isolated from a meat processing surface. The authors showed that this mixture of bacteria formed stable biofilm communities and that treatments with PAA and quaternary ammonia compounds at recommended dosages were unable to eliminate the *Lm* strains within these communities. The study also showed that *Lm* can locate to

preferential sites within a biofilm. Earlier studies with less complicated mixed-species biofilms such as those containing one *Lm* isolate and one or two other species, such as a *Pseudomonas* sp. (Fatemi and Frank, 1999), *Salmonella enterica* (Kostaki et al., 2012), *Pseudomonas aeruginosa* (Lourenco et al., 2011), or *Pseudomonas fragi* and *Staphylococcus xylosus* (Nowood and Gilmour, 2000), showed that such biofilms can exhibit increased resistance to antimicrobials compared with pure *Lm* surface-attached communities.

Given the observed difficulties in completely eliminating *Lm* from surface-attached communities, it is possible that such communities can persist over more or less extended periods within produce processing plants and release *Lm* into wash or sanitation solutions. Since *Lm* in surface-attached communities has been shown to be considerably more resistant to commonly used antimicrobials than cells that grew in liquid culture, it was of interest to examine whether *Lm* released from such surface-attached communities retained this resistance level or were as sensitive as planktonic cells that can easily be killed by antimicrobials.

Research Methods and Results

1. Lm *strain collection*

Five produce-related *Lm* strains and one laboratory strain were used in this study. Four strains (*Lm* FSL J1-107, FSL J1-108, FSL R9-0506 and FSL S10-2161) were provided by Dr. Martin Wiedmann, Cornell University. The first two strains were isolated during a coleslaw-related listeriosis outbreak in Canada in 1981. The third strain was isolated during the listeriosis outbreak associated with cantaloupe in 2011. The fourth strain was obtained from soil in a spinach field. Strains *Lm* 390-1 and Scott A were from the collection of strains in the Department of Animal and Food Sciences, University of Delaware. *Lm* Scott A is a widely used laboratory strain. *Lm* 390-1 is one of the strains isolated during the investigations of the listeriosis outbreak associated with cantaloupe in 2011 (Goodridge, 2014). The strains were maintained on TSBYE (tryptic soy broth/yeast extract) medium but grown in R2B medium for surface colonization experiments. When plating on selective media was required, Brilliance™ Listeria Agar (BLA) Base with supplement (Thermo Fisher Scientific, Inc.) was employed. Recovery and selection of *Lm* in liquid media employed Listeria Enrichment Broth (LEB) with supplement (Thermo Fisher Scientific, Inc.).

2. Collection and identification of bacteria from surfaces in produce processing facilities

2.1. Media selection

The purpose of collecting bacteria from processing facilities was to obtain isolates that could be used to create multi-species biofilms with relevance to produce processing environments. Since the nutrient conditions found on the different surfaces within produce processing plants differ with the processing step and the type of produce being processed, the decision was made to isolate the bacteria on a commercially available, standardized low-nutrient medium, rather than on media with produce extracts. The medium chosen, R2B, contains only 2.8 g of organic compounds per liter, a value that is likely not exceeded in many produce wash waters. The medium would not be representative of organic loads that can potentially be found at such places as cutting, peeling or shredding sites.

2.2. Strain collection and identification

Swabs were taken from various locations at a processing facility that was washing and packing peaches and corn at the time of sampling. The facility was operating at ambient air temperature and used water without cooling. The swabs were streaked onto R2B medium solidified with agar (R2A), and plates were incubated at 21°C in the laboratory for two days to obtain single colonies. Colonies were purified and unique isolates, as determined by digestion of their amplified 16S rDNA with restriction enzyme *Hha*I, were identified by sequencing of a variable region of 16S rDNA.

A second set of isolates was obtained from nine swab cultures provided by a produce processing facility operating entirely at refrigeration temperature. One single colony derived from each of the swab cultures was picked from R2A plates incubated at 4°C for 7 days, then purified, and part of their 16S rRNA genes was sequenced to provide identification.

Four strains (*Pedobacter* sp., *Acinetobacter* sp., *Pantoea agglomerans* and *Chrysobacterium* sp.) were chosen from among the isolates obtained from the facility operating at ambient temperature for use in mixed-species biofilm experiments because preliminary experiments showed that they were compatible with the *Lm* strains, were easily distinguishable from the *Lm* strains on R2A medium, and either did not grown on BLA or formed small white colonies on this medium. For mixed-species biofilm growth at refrigeration temperature, a *Pseudomonas* sp., and a *Flavobacterium* species were chosen for similar reasons.

3. Colonization of surfaces with Lm *or multiple species*

For simplicity reasons, the term “biofilm” is used in this report for cells attached to a surface from which they could not be removed by three to five washes with phosphate-buffered saline (PBS). The surfaces that were used in the current study were wells of 6-, 24- and 96-well microtiter plates (polystyrene), pegs of the Calgary Biofilm Device (polystyrene) (**Fig. 1**), and 1×1 inch coupons of stainless steel, PVC, HDPE or a commercial plastic used in a produce processing plant.

For experiments involving surface colonization with *Lm* only, a cocktail of the six *Lm* strains was employed. For multi-species biofilms to be grown at room or refrigeration temperatures, the *Lm* cocktail was mixed with the respective four or two strains listed in section 2.2. Specifically, the strains to be employed in surface colonization experiments were grown individually overnight in R2B medium at room temperature. The optical density (OD) at 600 nm of each culture was measured, and cultures were diluted in R2B medium to achieve an OD_{600} of 0.05. For surface colonization with *Lm* strains only, equal volumes of the six OD-adjusted *Lm* cultures were combined and diluted with an equal volume of R2B medium prior to pipetting the mixture into microtiter well plates (150 µl for 96-well plates, including Calgary Biofilm Devices (CBDs), 500 µl for 6-well plates) or onto stainless steel or plastic chips (100 µl/chip). For mixed-species colonization, the *Lm* strain mixture was combined with an equal volume of the four-strain mixture (adjusted to OD_{600}=0.05) or

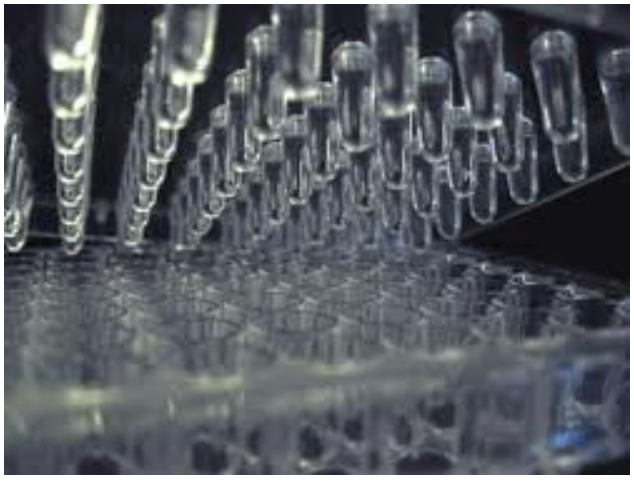


Fig. 1. Image of microtiter plate system for biofilm formation assays. The bottom part is a 96-well microtiter plate; the top part of the image shows the lid with pegs.

the two-strain mixture for growth at room temperature or 4°C, respectively. The same volumes of the final mixtures as those prepared for the *Lm* mixtures were pipetted into microtiter plate wells or onto chips. Cells were allowed to adhere to the surfaces for 4 h at room temperature or 4°C, and then the liquid was pipetted out of the wells or off the chip surfaces and replaced with fresh R2B medium. On some occasions, biofilms were grown in TSBYE medium and in produce extracts (for example, see section 6). The produce extracts were obtained by stomaching a 10% (w/v) mixture of chopped produce and water for 2 min, filtering the slurry through cheesecloth and filter-sterilizing the filtrate with a 0.2-µm filtration device. For room-temperature biofilm generation, the medium was replaced daily, whereas the medium for biofilms grown at 4°C was replaced every other day. Surfaces with adherent cells were obtained by rinsing the wells five times with PBS.

4. Colonization of soil particles with Lm *and multiple species*

Cultures were grown and strain mixtures were obtained as described in section 3. Soil obtained from a field in southern Delaware was sterilized by autoclaving and then distributed in 1-g portions into the wells of a 6-well microtiter plate. Two ml of water or R2B medium was added to each well together with 100 µl of the OD-adjusted strain mixture. The plate was kept at room temperature for five days. Prior to experiments, the liquid was removed and the soil particles were washed five times with PBS to remove suspended cells and small soil particles.

5*. Enumeration of viable* Lm *cells*

Lm counts of cells in suspension were obtained by plating appropriate dilutions on BLA and counting of typical green colonies after 48 h at 37°C. *Lm* counts in surface-attached communities were obtained after the addition of either R2B medium or D/E Neutralizing Broth (Thermo Fisher Scientific) (for biofilms treated with antimicrobials), sonication in a 1.9-l sonicator bath (Thermo Fisher Scientific) for 10 min and plating on BLA. To test for the presence/absence of viable *Lm* cells, liquid samples were mixed with at least 1 volume of LEB, and surfaces with attached cells were covered with LEB, incubated for 48 h at 37°C, and 10-µl aliquots were spotted on BLA. The appearance of green colonies indicated the presence of *Lm* in the samples.

6. Coexistence of Lm *with produce processing plant isolates*

Since it is possible that the *Lm* strains could be inhibited by one or more of the produce processing plant strains, viable counts of *Lm* on BLA and total counts on R2A were determined for the mixed-species biofilms growing on the pegs of the CBD in the presence of the rich medium (TSBYE), the low-nutrient medium (R2B), and four produce extracts with various organic contents. **Fig. 2** shows the counts for biofilms grown at room temperature (~21°C) for 4 and 8 days and at 4°C for 4, 8 and 14 days. *Lm* was able to achieve counts of between 10^5 and 10^7 CFU/peg in the presence of the four other strains. The counts for *Lm* in mixed-species biofilms developed in different media at refrigeration temperature were in the range of 10^3 to 10^5 CFU/peg, thus lower than those found for the biofilms grown at room temperature.

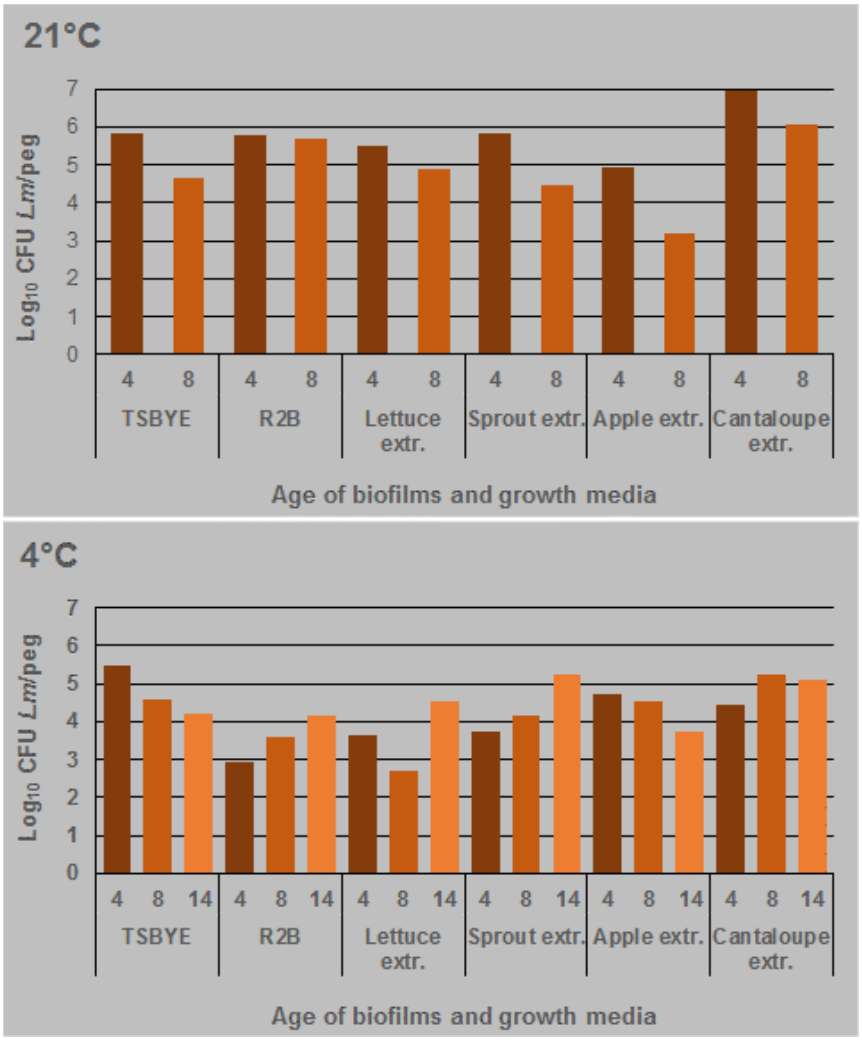


Fig. 2. *Lm* counts in **mixed species biofilms** grown on polystyrene pegs for 4 and 8 days at **21°C** (top panel) and for 4, 8, and 14 days at **4°C** (bottom panel) in the presence of different media: TSBYE, tryptic soy broth/yeast extract, COD=20,780 mg/l; R2B, standard low-nutrient medium, COD=3,140 mg/l; Lettuce, lettuce extract, COD=1,410 mg/l; Sprouts, bean sprout extract, 1,766 mg/l; Apple, apple extract, COD=12,130 mg/l; Cantaloupe, cantaloupe extract, COD=8,310 mg/l.

7. Susceptibility of Lm *in surface-attached communities to antimicrobial agents*

The concentrations of antimicrobial agents or the times required for complete inactivation of *Lm* in mono- and multi-species biofilms was determined with cells attached to the pegs of the CBD. Antimicrobial solutions were prepared with autoclaved tap water (Hardness: 240; alkalinity: 40). Therefore, the reported concentrations for inactivation of *Lm* only apply to conditions without organic load of wash solutions. Sodium hypochlorite (NaOCl) solutions were prepared from a 6% commercially available solution by appropriate dilution with sterile tap water and adjustment of the pH to 6.5 to 6.8. Peracetic acid solutions were prepared from a 17% peracetic acid stock (FMC), quaternary ammonium solutions were prepared with Madisan-75 (Madisan Chemical Co.), and alkaline oxychloride solutions were prepared by appropriate dilution from stock (Safe-Zone LOD, GreenAgri Solutions) and adjustment of the pH to 9.4. The ORP level was checked with a calibrated probe. Available chlorine, PAA and quaternary ammonium concentrations were checked with appropriate test strips.

Pegs were removed from the culture media (R2B, unless otherwise noted) and dipped into 150 µl of PBS for 30 sec to allow removal of loosely attached cells. The pegs were then completely submerged in 330 µl of sterile tap water containing various concentrations of antimicrobials for a specified amount of time. Alternatively, the pegs were lowered into tap water

with a single concentration of the antimicrobial and removed at various time points. Following exposure to the antimicrobial, the pegs were submerged in 200 µl of Neutralizing Broth for 1 min, before being submerged in 150 µl of LEB. After 24 to 48 h, aliquots of the LEB cultures were spotted onto BLA, and the appearance of green colonies indicated the presence of survivors of the antimicrobial treatments.

Testing of susceptibility of biofilms grown at 4°C to antimicrobials was done with the same volumes of solutions, except that PBS and antimicrobial solutions were at refrigeration temperature and exposure was carried out at 4°C.

7.1. Susceptibility to sodium hypochlorite (NaOCl)

7.1.1. Biofilms grown at room temperature

Fig. 3 shows the results of assays done with mixed-species biofilms grown on pegs in R2B medium at room temperature. To inactivate *Lm* in the biofilms required 30-min exposure to 25 ppm NaOCl in tap water at pH 6.8, or 7.5 min exposure at 250 ppm. These times and concentrations are markedly higher than those required for inactivation of planktonic cells. For example, an assay with cell suspensions consisting of six *Lm* strains (the six *Lm* and the four isolates from the processing plant operating at ambient temperature and the six *Lm* strains with the two isolates from the refrigerated processing environment) showed that it took only seconds to completely inactivate planktonic *Lm*, regardless of the presence of other bacteria **(Fig. 4)**.

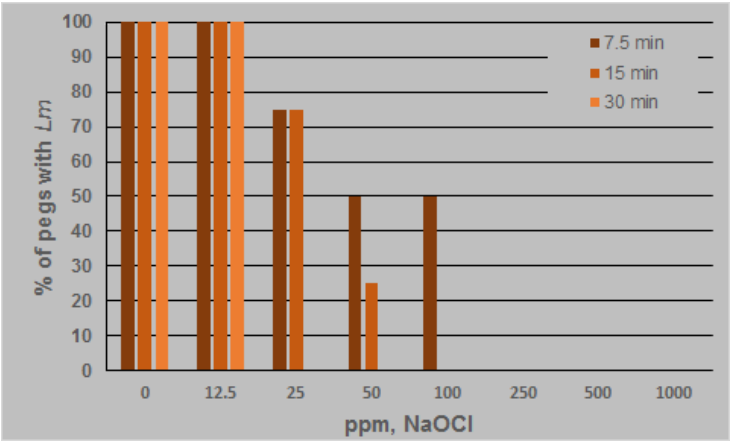


Fig. 3. Percent of pegs with **mixed-species biofilms** that tested positive for *Lm* following treatments with 0 – 1,000 ppm **NaOCl** in tap water for 7.5, 15 and 30 min. Biofilm growth and treatment were done at **room temperature**. Data are from four replicate tests.

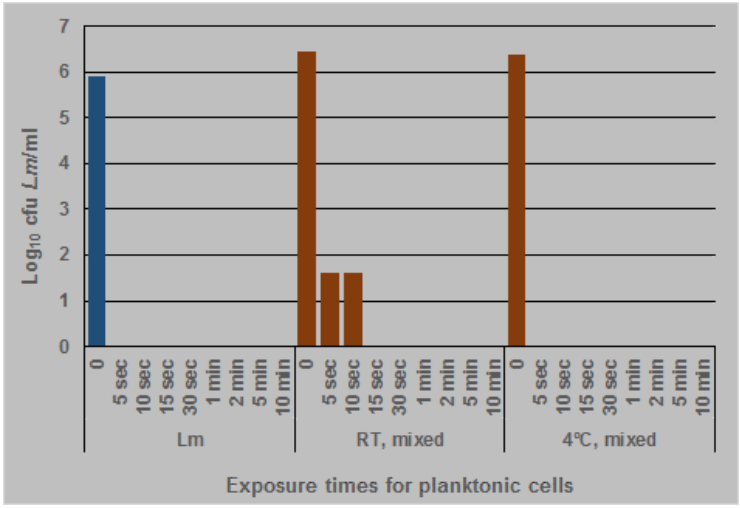


Fig. 4. Effect of exposure to **100 ppm NaOCl** of planktonic cells. Lm, mixture of six *Lm* strains; RT, mixed, mixture of *Lm* strains and four strains isolated from processing plant operating at ambient temperature; 4°C, mixed, *Lm* strain cocktail plus the two strains from processing plant operating at refrigeration temperature.

Fig. 5 shows the corresponding data for pure *Lm* biofilms. In this example, as in other assays, surface-associated *Lm* are slightly more sensitive to the antimicrobial than their counterparts in mixed-species biofilms.

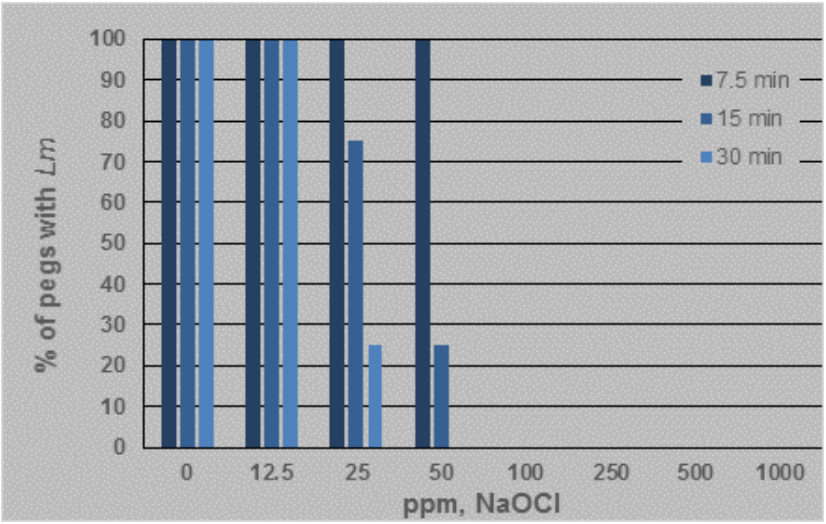


Fig. 5. Percent of pegs with ***Lm*-only** biofilms that tested positive for *Lm* following treatments with 0 – 1,000 ppm **NaOCl** in tap water for 7.5, 15 and 30 min. Biofilm growth and treatment were done at **room temperature**. Data are from four replicate tests.

7.1.2. Biofilms grown at 4°C

Fig. 6 shows data for mixed-species biofilm grown and treated at 4°C. In this example, higher concentrations of NaOCl were required for *Lm* inactivation at refrigeration temperature than was observed at room temperature (Fig. 3).

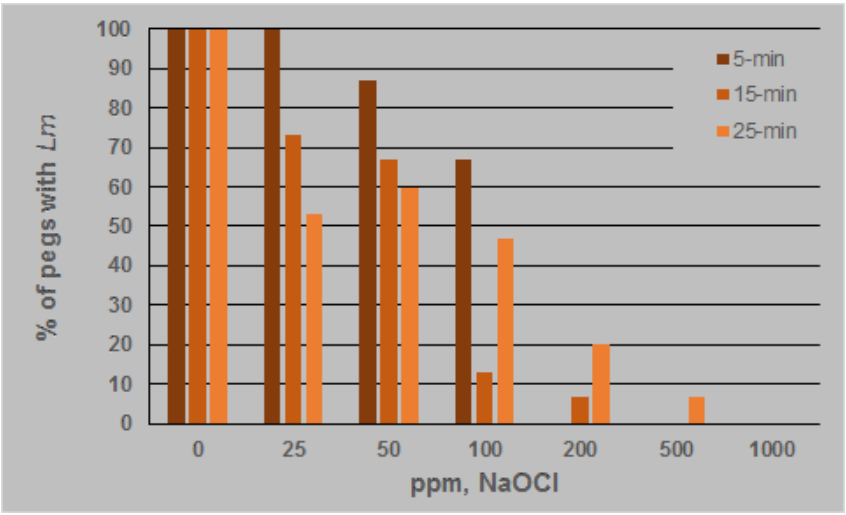


Fig. 6. Percent of pegs with **mixed-species biofilms** that tested positive for *Lm* following treatments with 0 – 1,000 ppm **NaOCl** in tap water for 7.5, 15 and 30 min. Biofilm growth and treatment were done at **4°C**. Data are from 15 replicate tests.

As observed for the room temperature assays, *Lm* in mono-species biofilms (**Fig. 7**) exhibited a slightly higher sensitivity to NaOCl than *Lm* in mixed-species biofilms.

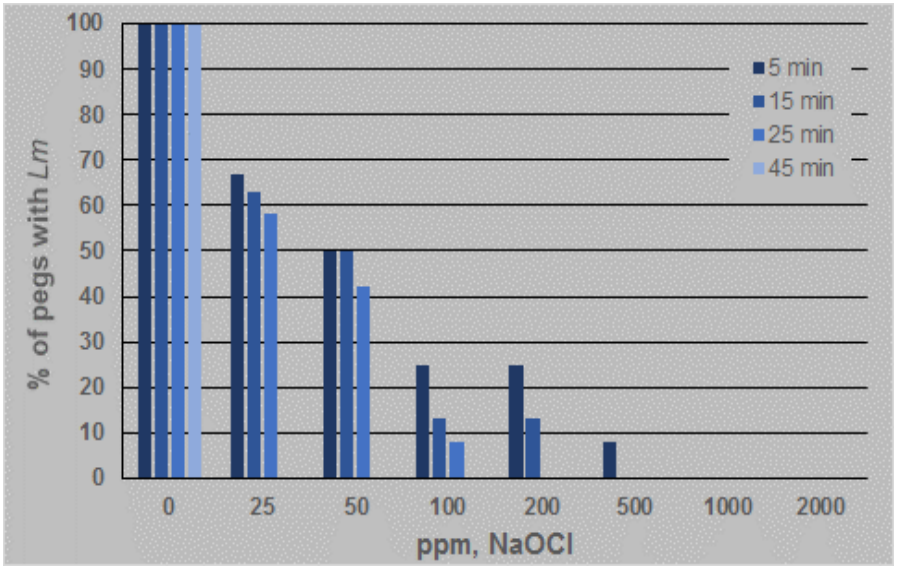


Fig. 7. Percent of pegs with ***Lm*-only** biofilms that tested positive for *Lm* following treatments with 0 – 1,000 ppm **NaOCl** in tap water for 7.5, 15 and 30 min. Biofilm growth and treatment were done at **4°C**. Data are from 12 replicate tests.

7.2. Susceptibility to peracetic acid (PAA)

7.2.1. Biofilms grown and treated at room temperature

The effect of PAA on mixed-species and mono-species biofilms grown and treated at room temperature is shown in **Fig. 8** and **Fig. 9**, respectively. As observed for NaOCl, a slightly higher concentration/exposure time was required for complete inactivation of *Lm* in the mixed-species biofilms than in pure *Lm* biofilms.

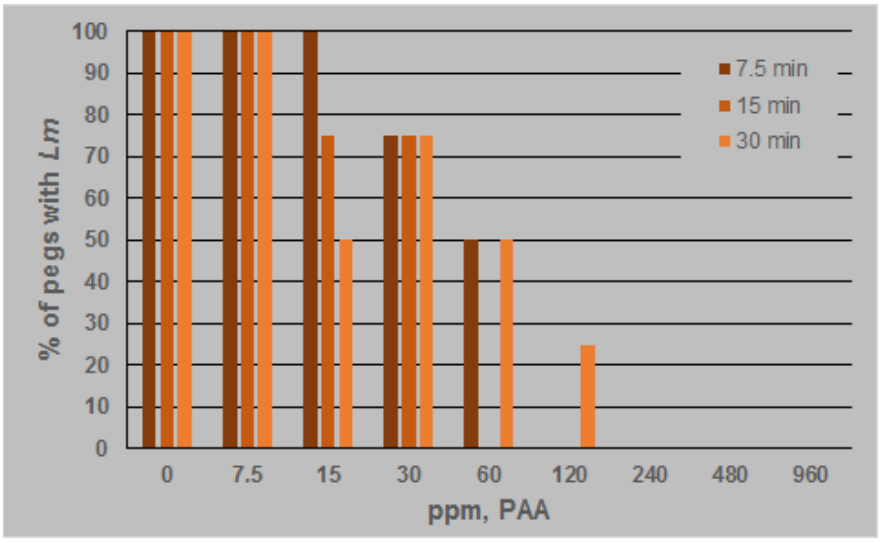


Fig. 8. Percent of pegs with **mixed-species biofilms** positive for *Lm* after treatment with different concentrations of **PAA** for different times at **room temperature**. Data are from 4 replicates.

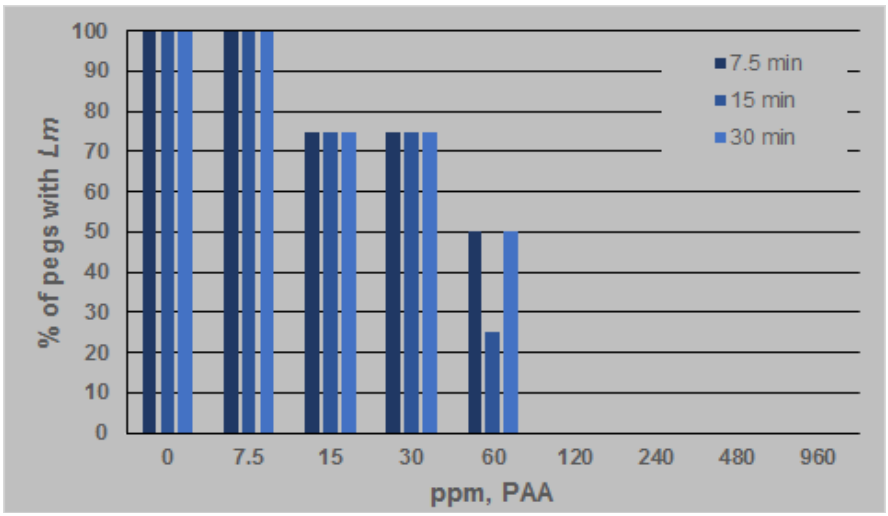


Fig. 9. Percent of pegs with ***Lm*-only biofilms** positive for *Lm* after treatment with different concentrations of **PAA** for different times at **room temperature**. Data are from 4 replicates.

7.2.2. Biofilms grown and treated at 4°C

Exposure of biofilms to PAA concentrations of 50 ppm for 45 min was able to inactivate *Lm* in mixed-species biofilms grown and treated at 4°C (**Fig. 10**); however, short exposure time (5 min) was not able to inactivate *Lm* in all cases, even at very high NaOCl concentrations.

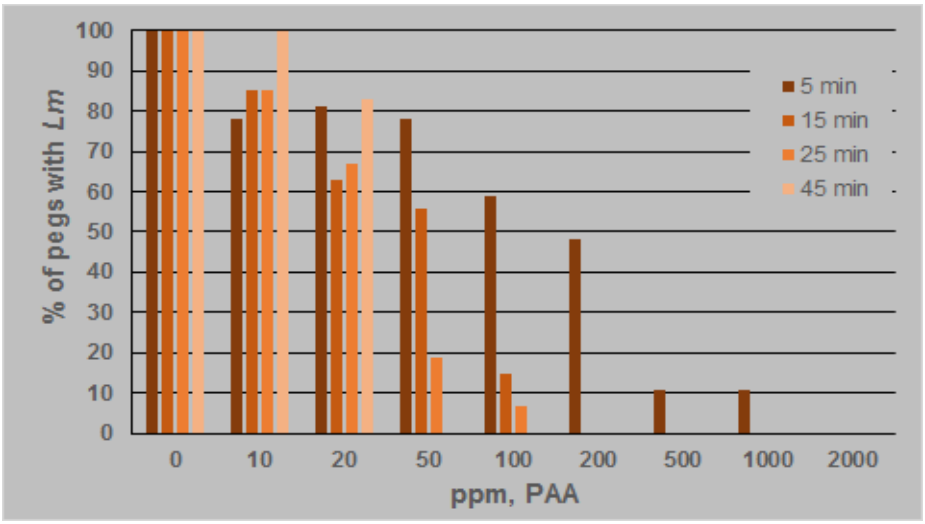


Fig. 10. Percent of pegs with **mixed-species biofilms** positive for *Lm* after treatment with different concentrations of **PAA** for different times at **4°C**. Data are from 27 replicates.

Even for *Lm*-only biofilms, it took high concentrations of PAA to reliably inactivate *Lm* on the pegs, although a 45-min exposure to 10 ppm was sufficient for complete inactivation. *Lm* in *Lm*-only biofilms were inactivated more easily than those in multi-species biofilms, but 15-min exposure required the presence of an initial concentration of PAA of more than 500 ppm to reliably inactivate all *Lm* (**Fig. 11**).

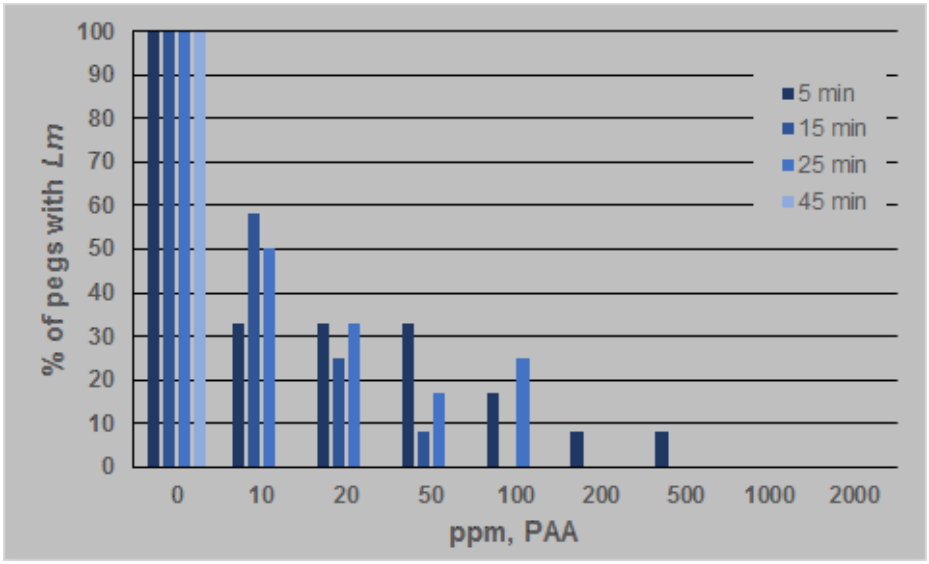


Fig. 11. Percent of pegs with ***Lm*-only biofilms** positive for *Lm* after treatment with different concentrations of **PAA** for different times at **4°C**. Data are from 4 replicas.

7.3. Susceptibility to quaternary amines (Madisan-75)

7.3.1. Biofilms grown and treated at room temperature

Fig. 12a illustrates the outcome of treatments of mixed-species biofilms growing on pegs at room temperature with the quaternary ammonium product, Madisan-75. The data for the corresponding *Lm*-only biofilms can be viewed in **Fig. 12b**. In the case of the mixed-species biofilms, a concentration of 75 ppm and exposure for 45 min was required to inactivate *Lm* on all pegs. A lower concentration (18.75 ppm) applied for 45 min was sufficient for the inactivation of *Lm* in *Lm*-only biofilms, but shorter exposure times required more than 600 ppm for reliable inactivation.

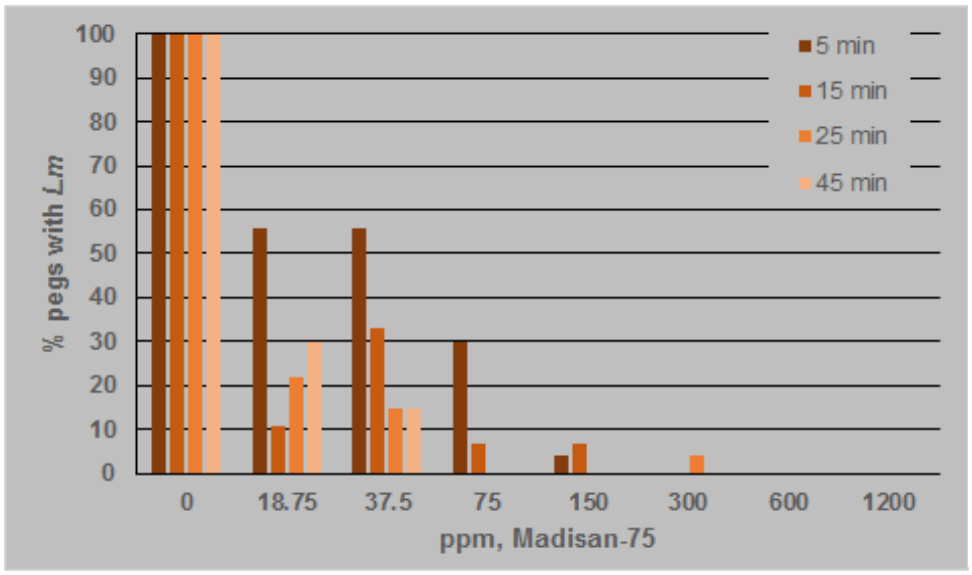


Fig. 12a. Percent of pegs positive for *Lm* after treatment of peg-associated **mixed-species biofilms** with different concentrations of **Madisan-75** for 0 to 45 min at **room temperature**. Data are from four replicates.

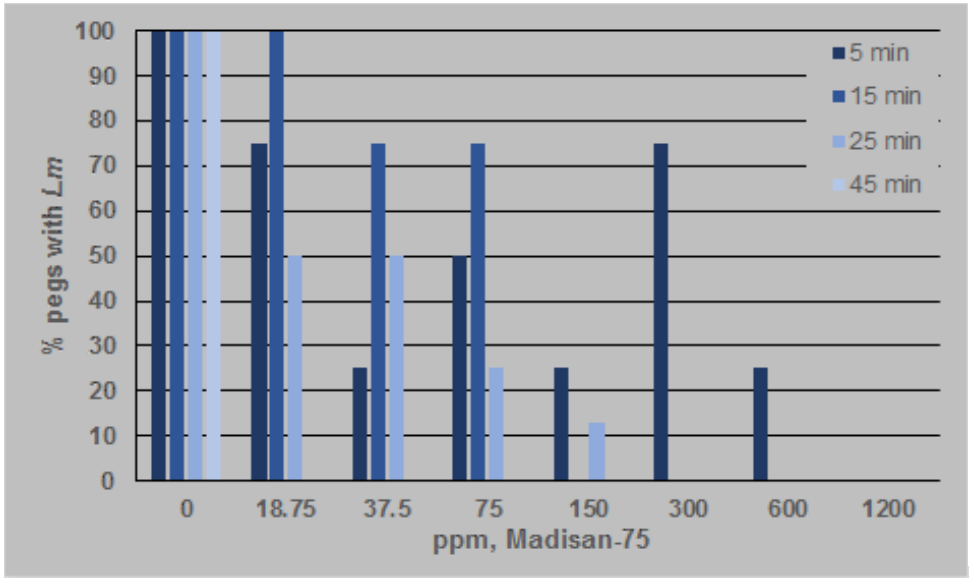


Fig. 12b. Percent of pegs positive for *Lm* after treatment of peg-associated *Lm*-only biofilms with different concentrations of **Madisan-75** for 0 to 45 min at **room temperature**. Data are from four replicates.

7.3.2. Biofilms grown and treated at 4°C

As seen in **Fig. 13**, an initial concentration of 18.75 ppm of quaternary amines was able to completely inactivate *Lm* from some pegs coated with mixed-species biofilms, but reliable inactivation required considerably higher concentrations. The differences in susceptibility could be due to variability in the peg surfaces or other unknown variables. In contrast, the susceptibility of *Lm* in *Lm*-only biofilms was considerably higher (**Fig. 14**), although reliable inactivation was not achieved until high concentrations were applied.

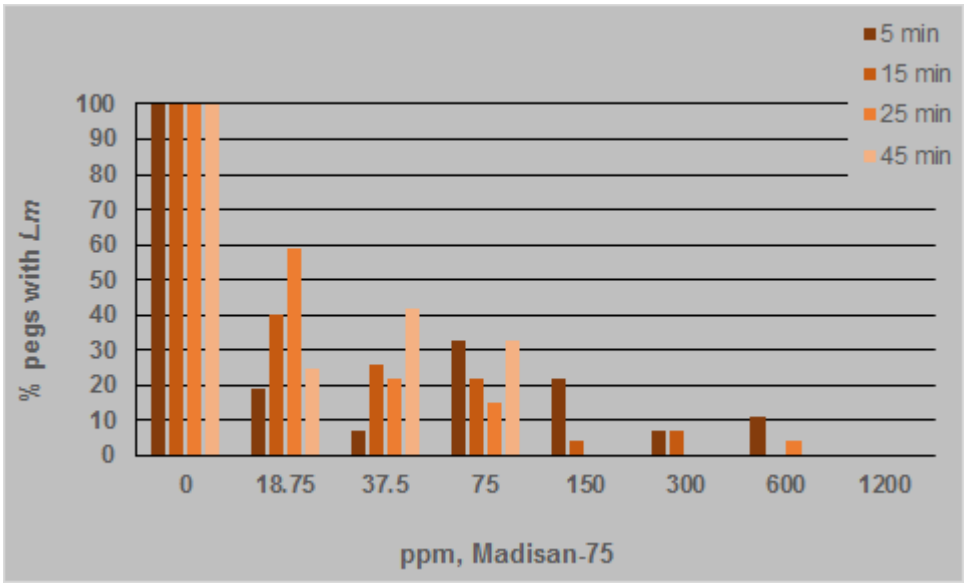


Fig. 13. Percent of pegs positive for *Lm* after treatment of peg-associated **mixed-species biofilms** with different concentrations of **Madisan-75** for 0 to 45 min at **refrigeration temperature**. Data are from 12–27 replicates.

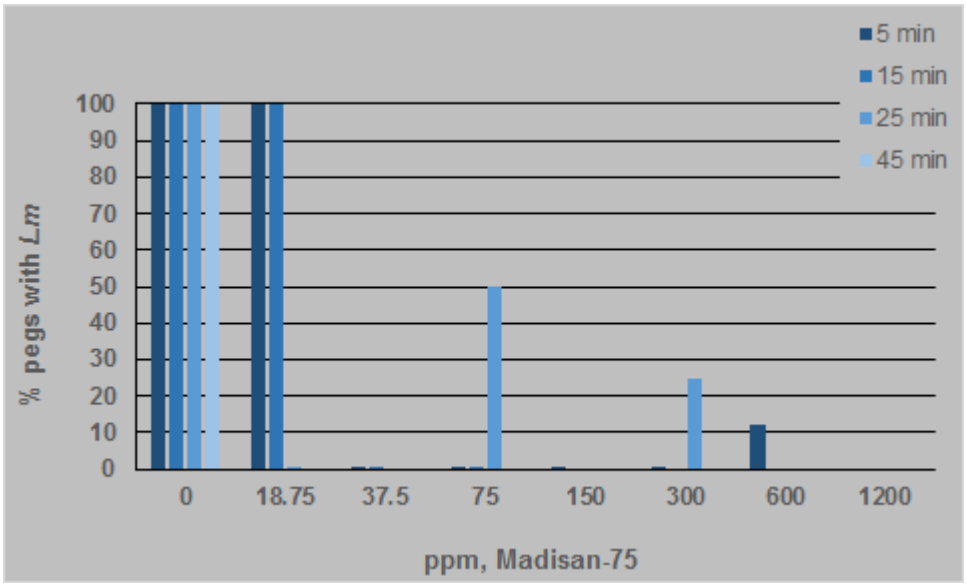


Fig. 14. Percent of pegs positive for *Lm* after treatment of peg-associated ***Lm*-only biofilms** with different concentrations of **Madisan-75** for 0 to 45 min **at refrigeration temperature**. Data are from 12–27 replicates.

7.4. Susceptibility to alkaline oxychloride (Safe-Zone LOD)

At the recommended initial target ORP level of around 700, a 30-min exposure to Safe-Zone LOD was able to reduce *Lm* in *Lm*-only and in mixed-species biofilms grown and treated at room temperature in microtiter plate wells by ~5 log, but complete inactivation was not achieved (**Fig. 15**).

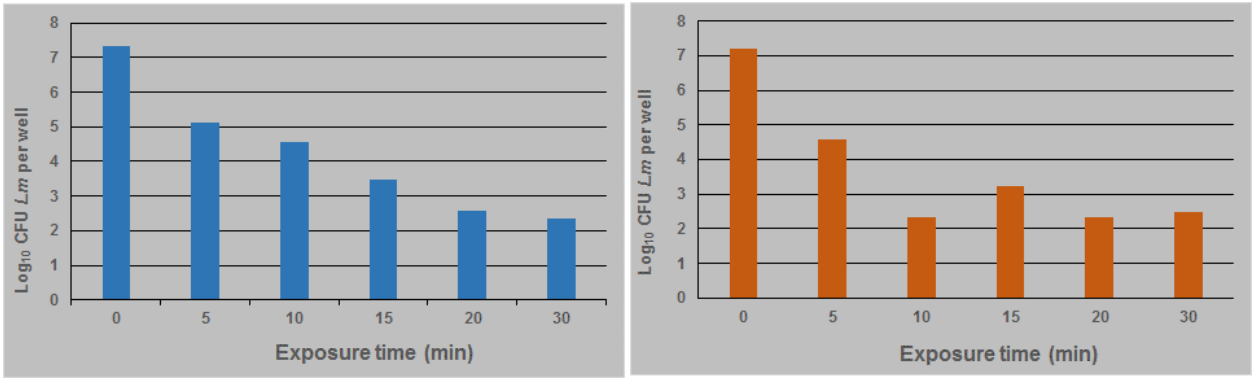


Fig. 15. Log_{10} CFU of *Lm* recovered after treatment of biofilms consisting of ***Lm* strains** (blue bars) or *mixed strains* (orange bars) with **Safe-Zone LOD** for 0–30 min. Three separate experiments were carried out in 96-well plates at **room temperature** with Safe-Zone levels of **700–735 ORP**.

7.5. Differences in biofilm formation and antimicrobial susceptibility due to growth medium

7.5.1. Lm *counts in mixed-species biofilms grown at room temperature in different media*

Most experiments for the current project were done with biofilms grown in the presence of the low-nutrient medium, R2B, as it was considered a more realistic reflection of the nutrient levels to be expected in various locations in produce processing plants; however, a number of experiments were carried out with biofilms grown in other nutrient conditions. As can be seen in **Fig. 16**, *Lm* was able to persist, and likely grow, in mixed species biofilms at room temperature and at 4°C in the presence of different nutritional conditions. Interestingly, in this particular experiment, the *Lm* count in the mixed biofilms grown in TSBYE (COD=20,780 mg/l) was not different from that in the the standard low-nutrient medium, R2B (COD=3,140 mg/l). Overall, in this particular experiment, the COD level of the growth medium did not correlate well with the *Lm* CFU observed on the pegs. For example, the counts in biofilms grown in cantaloupe extract with a COD of 8,310 mg/l were as high or higher than those obtained with TSBYE (COD=20,780 mg/l). The low COD levels of lettuce extract (COD=1,410 mg/l) and bean sprout extract (1,766 mg/l) still allowed for the presence of *Lm* in numbers not too different from those in the biofilms grown in media with higher CODs, such as apple extract (COD=12,130 mg/l). Therefore, it appears that specific nutrients rather than the overall nutrient content might determine how well *Lm* survives or growth in the mixed species biofilms.

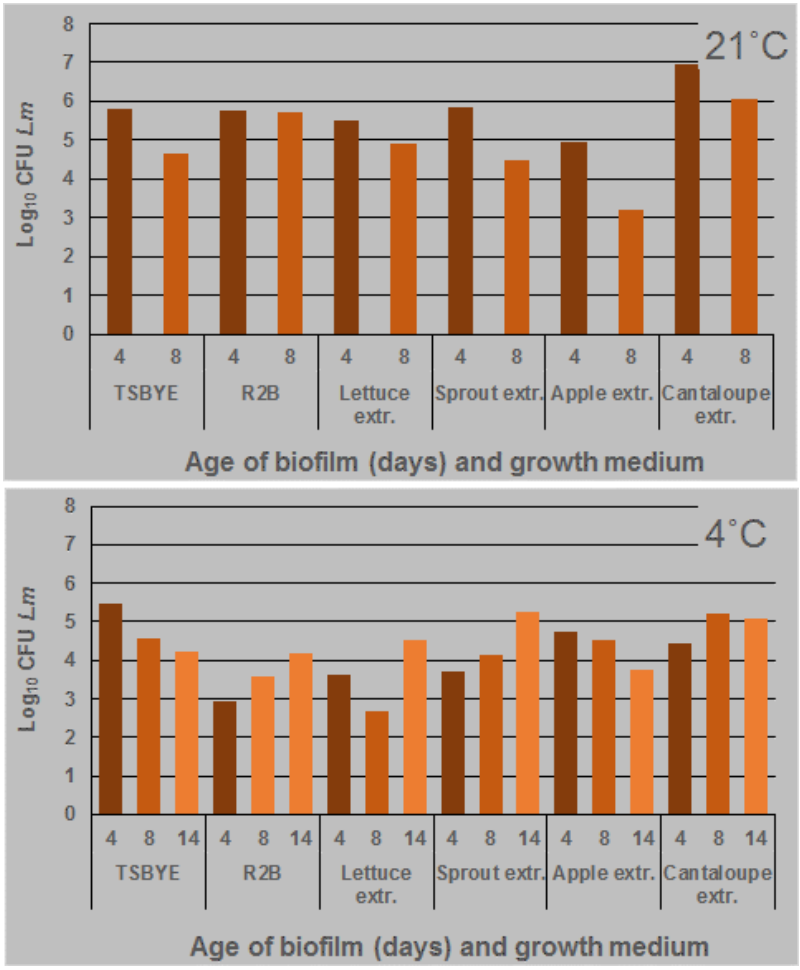


Fig. 16. Log_{10} CFU of *Lm* in mixed-species biofilms grown on pegs at **room temperature** (top panel) for 4 and 8 days or at **refrigeration temperature** (bottom panel) for 4, 8 and 14 days in the presence of TSBYE, R2B or produce extracts.

7.5.2. Susceptibility to 100 ppm NaOCl for Lm *in biofilms grown in the presence of high- and low-nutrient media*

In these experiments, the initial *Lm* counts in the biofilms on pegs were generally ~1 log lower for biofilms grown in R2B than in TSBYE medium (**Fig. 17**). This difference could, at least in part, explain some of the faster inactivation times observed for the R2B-grown biofilms, but it is also possible that the non-*Lm* strains or the *Lm* strains themselves were able to produce higher amounts of protective materials under the nutrient-rich conditions than under the low-nutrient conditions.

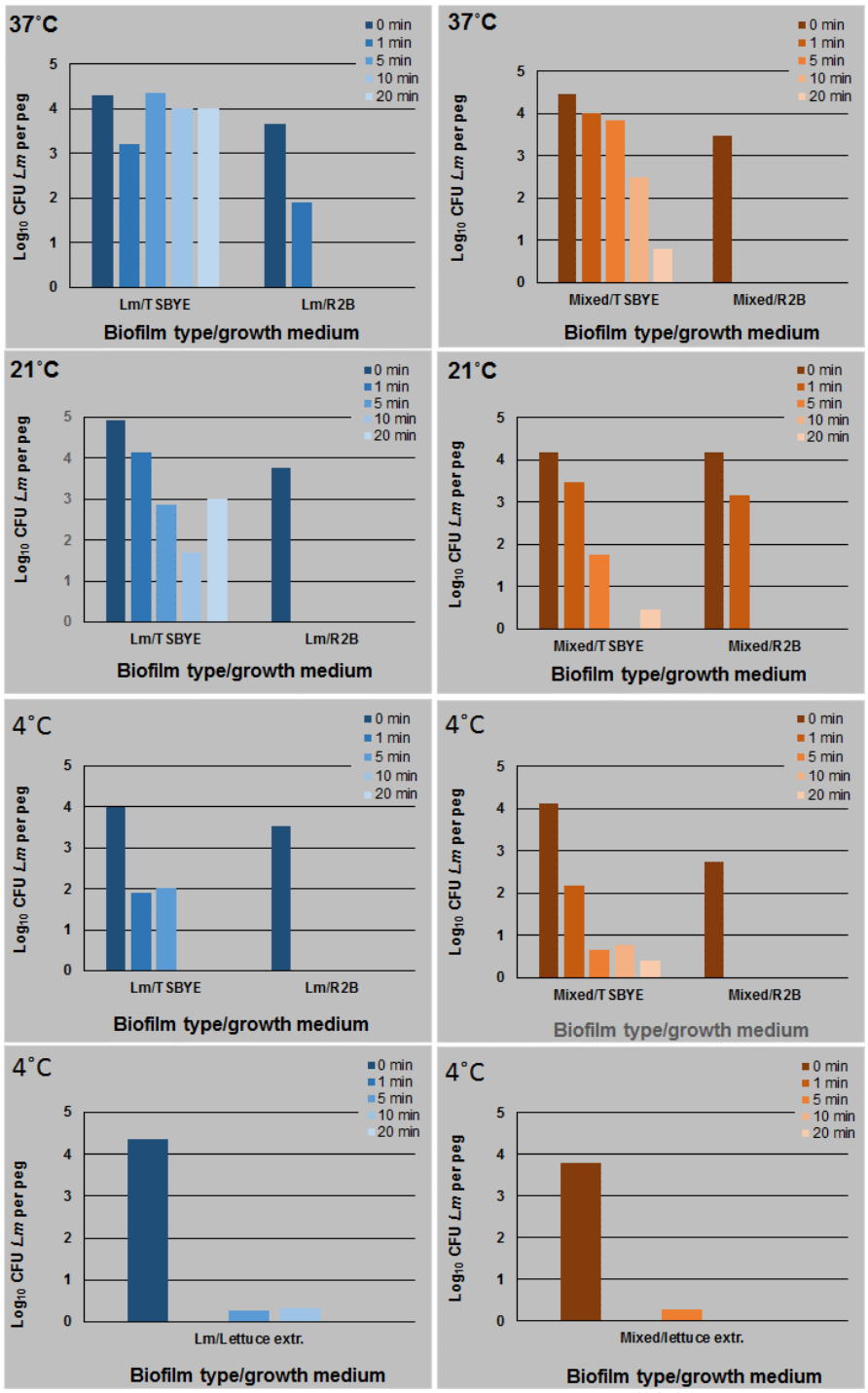


Fig. 17. Log_{10} CFU of *Lm* in ***Lm*-only** (blue bars) and in **mixed-species** biofilms (orange bars) after treatment with **100 ppm NaOCl** for 0 to 20 min. The biofilms were grown in microtiter plate wells at **37**, **21** and **4°C** in the presence of either **TSBYE** or **R2B** medium (top three rows) or in lettuce extract (bottom two panels). Biofilm ages were 5 and 8 days for those grown at 37 and 21°C, and 4°C, respectively. Data are averages from two wells.

7.5.2. Susceptibility to PAA, Madisan-75, or Safe-Zone LOD of Lm *in biofilms grown in the presence of different media*

As a rule, *Lm* in biofilms grown in TSBYE medium was more resistant to antimicrobials than those grown in R2B, and *Lm* in *Lm*-only biofilms was easier to inactivate than *Lm* in mixed-species biofilms. This observation held true for the trial whose outcome is illustrated in **Fig. 18**. In this trial, Safe-Zone LOD was employed to treat pure *Lm* and mixed species biofilms grown in either TSBYE or R2B at room temperature or 4°C.

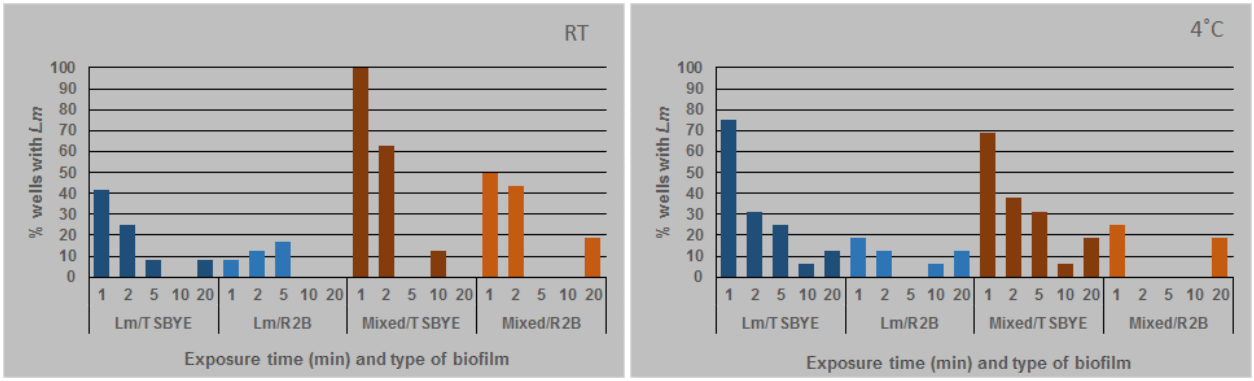


Fig. 18. Percent of wells positive for *Lm* after treatment of *Lm*-only biofilms (blue bars) and mixed-species biofilms (orange bars) grown in either TSBYE or R2B with **Safe-Zone LOD** at 700 ORP for 1 to 20 min. Left panel: Biofilms grown and treated at room temperature; right panel, biofilms grown and treated at 4°C.

Fig. 19 shows the results of a trial with mixed-species biofilms on pegs exposed to three different antimicrobials at concentrations similar to those that could be encountered in processing environments. Except for Madisan-75 at an initial concentration of 150 ppm, the antimicrobials were unable to inactivate *Lm* in all cases even within 20 min. Exposure to 100 ppm PAA resulted in the inactivation of *Lm* from at least one of the pegs at 2 min, but even a 20-min exposure was unable to inactivate *Lm* on both pegs in all cases. *Lm* in TSBYE-grown multi-species biofilms was particularly resistant to Safe-Zone LOD as both pegs were still positive for *Lm* at an exposure time of 20 min.

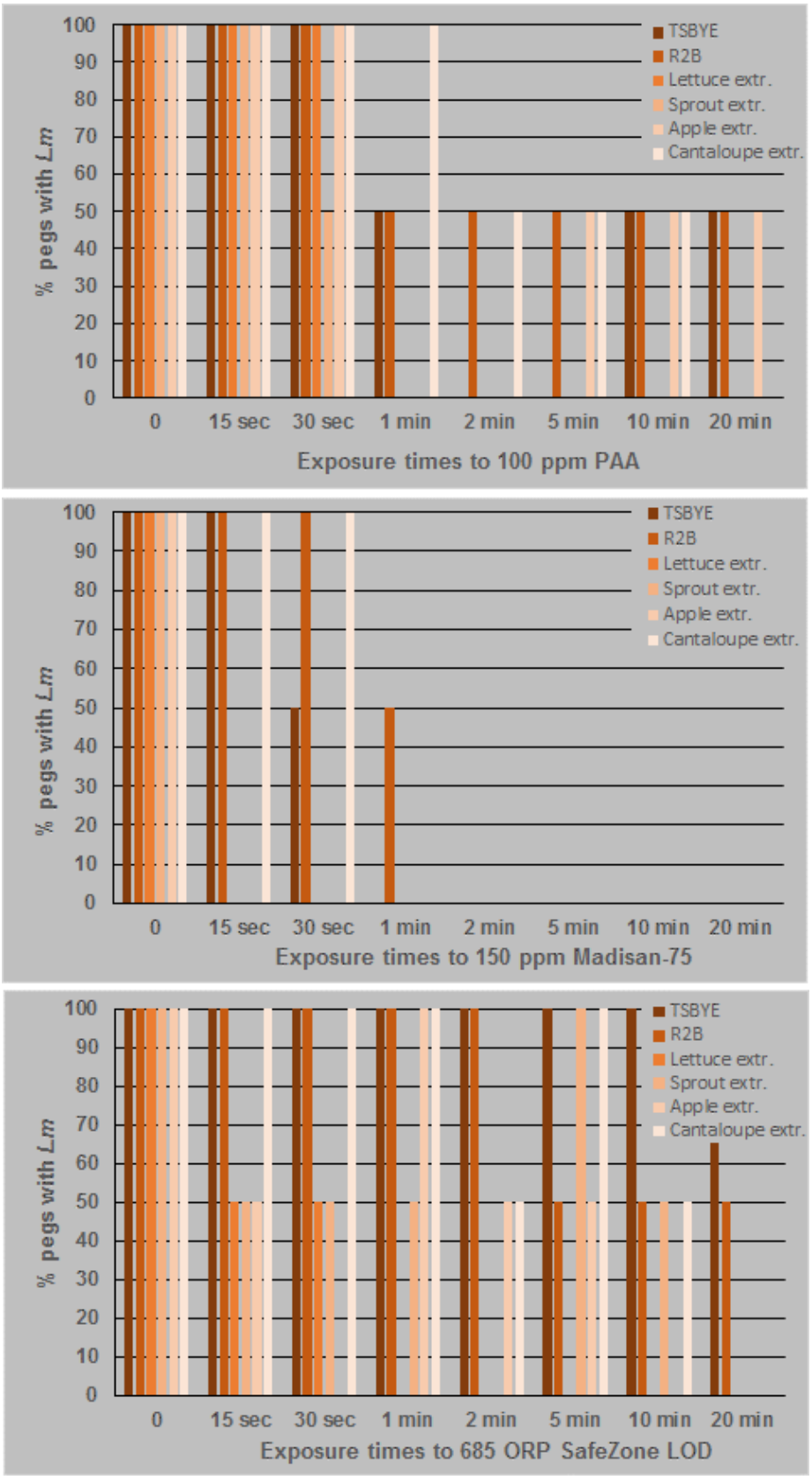


Fig. 19. Percent of pegs positive for *Lm* in biofilms grown in different media at room temperature after treatment with **100 ppm PAA** (top panel), **150 ppm Madisan-75** (middle panel) or **Safe-Zone LOD** at **685 ORP** for 0 to 20 min. Data are from two replicates only.

7.6 Susceptibility of Lm added to existing biofilm to NaOCl and PAA

To mimic a situation in which planktonic *Lm* encounters and interacts with an existing biofilm, trials were conducted at room temperature in which the four non-*Lm* strains were allowed to form a biofilm for two days, at which point the medium was replaced with a suspension of the *Lm* strains. *Lm* was allowed to interact with the biofilms for 4 h before the cell suspension was removed and replaced with fresh R2B medium.

Fig. 20 allows side-by-side comparisons of the effects of different concentrations and exposure times to PAA or NaOCl on *Lm* in peg-associated pure *Lm* biofilms, in mixed-species biofilms in which *Lm* was a component from the beginning and in biofilms to which *Lm* was added 48 h after the non-*Lm* strains started developing biofilms. *Lm* added to existing biofilms was about as sensitive to the antimicrobial as *Lm* that was part of the biofilm from the start.

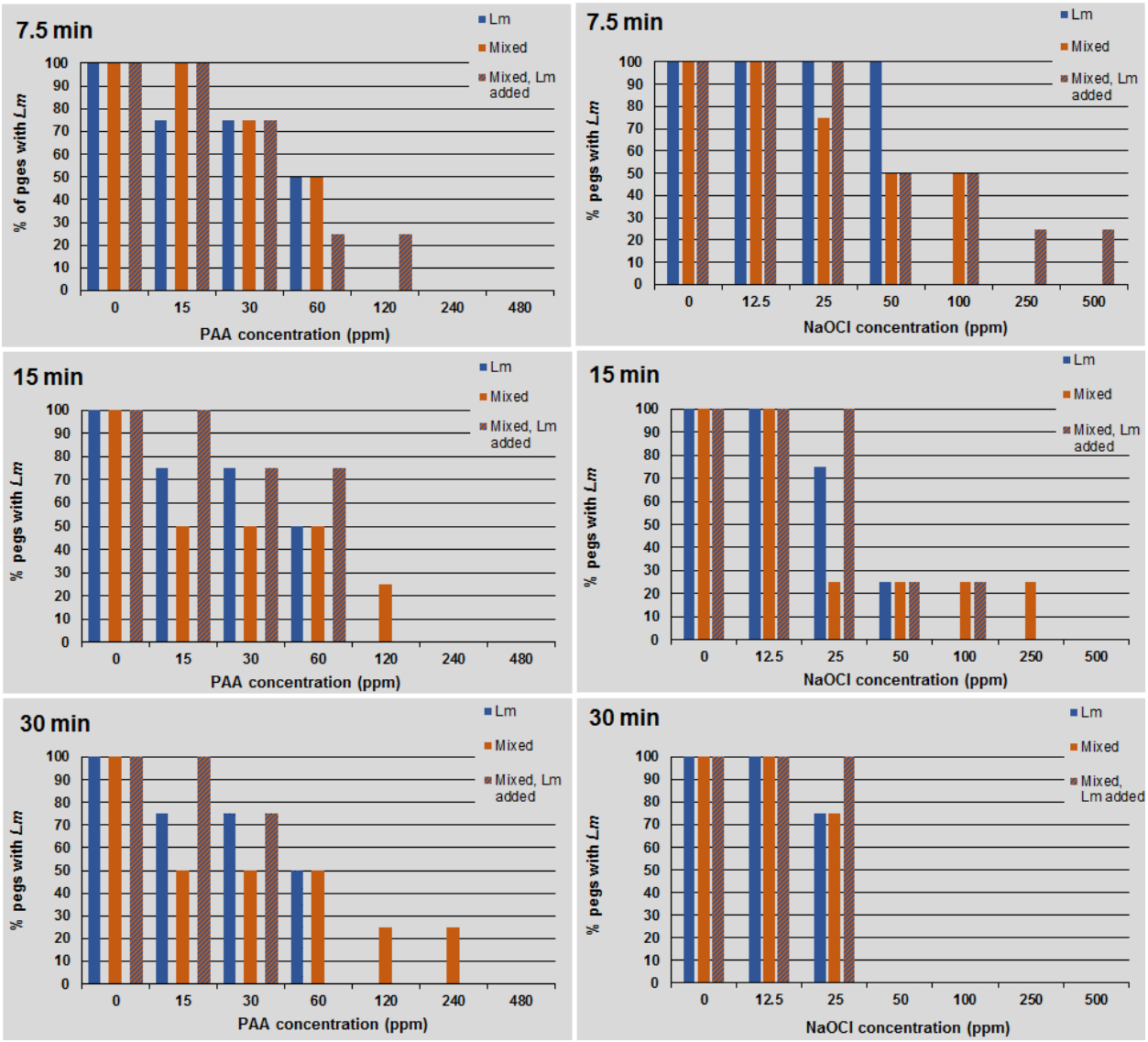


Fig. 20. Percent of pegs in CBDs positive for *Lm* after treatment with different concentrations of **PAA** or **NaOCl** for 7.5, 15, or 30 min. The blue bars indicate results for peg-associated pure *Lm* biofilms, the orange bars are for mixed species, room temperature-grown biofilms and the hatched bars represent results for mixed-species biofilms produced by adding *Lm* to a 2-day-old biofilm of the four produce processing plant isolates. Data are from four independent trials.

8. Effect of NaOCl and quaternary ammonium compounds on Lm *in desiccated biofilms*

Although most produce processing surfaces are likely wet for extended periods of time, periods when the surfaces can dry or are deliberately allowed to dry may exist. Therefore it was was of interest to test what effect antimicrobials would have on *Lm* in dried biofilms compared with hydrated biofilms. Pure *Lm* and mixed-species biofilms were grown at room temperature in R2B or TSBYE medium in microtiter plate wells and allowed to dry. The plates were kept at room temperature in an incubator. The susceptibility to 100 ppm NaOCl and 150 ppm Madisan-75 of *Lm* in these biofilms was assayed prior to initiation of drying, then at 6, 24, 120 and 240 h post drying. As seen in **Fig. 21** and **Fig. 22**, the longest time periods required to inactivate *Lm* completely in the biofilms were generally prior to initiation of drying (0 h). The longer the biofilms were kept in a dry state, the shorter the time period for complete inactivation by the two antimicrobials; however, the data are from two replicate samples only and the extent of the effect of desiccation on antimicrobial susceptibility requires further study.

According to **Fig. 21**, the differences between the times required to inactivate *Lm* in *Lm*-only and mixed-species biofilms by 100 ppm NaOCl were only minor whereas the differences due to the growth medium were more pronounced. In contrast, the differences between the inactivation times for *Lm* from *Lm*-only and mixed-species biofilms were more noticeable. Similarly, *Lm* from the desiccated multi-species biofilms originally grown in TSBYE was less sensitive to the quaternary ammonium compounds than *Lm* from the mixed-species biofilm grown in R2B (**Fig. 22**).

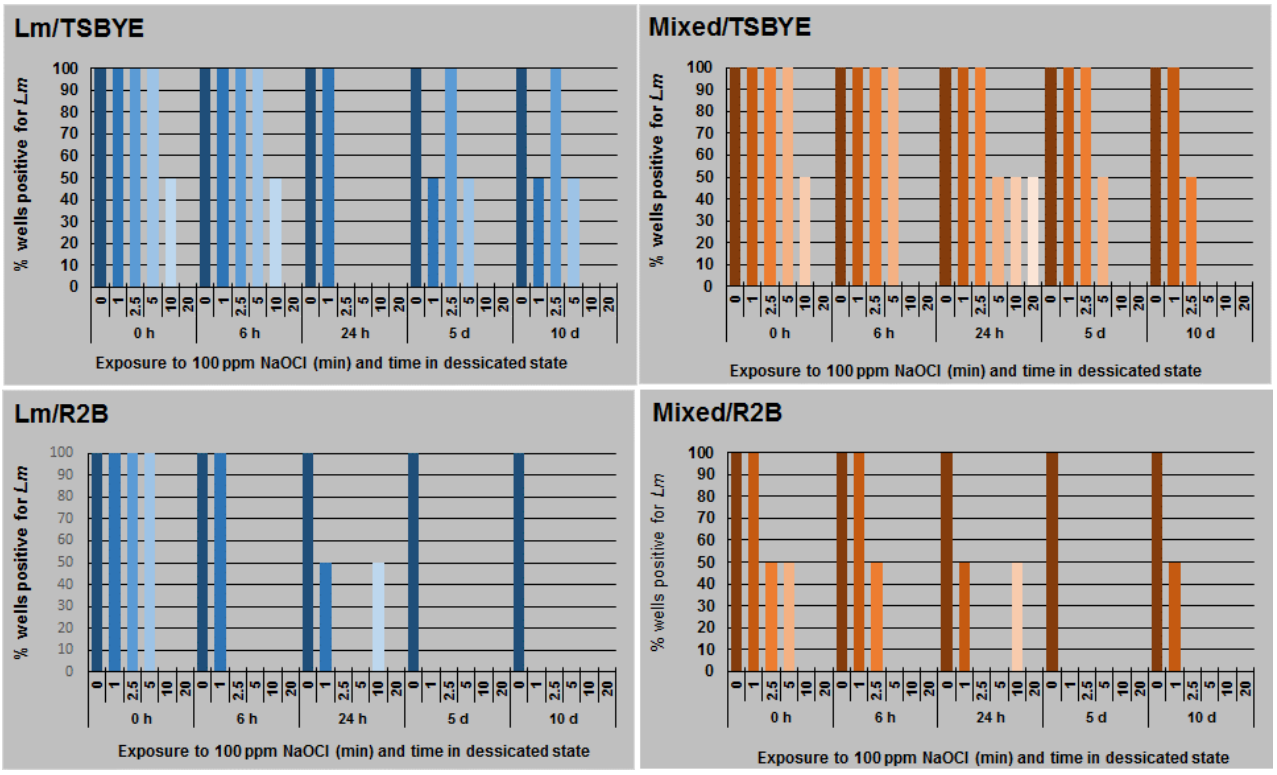


Fig. 21. Percent of wells positive for *Lm* in ***Lm*-only biofilms** (blue markers) and in **mixed-species biofilms** (orange markers) grown in either TSBYE or R2B after exposure to **100 ppm NaOCl** in tap water for 0 to 20 min.

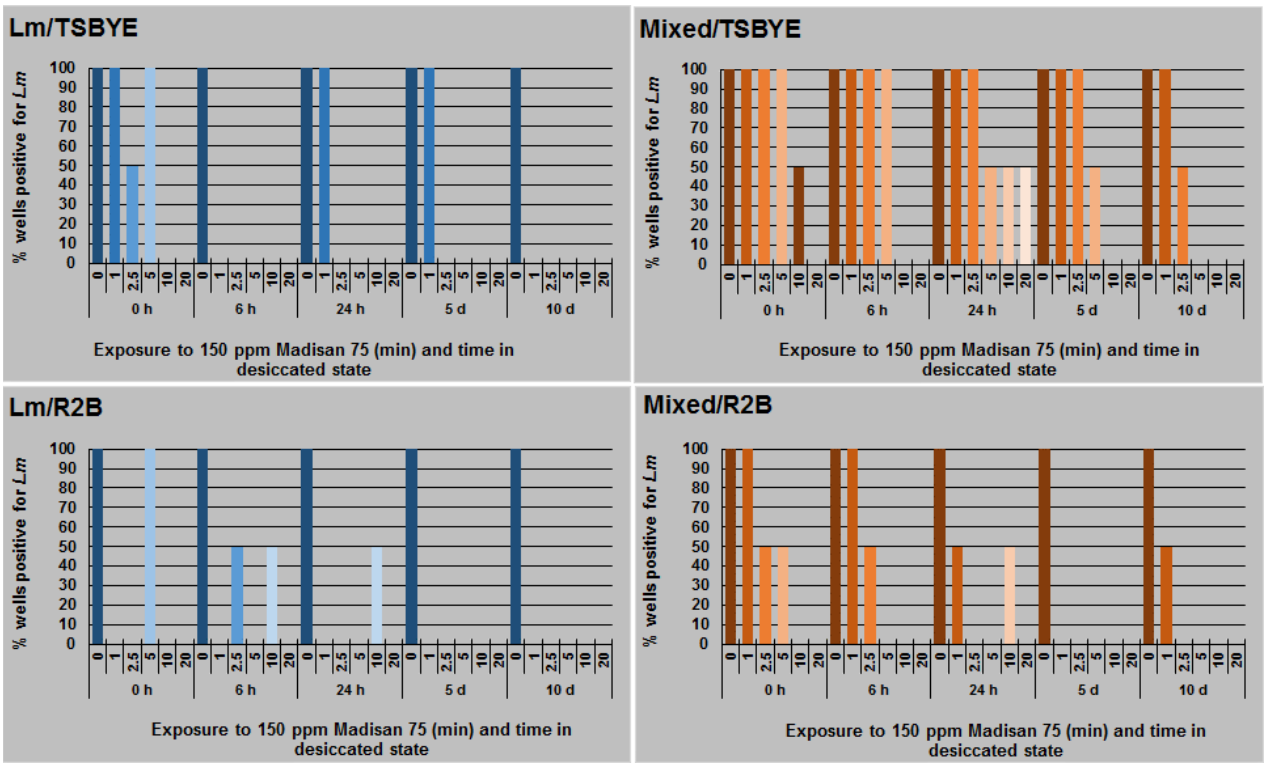


Fig. 22. Percent of wells positive for *Lm* in ***Lm*-only biofilms** (blue markers) and in **mixed-species biofilms** (orange markers) grown in either TSBYE or R2B after exposure to **150 ppm Madisan-75** in tap water for 0 to 20 min.

9. Susceptibility to antimicrobials of Lm *released from biofilms*

9.1 Lm *release characteristics*

The release of cells from biofilms depends on the structure of the biofilm and the mechanical forces exerted on the biofilm. In an experiment with *Lm*-containing biofilms grown in TSBYE or R2B medium at room temperature in microtiter plate wells, *Lm* cells were counted in the wash solutions in the wells. Specifically, the growth medium was removed from the wells and tap water was pipetted into the wells without directly pipetting onto the surfaces with the biofilms. This process was repeated five times, then a sample of the water was plated to obtain counts of *Lm* that were released from the biofilms. The wash process was continued and samples were plated. The results, illustrated in **Fig. 23**, indicate that *Lm* in pure *Lm* biofilms and in mixed-species biofilms is released by this washing procedure at similar numbers over as many as 25 washing steps.

In a second set of experiments, mixed-species biofilms were grown on different surfaces. The coupons with the biofilms were washed with PBS, then transferred to beakers containing sterile tap water. The water was agitated with a magnetic stirrer to simulate water flow over the surfaces of the coupons. *Lm* counts were obtained for the cells remaining on the coupons after the rinsing procedure: for the wash water after 10 and 20 min of agitation, and for the coupons in the agitated water at 10 and 20 min. The data shown in **Fig. 24** indicates that the *Lm* counts on rinsed coupons were similar for each of the four surfaces and high numbers of *Lm* CFUs were released into the wash water after 10 and 20 min. High numbers were also still present on the coupons after agitation for 10 and 20 min.

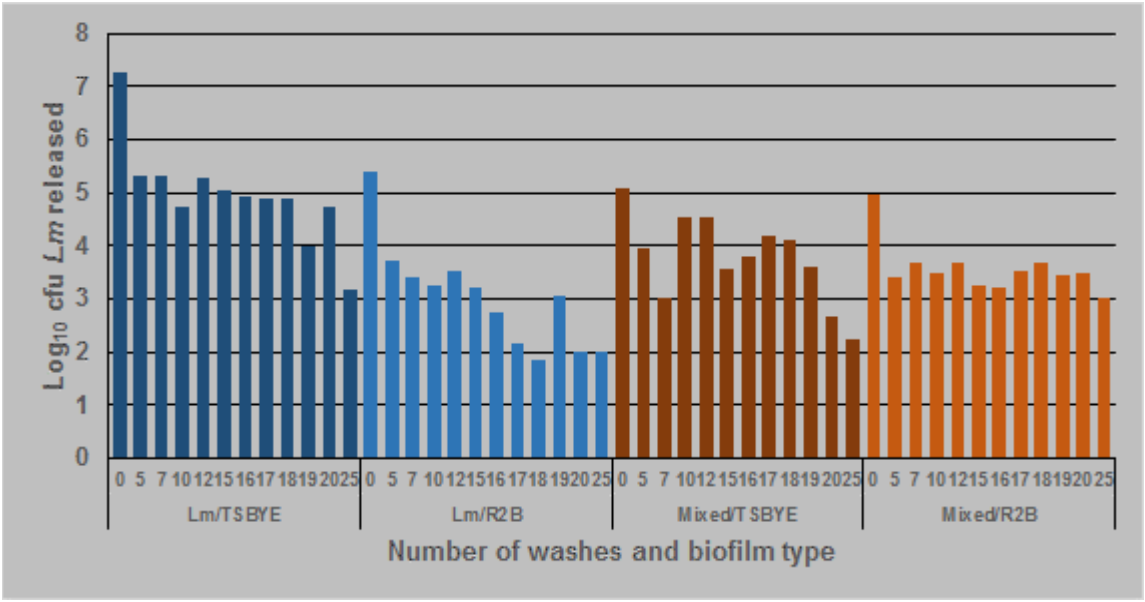


Fig. 23. *Lm* counts in 0.1 ml of wash water in microtiter plate wells after 5 to 25 wash steps. The blue bars represent counts of *Lm* released from pure *Lm* biofilms grown either in TSBYE or in R2B; the orange bars represent the corresponding counts of Lm released from mixed-species biofilms.

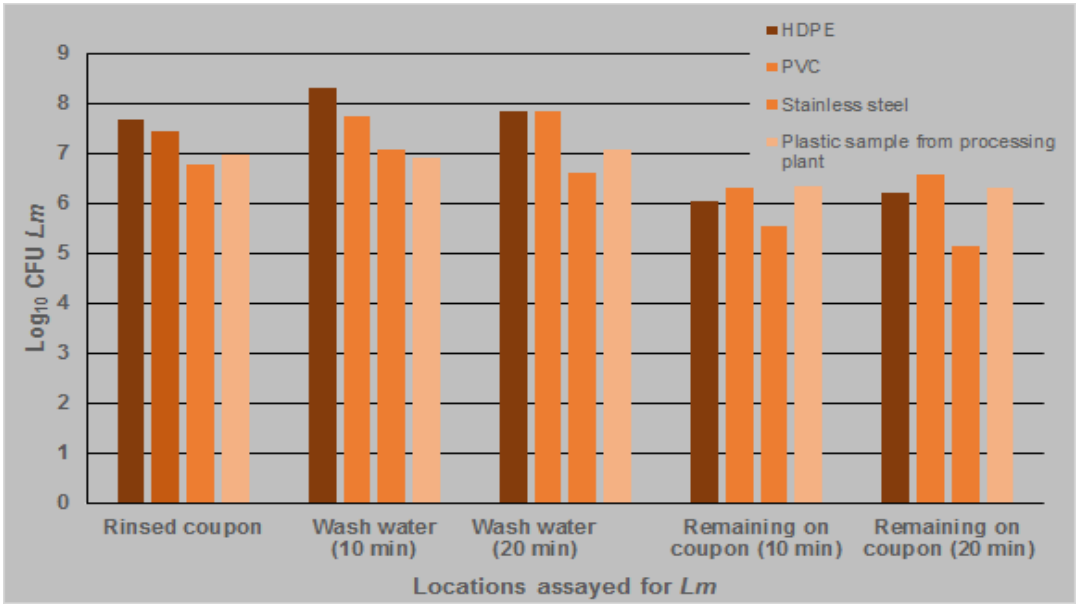


Fig. 24. *Lm* counts on PBS-rinsed coupons (initial counts), *Lm* released into wash water after 10 and 20 min of agitation, and *Lm* remaining on the coupons after 10 and 20 min washes. Data are for *Lm* originating from mixed-species biofilms grown in R2B medium at room temperature on HDPE, PVC, stainless steel or a commercial plastic sample from a produce processing plant.

9.2. Fate of Lm *released from* Lm*-only and mixed-species biofilms in the presence of 100 ppm NaOCl*

Lm-only and mixed-species cells suspensions were prepared as described in section 3. A portion of the cell mixtures was subjected to treatments with 100 ppm NaOCl, and the outcome of the treatment was presented in Fig. 4. *Lm* in the planktonic cell mixtures was completely inactivated in less than 15 seconds.

The cell mixtures were also used to grow biofilms in 6-well microtiter plates in TSBYE or R2B medium. Following biofilm development, the wells were washed five times with PBS. One set of plates was kept stationary, 1 ml of 100 ppm NaOCl was added to each well, and then removed from wells at different time points. The removed solution was replaced immediately with Neutralizing Broth. After 5 min, the wells were filled with LEB and incubated at 37°C. After 48 h, aliquots were spotted onto BLA, and the growth of green colonies indicated the presence of survivors of the antimicrobial treatments. It took stationary treatments lasting from 10 to more than 40 min to completely inactivate *Lm* in the biofilms. As observed before, *Lm* in TSBYE-grown biofilms and mixed-species biofilms was generally more difficult to inactivate than in pure *Lm* and R2B-grown biofilms (**Fig. 25**).

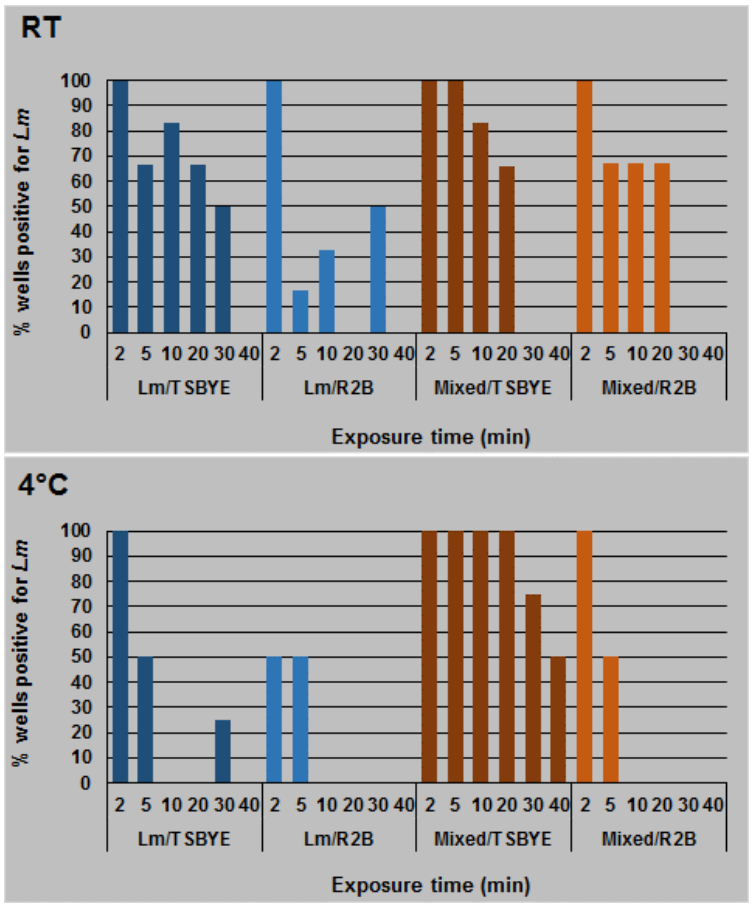


Fig. 25. Percent of wells with *Lm*-only or mixed species biofilms grown in TSBYE or R2B medium at 37 or 4°C and treated with **100 ppm NaOCl** for 2 to 40 min that were positive for *Lm* after enrichment. Blue bars represent results for *Lm*-only biofilms, orange bars show results for the respective mixed-species biofilms.

The other set of plates was used to test the effect of 100 ppm NaOCl on cells that were released from biofilms mechanically. The wells of the plates were washed and 500 µl of sterile tap water was added. Cell release from the biofilms was achieved by brushing the well surfaces with sterile nylon brushes for 30 sec or by shaking the plate on a shaker at 70 rpm. Fifty µl of the resulting cell suspension was used to determine the count of *Lm* released from the biofilms. Fifty µl of 1000 ppm NaOCl solution (adjusted to pH 6.8) was added to the remaining 450 µl of suspension to achieve a NaOCl concentration of 100 ppm. Aliquots were withdrawn at various time points, mixed with a 4-fold volume of Neutralizing Broth, and then plated to determine viable counts. Enrichment was done in LEB.

With two exceptions, the cells released from biofilms were inactivated by treatment with 100 ppm NaOCl for 1 min or less (**Fig. 26**). These data indicate that *Lm* released from biofilms is far more sensitive to the antimicrobial than *Lm* located within biofilms (**Fig. 27**). The sensitivity of *Lm* released from biofilms, especially from *Lm*-only biofilms, is similar to that of planktonic cells since inactivation occurred in most cases within 15 seconds.

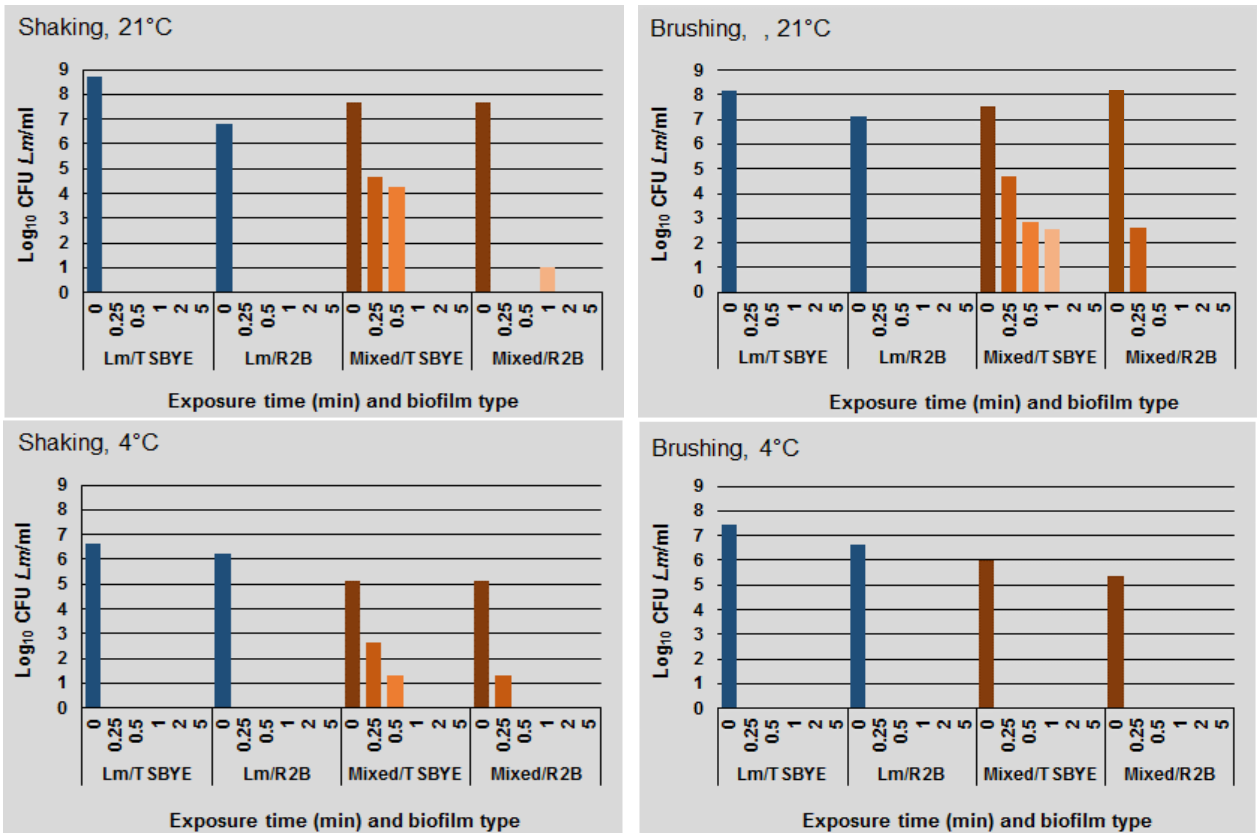


Fig. 26. Counts of ***Lm* released** from *Lm*-only and mixed-species biofilms grown under different conditions and exposed to **100 ppm NaOCl** for 0 to 5 min.

10. Test of transfer of Lm *from biofilms to other surfaces*

10.1. Transfer in CBD system

Lm-only, mixed-species biofilms and biofilms to which *Lm* was added on day two were developed in 96-well microtiter plate wells. The wells were washed, and PAA or NaOCl was added to the wells at various concentrations. The pegs of the CBD system were immediately lowered into the wells and the plates were shaken for 7.5 or 15 min. The pegs were removed, dipped into Neutralizing Broth and then lowered into LEB. After incubation for 48 h, the presence of *Lm* in the wells with the pegs was determined by plating on BLA.

Table 1 lists the concentrations of PAA or NaOCl that prevented the transfer of *Lm* from the biofilms to the pegs under the shaking conditions applied. Transfer was prevented by PAA at concentrations between 7.5 and 120 ppm, and by NaOCl at concentrations between 20 and 50 ppm.

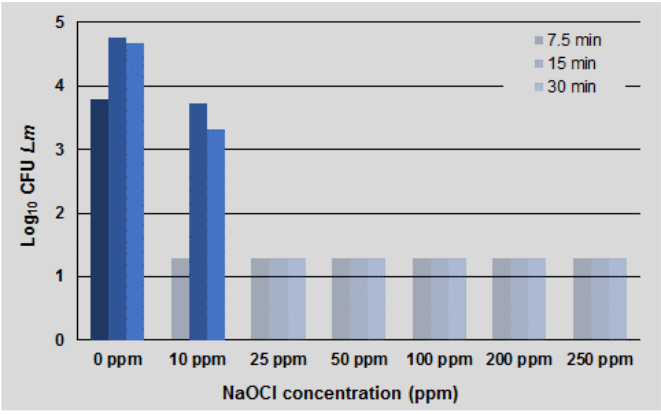
Table 1. Minimum concentrations[1] of antimicrobials needed to prevent transfer of *Lm* from pure and mixed-species biofilms to pegs of the Calgary Biofilm Device during exposure times of 7.5 and 15 min at room temperature.

Biofilm type	PAA concentration (ppm) required at		NaOCl concentration (ppm) required at	
	7.5 min	15 min	7.5 min	15 min
Lm only	15	7.5	25	25
Mixed	30	120	25	50
Lm added[2]	60	60	25	25

[1] Minimum concentration is defined as the concentration at which three replicate pegs did not show the presence of *Lm* after growth in LEB medium and plating on BLA.

[2] The four processing plant isolates were allowed to form biofilms for 48 h, then *Lm* was added, allowed to attach for 4 h, then the medium was replaced with fresh R2B medium and biofilm development was allowed to continue for another 72 h with daily medium changes.

The results of a quantitative assay are shown in **Fig. 27** and **Fig. 28**. In this assay, *Lm*-only or mixed-species biofilms grown at room temperature in microtiter plate wells in the presence of R2B medium were used as a source of cells that could be transferred to the pegs of the CBD device during shaking. NaOCl concentrations of 25 ppm or less were able to prevent the transfer of *Lm* from the biofilms to the pegs (**Fig. 27**). Similarly, low concentrations of PAA were able to prevent transfer of *Lm* from biofilms to the pegs (**Fig. 28**).



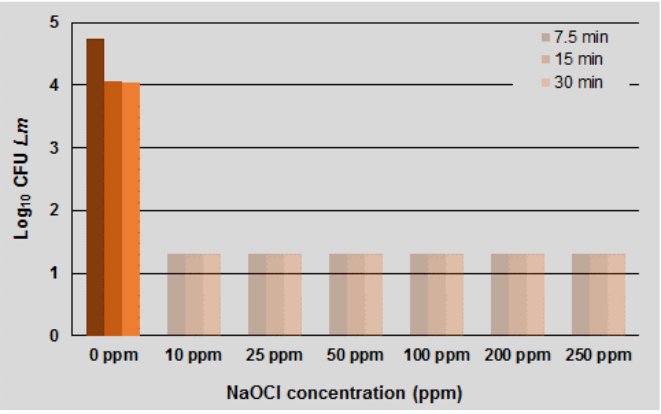
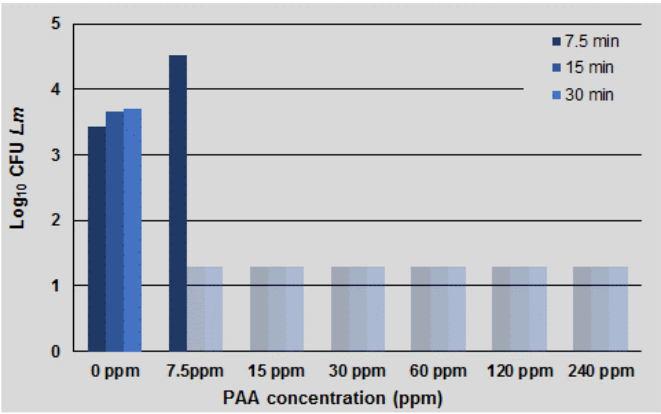


Fig. 27. *Lm* transfer from the surfaces of microtiter plate well to sterile pegs in the CBD in the presence of **0 – 250 ppm NaOCl**. Treatments and biofilm growth were done at room temperature. Blue bars, transfer from *Lm*-only biofilms; orange bars, transfer from mixed-species biofilms. Translucent bars indicate the detection limit for this assay.



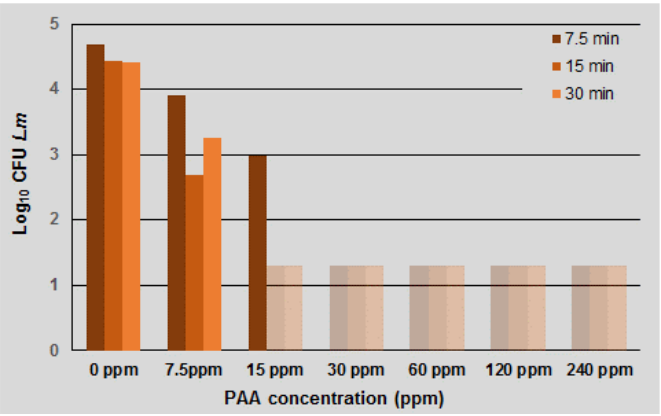


Fig. 28. *Lm* transfer from the surfaces of microtiter plate well to sterile pegs in the CBD in the presence of **0 – 240 ppm PAA**. Treatments and biofilm growth were done at room temperature. Blue bars, transfer from *Lm*-only biofilms, orange bars, transfer from mixed-species biofilms.

10.2. Transfer of Lm *from biofilms on stainless steel coupons to blueberries*

In this experiment, mixed-species biofilms were grown on stainless steel coupons, washed and then submerged in a beaker with sterile tap water. After 1 min of stirring with a magnetic stirrer, samples were removed to determine the number of cells released (0 min wash in **Fig. 29**). Subsequently, NaOCl solutions were added to achieve initial levels of 10, 20, 50 and 100 ppm, and 30 blueberries were placed in each beaker. Stirring was continued and samples of the wash solutions were removed at 5, 10, 20 and 30 min, mixed with 4 volumes of Neutralizing Broth and plated to detect viable *Lm*. Five blueberries were also removed each time point and immediately immersed in 5 ml of Neutralizing Broth. LEB was added and the berries in the LEB were incubated for 48 h, and samples were spotted onto BLA to determine the presence of *Lm*. After 30 min in the stirred wash solutions, the coupons were retrieved and immediately covered with Neutralizing Broth. Dilutions were plated and enrichment in LEB was done.

None of the NaOCl-containing solutions tested had *Lm* counts above the detection limit. Except for the no-NaOCl control and the treatment with 10 ppm NaOCl, *Lm* could not be detected on the coupons after 30 min. Transfer of *Lm* to blueberries was only observed in the water-only wash, the NaOCl-containing wash solutions prevented transfer.

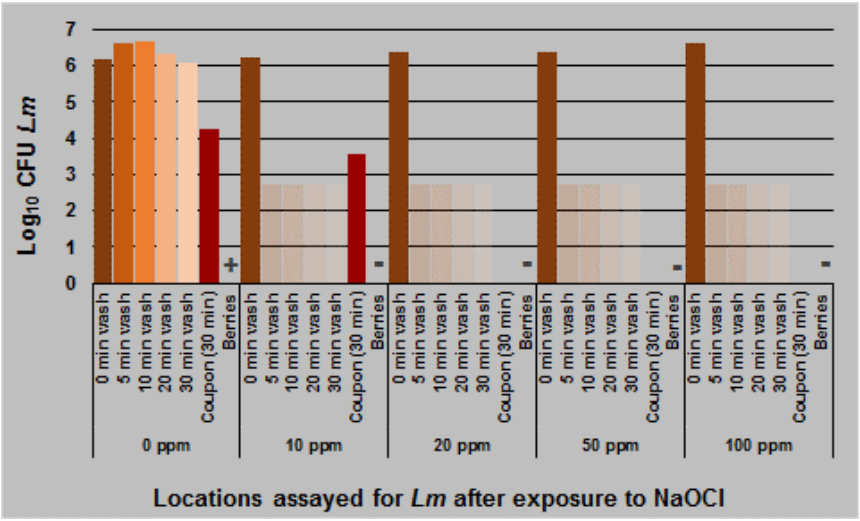


Fig. 29. *Lm* counts in wash water containing **0** – **100 ppm NaOCl** at various times (orange bars) and on stainless steel coupons after 30 min in the respective wash solutions (red bars). Mixed species biofilms were grown in R2B medium on stainless steel coupons for 5 days at room temperature. Transfer from the surfaces of stainless steel coupons to blueberries is indicated by a "+" sign, no transfer by a "–" sign.

10.3. Transfer of Lm *from biofilms on stainless steel and plastic coupons to blueberries*

In another set of experiments, mixed-species biofilms were grown at room temperature on plastic and stainless steel coupons in the presence of R2B medium. The coupons were washed and added to beakers containing sterile tap water. The addition of antimicrobials and blueberries was done as described in section 10.2; however, in this case, the antimicrobials used were NaOCl (100 ppm), PAA (100 ppm), Madisan-75 (150 ppm) and Safe-Zone LOD (700 ORP). Sampling times were 10 and 20 min.

Lm counts were not obtained for any of the samples; however, as seen in **Table 2**, *Lm* was detected after enrichment in LEB medium in a number of wash water samples at sampling times of 10 and 20 min. Only one coupon tested positive for *Lm* after 20 min in the wash solution. *Lm* was only transferred from the biofilms on coupons in two cases at 10 min. No transfer was observed after 20 min in the wash solutions.

Table 2. Presence/absence of *Lm* in wash solutions, on coupons and on blueberries after exposure of coupons carrying mixed-species biofilms and blueberries to antimicrobial wash solutions under stirring conditions.

Antimicrobial	Surface with mixed-species biofilm	*Lm* present in wash solution after		*Lm* present on coupons after 20 min in wash solution	*Lm* transferred to blueberries after	
		10 min	20 min		10 min	20 min
NaOCl (100 ppm)	HDPE	No	Yes	No	No	No
	PVC	Yes	Yes	No	No	No
	Stainless steel	Yes	Yes	No	No	No
	Comm. cutting surface	Yes	No	No	No	No
PAA (100 ppm)	HDPE	Yes	Yes	No	No	No
	PVC	Yes	Yes	No	No	No
	Stainless steel	Yes	Yes	No	Yes	No
	Comm. surface	Yes	Yes	No	No	No
Madisan-75 (150 ppm)	HDPE	Yes	No	No	Yes	No
	PVC	Yes	No	No	No	No
	Stainless steel	No	No	No	No	No
	Comm. surface	No	No	No	No	No
Safe-Zone LOD (700 ORP)	HDPE	Yes	Yes	Yes	No	No
	PVC	Yes	Yes	No	No	No
	Stainless steel	Yes	No	No	No	No
	Comm. cutting surface	Yes	Yes	No	No	No

10.4. Transfer of Lm *from soil particles to mung beans in the presence of NaOCl or PAA*

Soil particles with *Lm* and the four isolates from a produce processing were obtained as described in section 4. After the soil was washed, tap water was added and the plates with the soil were agitated on a rotary shaker for 1 min. Aliquots of the suspensions were removed to obtain counts of *Lm* released from the washed particles. NaOCl or PAA was added to achieve initial concentrations of 20, 100 or 250 ppm. Nine mung beans were added and shaking was continued for 5, 10 and 20 min. (Mung beans were used for this set of experiments as it was possible to utilize them in the wells of 6-well microtiter plates and because they have both smooth and rough surface structures.) At these time points, three beans and an aliquot of the wash solution were removed from the wells for tests of the presence of *Lm*.

As illustrated in **Fig. 30**, *Lm* could be detected in all wash solutions at each time point and in the soil exposed to the antimicrobial solutions for 20 min. Transfer of *Lm* to beans occurred in this experimental set-up even at the highest initial NaOCl concentration (**Fig. 31**).

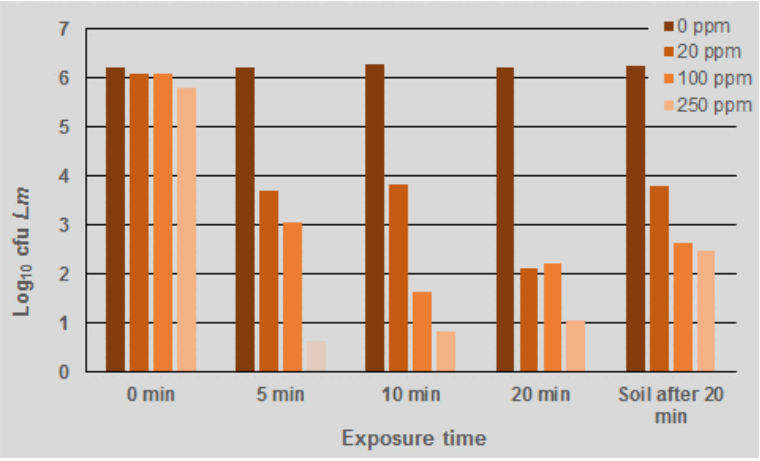


Fig. 30. Soil-derived *Lm* counts in **wash water** containing 0 – 250 ppm **NaOCl** at 0, 5, 10 and 20 min and viable counts in soil after 20 min in wash water. Counts are per ml of wash solution and are averages from two independent trials.
The transparent bar at 5 min/250 ppm indicates that the count was at or below this value as *Lm* was only detectable after enrichment.

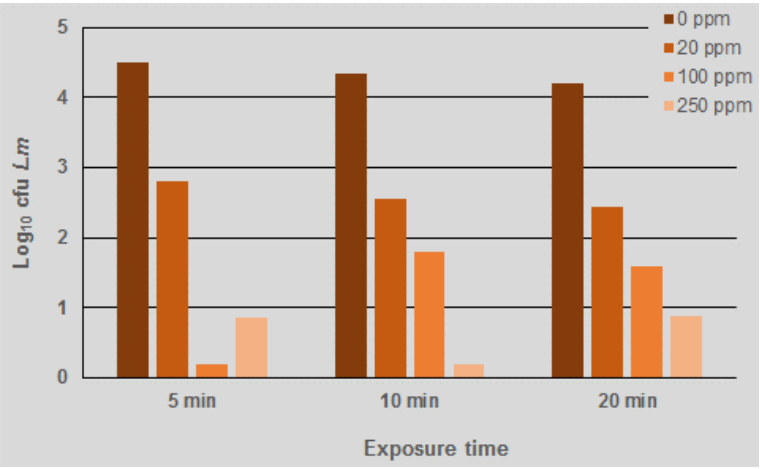


Fig. 31. *Lm* counts on **mung beans** agitated in soil-containing wash water with an initial concentration of 0 – 250 ppm **NaOCl**. Data are averages from two independent trials and counts are expressed as cfu per cm^2 of bean surface.

Initial PAA concentrations of 100 and 250 ppm were sufficient to inactivate *Lm* released from the soil particles as *Lm* could not be detected even after enrichment (**Fig. 32**); however, low counts of *Lm* were observed on the beans, even at the high concentrations (**Fig. 33**). It is conceivable that the soil matrix, even after five washes, contained sufficient organic materials that together with the surfaces of the beans could have reduced the effective concentrations of the antimicrobials, thus ensuring survival and transfer of *Lm*. Shielding of *Lm* associated with soil particles from the antimicrobials in crevices or other structures could also have contributed to the ineffectiveness of the treatments.

Further studies on the fate of *Lm* associated with soil particles will need to be carried out to determine conditions required to prevent transfer of *Lm* from soil particels or on soil particles to produce or other surfaces.

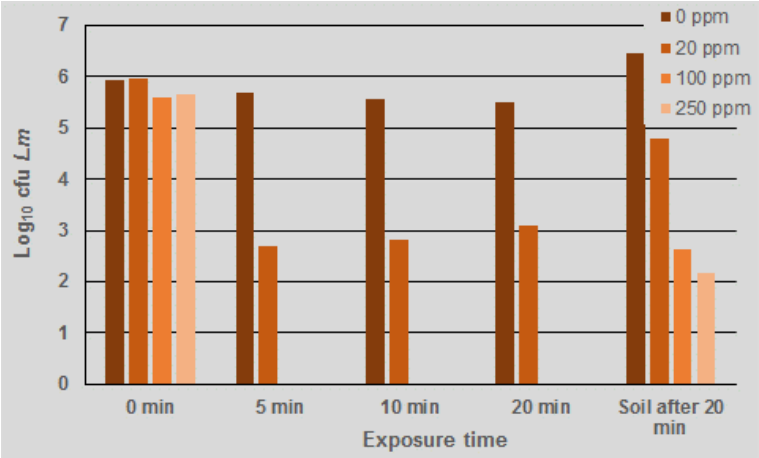


Fig. 32. Soil-derived *Lm* counts in wash water containing 0 – 250 ppm **PAA** at 0, 5, 10 and 20 min and viable counts in soil after 20 min in wash water. Counts are per ml of wash solution and are averages from two independent trials.

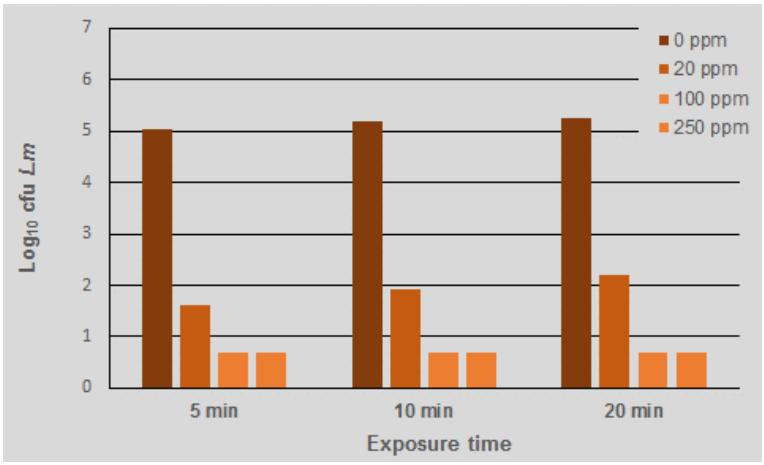


Fig. 33. *Lm* counts on **mung beans** agitated in soil-containing wash water with an initial concentration of 0 – 250 ppm **PAA**. Data are averages from two independent trials and counts are expressed as cfu per cm^2 of bean surface.

Outcomes and Accomplishments

- Bacterial strains were isolated from produce processing plants operating at ambient and refrigeration temperatures, identified to the genus level by genetic means and tested for their utility in establishing multi-species biofilms with *Lm* strains.
- Susceptibility of *Lm* in *Lm*-only biofilms and in mixed-species biofilms to sodium hypochlorite (NaOCl), peracetic acid (PAA), quaternary ammonium compounds and alkaline sodium chloride was studied. Overall, the concentrations of antimicrobials needed to completely inactivate *Lm* attached to surfaces was more than 10 times that required for inactivation of *Lm* in a planktonic state.
- *Lm* in mixed-species biofilms enjoys a higher level of protection from antimicrobials than *Lm* in *Lm*-only biofilms. When data from all applicable experiments were combined, it took 1.2 times the concentration of NaOCl to completely inactivate *Lm* from multi-species biofilms than from *Lm*-only biofilms, at the same exposure times; the corresponding value for PAA was 1.9. *Lm* interacting with existing non-*Lm* biofilms also showed increased resistance to antimicrobials.
- *Lm*-only and mixed-species biofilms can develop in low-nutrient medium (R2B) as well as in produce-derived media and in high-nutrient medium (TSBYE). Considering all relevant data from the study, it took 2.8 times as long to completely inactivate *Lm* from TSBYE-grown *Lm*-only biofilms than from corresponding biofilms grown in R2B. It took 4 times as long to inactivate *Lm* from mixed-species biofilms grown in TSBYE than *Lm* from biofilms grown in R2B. This media effect is likely due to a combination of higher cell counts in the TSBYE-grown biofilms and possibly higher production of protective exopolymeric substances.
- Desiccation seems to make *Lm* in biofilms more susceptible to antimicrobials. The extent of the increase in susceptibility remains to be explored further.
- *Lm* from both *Lm*-only and mixed-species biofilms was released continuously into the surrounding medium, even after multiple washes. It appears that *Lm* can adhere to different surfaces but does not form biofilms that strongly bind cells to each other or to an abiotic surface; however, since comparable wash experiments were not done with non-*Lm* strains, it is not possible to state at this time if the release rates are different from those of other bacterial biofilms.
- Although *Lm* in *Lm*-only and mixed-species biofilms is considerably more resistant to antimicrobials than cells in a planktonic state, cells released from such biofilms are nearly as susceptible or as susceptible to the antimicrobials as cells grown in a planktonic state.
- Transfer of *Lm* from *Lm*-only and mixed-species biofilms to new sites was prevented by antimicrobials at concentrations that are lower than those generally used for produce washes or surface cleaning.
- Inactivation of *Lm* associated with soil particles required concentrations of NaOCl and PAA that were close to or even exceeded concentrations generally in use. Transfer of *Lm* from soil particles to test objects (e.g., mung beans) was observed even in the presence of an initial NaOCl concentration of 250 ppm or PAA. The susceptibility of soil-associated *Lm* to antimicrobials should be studied further.

Summary of Findings and Recommendations

The data collected in this study indicate that *Listeria monocytogenes* in biofilms cannot be reliably inactivated by antimicrobials at concentrations usually found in produce wash water. This observation was made when the wash medium was essentially free of organic compounds, except for those released by the biofilms. Therefore, it can be expected that the required concentrations in systems with organic loads will be even higher. Despite the observed survival of *Lm* in biofilms in the presence of relatively high concentrations of antimicrobials, *Lm* released from such biofilms did not show the same level of resistance as those associated with a surface. *Lm* appears to be released from biofilms in a state that makes them susceptible to inactivation by low concentrations of antimicrobials.

The data stress the need to prevent the formation of biofilms as much as possible to prevent the creation of reservoirs for *Lm* in a facility; however, the data also indicate that as long as recommended concentrations of antimicrobials are maintained in wash water, it should be possible to prevent *Lm* from reaching existing biofilms or to transfer to produce being washed. *Lm* associated with soil appears to be difficult to inactivate, but further studies need to be done with different soils and soil levels to determine concentrations of antimicrobials that are effective against *Lm* associated with soil particles.

References

Aarnisalo K, Salo S, Miettinen H, Suihko M-L, Wirtanen G, Autio T, Lunden J, Korkeala H, Sjoberg A-M. 2000. Bactericidal efficiencies of commercial disinfectants against *Listeria monocyotgenes* on surfaces. J Food Safety 20:237-250.

Carballo J, Araujo A-B. 2012. Evaluation of the efficacy of commercial sanitizers against adhered and planktonic cells of *Listeria monocytogenes* and *Salmonella* spp. Cienc Tecnol Aliment 32:606-612.

Cruz CD, Fletcher GC. 2012. Assessing manufacturers' recommended concentrations of commercial sanitizers on inactivation of *Listeria monocytogenes*. Food Control 26:194-199.

FDA. 2018. 2017 Recalls, Market Withdrawals & Safety Alerts. https://www.fda.gov/Safety/Recalls/ArchiveRecalls/2017/default.htm. Accessed Jan 23, 2018.

Fagerlund A, Møretrø T, Heir E, Briandet R, Langsrud S. 2017. Cleaning and disinfection of biofilms composed of *Listeria monocytogenes* and background microbiota from meat processing surfaces. Appl Environ Microbiol 83:e01046-17. https://doi.org/10.1128/AEM.01046-17.

Fatemi P, Frank JF. 1999. Inactivation of *Listeria monocytogenes*/*Pseudomonas* biofilms by peracetic acid. J Food Prot 62:761-765.

Ferreira V, Wiedmann M, Teixeira P and Stasiewics MJ. 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J Food Prot 77:150-170.

Goodridge L. 2014. Case Study: Foodborne Outbreak (Cantaloupe) https://arssymposium.absa.org/wp-content/uploads/2014/06/2013_USDA_ARS_I-Goodridge.pdf.

Hoelzer K, Pouillot R, van Doren JM, Dennis S. 2014. Reduction of *Listeria monocytogenes* contamination on produce – A quantitative analysis of common liquid fresh produce wash compounds. Food Control 46:430-440.

Kostaki M, Chorianopoulos N, Braxou E, Nychas G-J, Giaouris E. 2012. Differntial biofilm formation and chemical disinfection resistance of sessile cells of *Listeria monocytogenes* strains under monospecies and dual-species (with *Salmonella enterica*) conditions. Appl Environ Microbiol 78:2586-2595.

Lourenco A, Machado H, Brito L. 2011. Biofilms of *Listeria monocytogenes* at 12 °C either in pure culture or in co-culture with *Pseudomonas aeruginosa* showed reduced susceptibility to sanitizers. J Food Sci 76:M143-M148.

McLaughlin HP, Casey PG, Cotter J, Gahan CG, Hill C. 2011. Factors affecting survival of *Listeria monocytogenes* and *Listeria innocua* in soil samples. Arch Microbiol 193(11):775-85. doi: 10.1007/s00203-011-0716-7.

Norwood DE, Gilmour A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. J Appl Microbiol 88:512-520.

Olmez H, Temur SD. 2010. Effects of different sanitizing treatments on biofilms and attachment of *Escherichia coli* and *Listeria monocytogenes* on green leaf lettuce. LWT-Food Sci Technol 43:964-970.

Olszewska M, Zhao T, Doyle MP. 2016. Inactivation and induction of sublethal injury of Listeria monocytogenes in biofilm treated with various sanitizers. Food Control 70:371-379.

Robbins JB, Fisher CW, Moltz AG, Martin SE. 2005. Elimination of *Listeria monocytogenes* biofilms by ozone, chlorine, and hydrogen peroxide. J Food Prot 68:494-498.

Rodrigues D, Cerca N, Teixeira P, Oliveira R, Ceri H, Azeredo J. 2011. *Listeria monocytogenes* and *Salmonella enterica* Enteritidis biofilms susceptibility to different disinfectants and stress-response and virulence gene expression of surviving cells. Microbial Drug Resistance 17:181-189.

Srey S, Park SY, Jahid IK, Oh S-R, Han N, Zhang C-Y, Kim S-H, Cho J-I, Ha S-D. 2014, Evaluation of the removal and destruction effect of a chlorine and thiamine dilaurylsulfate combined treatment on *L. monocytogenes* biofilm. Foodborne Path Dis 11:658-663.

Vaid R, Linton RH, Morgan MT. 2010. Comparison of inactivation of Listeria monocytogenes within a biofilm matrix using chlorine dioxide gas, aqueous chlorine dioxide and sodium hypochlorite treatments. Food Microbiol 27:979-984.

APPENDICES

Publications and Presentations

- Manuscripts will be submitted to appropriate journals after completion of the research by the graduate student involved in the project.

- The PI presented interim results at the CPS Research Symposium in 2016 (Lightning Round and poster) and in 2017.

Acknowledgment

Part of the experiments presented in this report were carried out by Juliet Wachira and Quincy Hardy in partial fulfillment of the requirements for a Master of Science degree. Some of the other experiments were carried out by Arpeeta Ganguly, Ph.D., a visiting scientist.

Budget Summary

	Budgeted	Spent
Total personnel costs (including graduate student salaries)	49,890	46,762
Travel	7,086	3,468
Supplies	50,077	*35,615
Overhead	2,495	2,338
Total	109,548	88,183

*The money for supplies was originally budgeted for graduate students working on the project for a full two years. Since the graduate students did not join our lab until 9 months after the start of the project term, only limited funds were spent on supplies during these first months.