Project Title: Control of *Listeria* on processing surfaces in apple packing facilities

Report Type: Final Project Report

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Cooperators: Stemilt Growers LLC.; McDougall & Sons; Hansen Fruit; Washington Fruit; Allan Bros Fruit; Pace International; Guardian Manufacturing, Inc.

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $98,447
Total Project Request for Year 2 Funding: $101,752
Total Project Request for Year 3 Funding: $105,882

WTFRC Collaborative Costs:

Item	2017	2018	2019
Salaries	1,573	2,172	2,172
Benefits	1,049	1,305	1,305
Wages	2,750	2,750	2,750
Benefits	825	825	825
Total	6,197	7,052	7,052

Budget 1
Primary PI: Meijun Zhu
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Contract Administrator: Anastasia Kailyn Mondy
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Contract administrator email address: arcgrants@wsu.edu

Item	2017	2018	2019
Salaries	13,562	19,889	20,685
Benefits	4,386	6,094	6,338
Wages	38,054	30,773	32,003
Benefits	3,248	3,300	3,432
Supplies	26,000	26,644	27,872
Travel	2,000	3,000	3,500
Miscellaneous	5,000	5,000	5,000
Total	92,250	94,700	98,830

OBJECTIVES

1. Assess antimicrobial efficacies of different commonly used chemical sanitizers against *L. monocytogenes* biofilm on the main food-contact surfaces.
2. Examine antimicrobial efficacies of steam against *Listeria* biofilm on different food-contact surfaces.
3. Evaluate the antimicrobial efficacies of steam in combination with the selected sanitizer against biofilm on the common food-contact surface using optimized parameters.

SIGNIFICANT FINDINGS

1. Efficacies of all tested sanitizers against aged (7-day-old) *Listeria* biofilm were reduced when compared to 2-day-old biofilm.
2. In general, efficacies against *L. monocytogenes* (*Lm*) biofilms on food-contact surfaces including stainless steel (SS), low-density polyethylene (LDPE), polyvinyl chloride (PVC), polyester (PET), and rubber were enhanced by increasing concentrations of quaternary ammonium compound (QAC), chlorine, and chlorine dioxide, or extending treatment time from 1 min to 5 min.
3. A 5 min treatment of 400 ppm QAC, 5.0 ppm chlorine dioxide, or 200 ppm chlorine reduced 3.0-3.7, 2.4-2.7, and 2.6-3.8 $\log_{10}$ CFU/coupon *Lm* biofilms depending on surfaces.
4. Peroxyacetic acid (PAA) at 160 - 200 ppm and 1-5 min contact showed similar antimicrobial efficacies against *Lm* biofilms on all tested food-contact surfaces, causing a 4.0-4.6 $\log_{10}$ CFU/coupon reduction of *Lm* biofilms on tested surfaces.
5. The cell counts of *Lm* biofilm on SS were not impacted by finish type and wear degree of SS surface.
6. *Lm* counts on worn non-SS surfaces (LDPE, PET, PVC, and rubber) were significantly higher than that on new ones
7. Abrasion on surfaces reduced the efficacies of chlorine, QAC, and PAA against *Lm* biofilm
8. Saturated steam caused a rapid kill of *L. innocua* biofilms on food contact surfaces. A 6-sec steam treatment attained a 2.4 - 3.2 $\log_{10}$ CFU/coupon reduction depending on the type of surface.
9. Saturated steam was more effective against *Listeria* biofilms on stainless steel surfaces than those on PET and rubber surfaces.
10. The effectiveness of both saturated and superheated steam in eliminating *L. innocua* biofilms decreased dramatically during prolonged steam treatment.
11. Organic matter soiling, regardless of sources, impaired sanitizer efficacies against *L. monocytogenes* biofilms independent of food-contact surfaces (new or worn) but did not negatively impact the efficacy of steam against *Listeria* biofilm on different surfaces.
12. Saturated steam exposure had no impact on the hydrophobicity and surface roughness of SS, PET, and rubber surfaces.
13. PAA at 40 ppm in combination with 6-sec saturated steam exposure provided > 6 log reduction of *L. innocua* biofilm on SS and PET surfaces.
14. PAA at 80 ppm and 6-sec saturated steam hurdle intervention resulted in ~ 5 log reduction on the rubber surface.
15. The efficacy of PAA and steam hurdle treatments was not impacted by the treatment order.
16. Organic soiling and/or surface defects, regardless of surface type, reduced the effectiveness of PAA and steam hurdle treatment in removing *Listeria* biofilm on surfaces.
17. Data on sanitizer interventions and saturated steam treatment have been published (Hua et al., 2019; Hua et al., 2021; Korany et al., 2018).

METHODS

Objective 1: Assess the antimicrobial efficacies of commonly used chemical sanitizers against *L. monocytogenes* biofilm on the main food-contact surfaces.

1. Strain selection

To elucidate the impact of strain variability on biofilm formation and sanitizer's antimicrobial efficacy, six strains of *Lm* were evaluated. These *Lm* strains were either outbreak strains or processing plant/food isolates. They have been stored at -80°C until used.

2. Selection and preparation of food-contact surfaces

Surface: SS, PVC, PET, LDPE and rubber along with polyester were selected.

Organic matter conditioning: The above surfaces were cleaned and exposed with diluted apple juice before being subjected to *Listeria* biofilm growth and sanitizer treatments.

3. *Listeria* biofilm formation on different surface materials

Inoculum preparation: Before inoculation, respective strains were twice activated in Tryptic Soy broth (TSB) with yeast extract (TSBYE), washed, and re-suspended in nutrient broth to achieve the target population density.

Biofilm formation on different surfaces: All surface coupons (conditioned with/without organic matter) were transferred to 6- strain *Listeria* suspension in culture media prepared as described above and incubated at room temperature (22°C/72°F) for 2 or 7 days statically to form biofilm.

4. Sanitizer intervention against *Listeria* biofilm on different surfaces.

Wells of polystyrene plates or coupons of the selected surface-bearing *Listeria* biofilm cells were rinsed with sterile distilled water, then subjected to respective sanitizer treatments (2.0/4.0 ppm ozonated water, 200/400 ppm quaternary ammonium compound (QAC), 100/200ppm chlorine, 2.0/5.0 ppm chlorine dioxide or 160/200ppm peroxyacetic acid (PAA)) at appropriate concentrations for 1- or 5-min. Untreated control wells with biofilm were subjected to distilled water instead of sanitizer solution treatments.

5. Microbiological analysis.

The biofilm on respective surfaces was detached from the surface per our established method. The detached cell suspensions were serially diluted in sterile PBS and plated in duplicate Tryptic Soy Agar (TSA) with yeast extract (TSAYE) agar plates. Colonies that had formed on the plates were counted after 48 h of incubation at 37°C (98°F).

Objective 2: Examine antimicrobial efficacies of steam against *Lm* biofilm on different food-contact surfaces

1. Strain selection

Three *L. innocua* isolates from produce packing facility/ processing plants were used to prepare a 3-strain cocktail of *Listeria* inoculum per our well-established method.

2. Food-contact surface selection and biofilm formation

The surface selection and biofilm formation were the same as in the objective 1 studies.

3. Steam generator and temperature monitoring

The steam generator was located at the Washington State University pilot plant due to power requirements. A stainless-steel chamber with three steam pipes and 25 steam nozzles was used to treat *L. innocua* biofilms formed on different food-contact surfaces. The temperature profile of food-contact coupons inside the steam brancher was monitored using a T-type self-adhesive thermocouple

(OMEGA, Norwalk, USA). Three-wire thermocouples were used to monitor the temperature profiles of steam at three different sites of the chamber (Fig. 8AB).

4. Steam intervention

The 7-day-old *L. innocua* biofilms on food-contact surfaces were treated with steam for 0-180 seconds. The treated surface coupons were immediately transferred to 50 ml Falcon tubes containing 2 ml sterile PBS immediately after treatments.

5. Microbiological analysis

It was conducted as described in Objective 1.

Objective 3: Evaluate the efficacies of steam in combination with the selected sanitizer against biofilm on food-contact surfaces using optimized parameters.

Methods developed in Objectives 1 and 2 are used for Objective 3 studies. The outcomes of Objective 1 & 2 studies guide the standardization of sanitizer concentrations in relation to residence times.

RESULTS AND DISCUSSION

Objective 1. Assess antimicrobial efficacies of different commonly used chemical sanitizers against L. monocytogenes biofilm on the main food-contact surfaces.

1. Impact of the age of biofilm on the efficacies of selected sanitizers against L. monocytogenes biofilm on polyester surface

We first compared the biofilm formation ability among the six *Lm* strains. There was no clear link between biofilm formation and the serotype of the selected strains (Fig. 1). NRRL B-33385, a 4b human clinic isolate had the lowest population density in the biofilm, while the *Lm* environmental isolate (NRRL B-33466) showed the highest biofilm forming ability among all strains tested (Fig. 1).

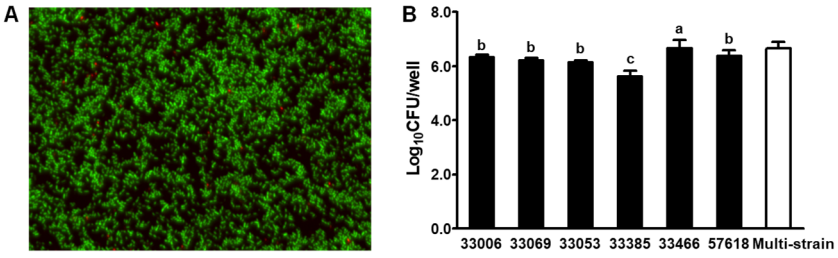


Figure 1. Biofilm forming ability of different *L. monocytogenes* strains on Polystyrene surface. A: BacLight Live/Dead staining B: *Lm* counts. Mean ± SEM. Bars topped with same letter are not different at $P < 0.05$.

Antimicrobial efficacies of all sanitizers except PAA against mixed strain *Lm* biofilm were reduced when compared to single strain *Lm* biofilm (data not shown). Antimicrobial efficacies of a sanitizers

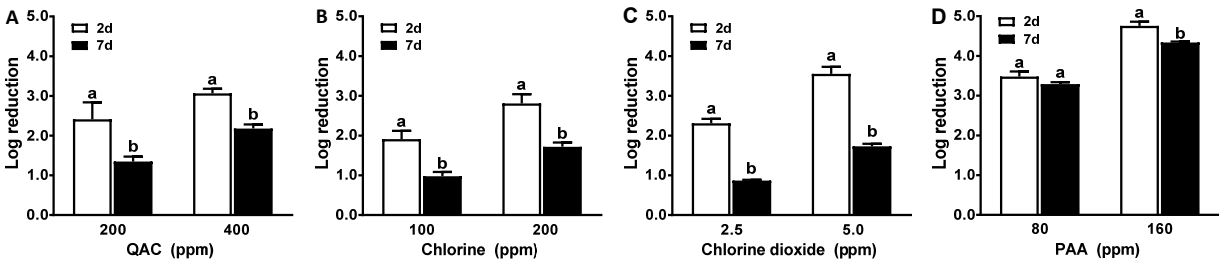


Figure 2. Efficacy of selected sanitizer intervention against mixed strain *L. monocytogenes* biofilm at different ages. 2d: 2-day-old biofilm; 7d: 7-day-old biofilm. A: QAC; B: Chlorine; C: Chlorine dioxide; D: PAA Mean ± SEM. Experiments were conducted independently three times, 6 replicates/treatment in each independent study.

against 7-day-old biofilm were reduced when compared to 2-day-old biofilm; antimicrobial efficacy of PAA was relatively less influenced by age of the biofilm (Fig. 2).

2. Efficacy of selected sanitizers against *L. monocytogenes* biofilms on food-contact surfaces

In general, increasing QAC concentration from 200 ppm to 400 ppm improved its efficacy against *Lm* biofilms on food-contact surfaces except for LDPE surfaces for both 1 min and 5 min exposures (Fig. 3). A 5 min exposure of QAC (200 or 400 ppm) showed a similar efficacy against *Lm* biofilms on SS coupons (Fig. 3A). Except for rubber surface, the efficacy of QAC against *Lm* biofilms on surfaces was enhanced when increasing treatment time increased from 1 min to 5 min (Fig. 3). Among all surfaces, QAC at 5 min exposure was the most effective on SS (Fig. 3), least effective on rubber (Fig. 3) and exhibiting a comparable efficacy against *Lm* biofilms on LDPE and PET (Fig. 3).

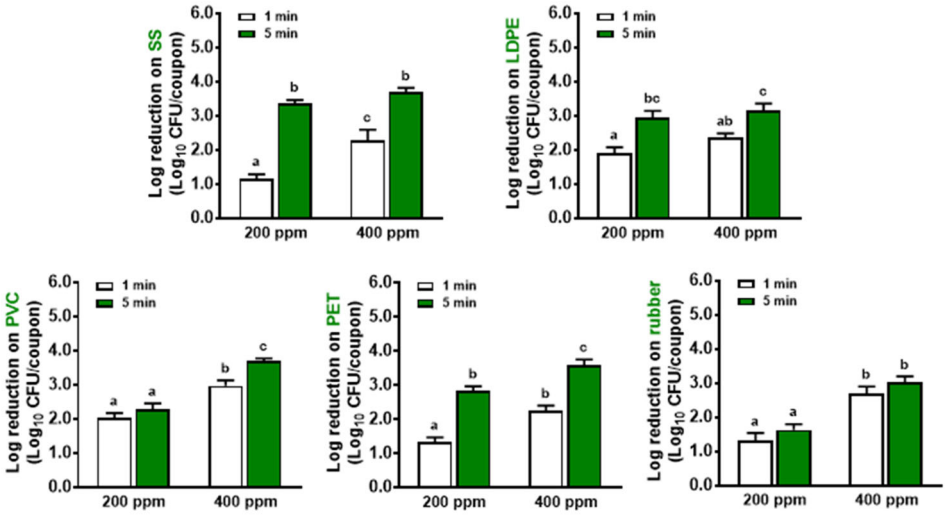


Fig. 3. Efficacies of QAC against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 200 or 400 ppm QAC. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

Chlorine dioxide solution at 2.5 ppm exhibited a limited efficacy against *Lm* biofilms on all surfaces tested; 1 min exposure reduced ~ 1.1, 0.6, 0.9, 1.1, and 0.9 $\log_{10}$ CFU/coupon *Lm* biofilms on SS, LDPE, PVC, PET, and rubber surfaces (Fig. 4). Though the efficacy of chlorine dioxide was enhanced with increased concentration and contact time, it displayed limited potency to inactivate *Lm* biofilms on food-contact surfaces. A 5 min treatment of 5.0 ppm chlorine dioxide caused similar bactericidal efficacy against *Lm* biofilms on all surfaces with 2.4-2.7 $\log_{10}$ CFU/coupon reductions (Fig. 4).

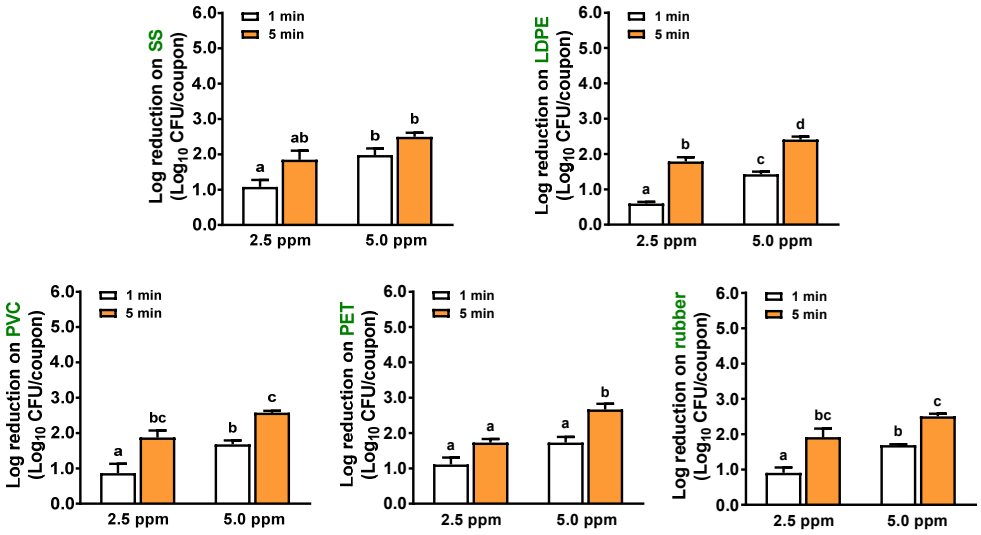


Fig. 4. Efficacies of chlorine dioxide against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 2.5 or 5.0 ppm chlorine dioxide. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

The efficacy of chlorine against *Lm* biofilms on all surfaces was enhanced at increased concentration and extended contact time except LDPE surface (Fig. 5). A 1 min treatment of 100 ppm chlorine showed a similar efficacy against *Lm* biofilms as 1 min exposure of 200 ppm QAC (Fig. 3) and was more effective than 1 min treatment of 2.5 ppm chlorine dioxide (Fig. 4), causing 1.0 - 2.0 log CFU/coupon reductions of *Lm* biofilms. Chlorine at 200 ppm for 5.0 min exposure caused 3.8, 2.7, 3.3, 3.6, and 3.0 $\log_{10}$ CFU/coupon reductions of *Lm* biofilms on SS, LDPE, PVC, PET and rubber surfaces (Fig. 5).

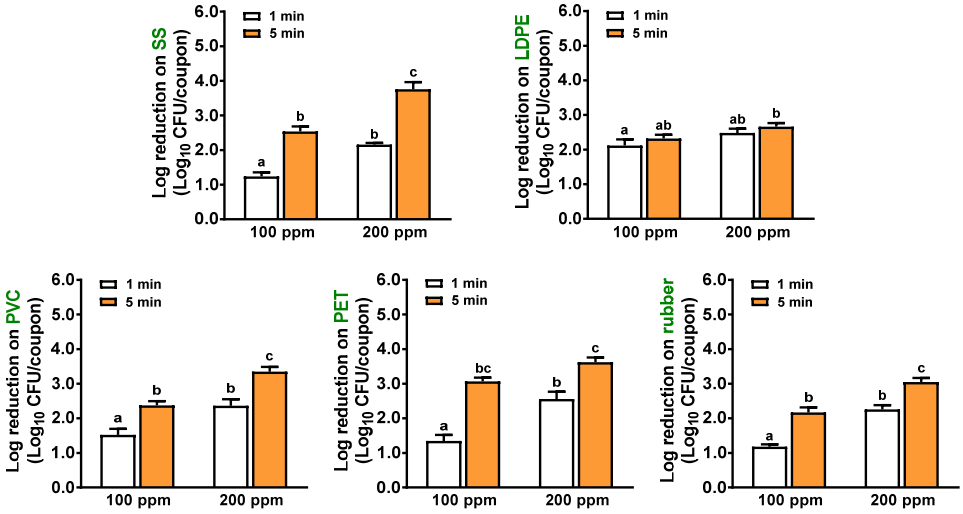


Fig. 5. Efficacies of chlorine against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 100 or 200 ppm chlorine. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

Among all selected sanitizers, PAA was the most effective against *Lm* biofilms on all food-contact surfaces (Fig. 6). One min treatment of 160 ppm PAA reduced ~ 4.3, 3.5, 3.8, 4.1, and 3.7 $\log_{10}$ CFU/coupon *Lm* biofilms on SS, LDPE, PVC, PET, and rubber surfaces, respectively (Fig. 6). In general, bactericidal effects of PAA against *Lm* biofilms on all surfaces was not improved when concentration of PAA increased from 160 ppm to 200 ppm or when the treatment time increased from 1 min to 5 min (Fig. 6). A 5 min treatment of 200 ppm PAA caused 4.5, 4.0, 4.4, 4.3, and 4.4 log reduction of *Lm* on SS, PET, PVC, LDPE, and rubber (Fig. 6).

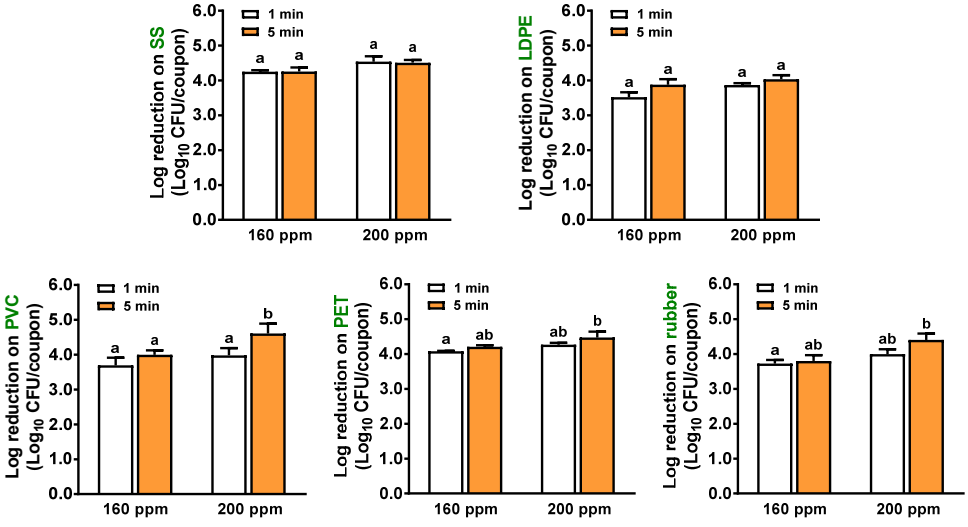


Fig. 6. Efficacies of PAA against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 160 or 200 ppm PAA. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

3. Effects of organic matter on sanitizer's efficacy

The anti-*Listeria* efficacies of tested sanitizers were compromised by organic soiling regardless of surface types. Food residues from apple juice or milk comparably impacted efficacies of the sanitizers (Fig. 7). Thought PAA efficacy was impaired by organic soiling, it was still the most effective sanitizer, which caused 3.0-37 log CFU/coupon reductions of *Lm* biofilm on different surfaces (Fig. 7)

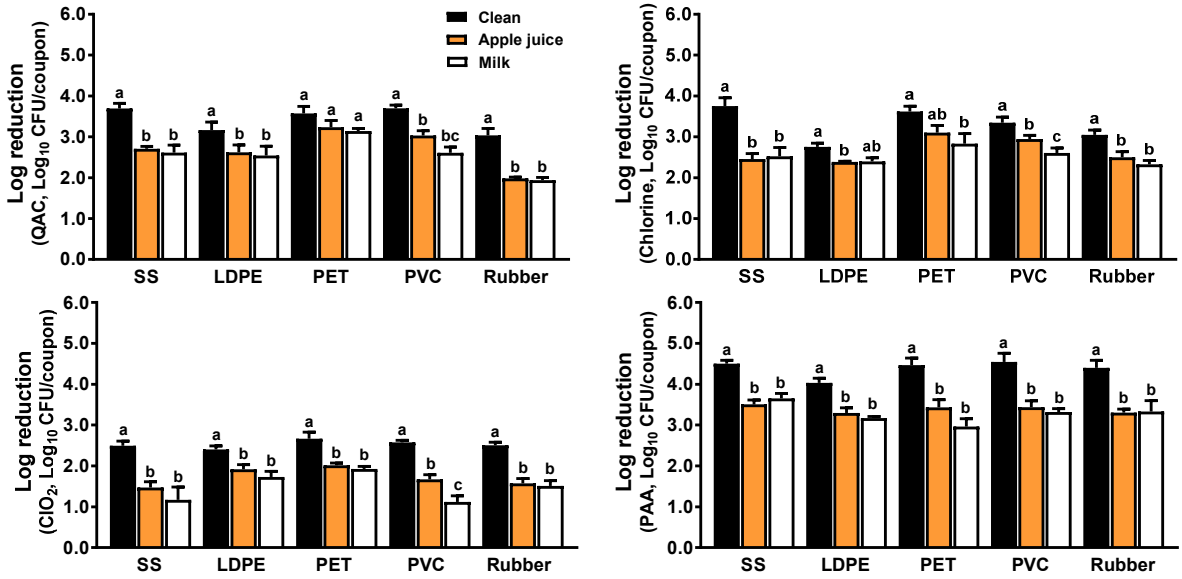


Fig. 7. Impacts of organic soiling on efficacies of tested sanitizers against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 400 ppm QAC, 200 ppm chlorine, 5 ppm chlorine dioxide, and 200 ppm PAA. A. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

4. Sanitizers efficacies against *L. monocytogenes* biofilm on new and worn surfaces

The food-contact surfaces are subjected to natural aging and abrasion with usage and time. *L. monocytogenes* was found on worn rubber surfaces (Tompkin, 2002) and damaged plastic cutting boards (Berzins et al., 2010) in ready-to-eat meat facilities. Yet, limited information is available about the practical efficacies of sanitizers against *Lm* biofilm formed on worn food-contact surfaces. In this study, we first compared the count of *Lm* in biofilm formed on new and worn surfaces. *Lm* counts of SS were comparable on the new and worn surface, and there was a 6.93 – 7.10 $\log_{10}$ CFU/coupon of *Lm*. *Lm* populations on worn LDPE, PVC, PET, and rubber were significantly ($P < 0.05$) higher compared to the corresponding new surfaces: 7.89 – 8.64 (worn) vs 7.05 - 7.50 (new) $\log_{10}$ CFU/coupon.

Lm in biofilm on SS-2B exhibited higher resistance than that on SS-4, and the surfaces with defects or damage compromised the efficacies of sanitizers in removing *Lm* from SS coupons (Table 1). The 5 min exposure of 400 ppm QAC caused 2.38 and 2.88 log reductions on worn SS-2B and SS-4 surfaces, respectively, which was less effective than that obtained on new coupons ($P < 0.05$).

Table 1 Sanitizer efficacies against *L. monocytogenes* biofilm on the stainless-steel surface

Treatment	Reduction ($\log_{10}$ CFU/coupon)			
	Surface	New	Defective	Worn
Chlorine	SS-2B	2.79 ± 0.14^{aA}	2.43 ± 0.16^{aA}	2.44 ± 0.11^{aA}
	SS-4	3.57 ± 0.09^{bA}	3.31 ± 0.13^{bA}	3.39 ± 0.16^{bA}
QAC	SS-2B	2.83 ± 0.21^{aA}	2.55 ± 0.18^{aAB}	2.38 ± 0.19^{aB}
	SS-4	3.65 ± 0.11^{bA}	3.17 ± 0.16^{bB}	2.88 ± 0.12^{bB}
PAA	SS-2B	4.05 ± 0.19^{aA}	3.53 ± 0.10^{aB}	3.41 ± 0.13^{aB}
	SS-4	4.32 ± 0.15^{aA}	3.85 ± 0.15^{aB}	3.83 ± 0.13^{bB}

SS-2B: stainless steel 2B finish, SS-4: stainless steel 4 finish. New: new surfaces. Defective: SS was bead blasted. Worn: SS was 80-grit sanded. [A-B] means within a row without the same letter differ significantly ($P < 0.05$). [a-b] means within a column without the same letter differ significantly for the same sanitizer treatment ($P < 0.05$). Mean ± SEM, n = 9.

Similarly, PAA at 160 ppm for 1-min contact is more effective against *Lm* biofilm on new SS surfaces than those on worn ones: 4.05 and 4.32 vs. 3.41 and 3.83 log reduction on SS-2B and SS-4 surfaces in new vs. worn conditions, respectively (Table 1). The effectiveness of sanitizer treatments on defective (moderate wear) and worn (severe wear) SS are comparable (Table 1).

The bactericidal effect of chlorine was significantly ($P < 0.05$) reduced on worn PVC, PET, and rubber surfaces compared to that on new surfaces, which removed 3.35 vs. 3.06, 3.23 vs. 1.84, 3.93 vs. 3.31, and 2.97 vs. 2.43 $\log_{10}$ CFU/coupon of *Lm* from LDPE, PVC, PET, and rubber in new vs. worn conditions, respectively (Table 2). QAC was more effective in removing *Lm* biofilm from new LDPE, PVC, and PET surfaces than from worn surfaces, but it caused a comparable reduction on new and worn rubber coupons (Table 2). PAA at 160 ppm for 1-min contact led to similar *Lm* reductions on new and worn LDPE, PVC, PET, and rubber surfaces (Table 2). Given that the population of *Lm* in biofilm on all tested worn surfaces is significantly ($P < 0.05$) higher than that on new surfaces, PAA was less effective in sanitizing the worn surfaces.

Table 2 Sanitizer efficacies against *L. monocytogenes* biofilm on non stainless surfaces

Surface	Condition	Initial levels	Reduction (Log_{10} CFU/coupon)		
			Chlorine	QAC	PAA
LDPE	**New**	7.05 ± 0.11^{a}	3.35 ± 0.11^{aA}	2.97 ± 0.15^{aB}	3.95 ± 0.15^{aC}
	Worn	7.89 ± 0.11^{b}	3.06 ± 0.14^{aA}	2.12 ± 0.12^{bB}	3.77 ± 0.19^{aC}
PVC	**New**	7.50 ± 0.12^{a}	3.23 ± 0.13^{aA}	3.37 ± 0.16^{aA}	3.80 ± 0.11^{aB}
	Worn	8.74 ± 0.03^{b}	1.84 ± 0.09^{bA}	2.05 ± 0.16^{bA}	3.93 ± 0.11^{aB}
PET	**New**	7.34 ± 0.12^{a}	3.93 ± 0.15^{aA}	3.66 ± 0.15^{aA}	4.64 ± 0.11^{aB}
	Worn	8.29 ± 0.09^{b}	3.31 ± 0.07^{bA}	2.47 ± 0.20^{bB}	4.35 ± 0.09^{aC}
Rubber	**New**	7.45 ± 0.07^{a}	2.97 ± 0.13^{aA}	2.51 ± 0.08^{aB}	3.68 ± 0.08^{aC}
	Worn	8.32 ± 0.19^{b}	2.43 ± 0.12^{bA}	2.55 ± 0.17^{aA}	3.95 ± 0.09^{aB}

New: new surfaces. Worn: surfaces were 80-grit sanded. $^{A-B}$ means within a row without the same letter differ significantly ($P < 0.05$). $^{a-b}$ means within a column without the same letter differ significantly for the same sanitizer treatment ($P < 0.05$). Mean ± SEM, n = 9.

Furthermore, the efficacies of QAC and PAA against *Lm* on worn SS and non-SS surfaces are compromised by organic matter conditioning. When the organic matter was present, QAC (400 ppm, 5 min) removed 1.69 and 1.38 $\log_{10}$ CFU/coupon of *Lm* on defective and worn SS-2B surfaces, 1.91 and 1.64 $\log_{10}$ CFU/coupon on defective and worn SS-4 surface, respectively, compared to 1.88 and 2.21 $\log_{10}$ CFU/coupon on new SS-2B and SS-4. PAA (160 ppm, 1 min) treatment reduced *Lm* by 2.78/2.58 and 3.11/2.93 $\log_{10}$ CFU/coupon on apple juice coated defective/worn SS-2B and SS-4 surfaces, respectively, compared to 3.24 and 3.50 $\log_{10}$ CFU/coupon reductions on new SS-2B and SS-4.

QAC (400 ppm, 5 min) decreased 2.12/1.37, 2.05/1.64, 2.47/1.00, and 2.55/1.52 $\log_{10}$ CFU/coupon *Lm* on clean/soiled worn LDPE, PVC, PET, and rubber surfaces. PAA (160 ppm, 1 min) removed 3.77/3.44, 3.93/3.80, 4.35/4.07, and 3.95/3.05 $\log_{10}$ CFU/coupon *Lm* on clean/soiled worn LDPE, PVC, PET, and rubber surfaces, respectively. Notably, up to ~7.0 and 4.5 $\log_{10}$ CFU/coupon of *Lm* were detected on all non-SS worn and soiled surfaces after QAC and PAA treatment, respectively.

In summary, the population of *Lm* in biofilms on all surface coupons except SS surfaces was significantly higher on the defective surfaces than on new ones. Worn food-contact surfaces reduced the effectiveness of all sanitizer treatments, especially when organic matter was present. Food residue/debris soiling, regardless of sources, reduced anti-*Listeria* efficacies of all sanitizers against biofilms on both new and worn surfaces regardless of types of surface coupons. Among all sanitizers, PAA was the most effective sanitizer against *Lm* biofilms on different surfaces. Data highlights the importance of surface maintenance and the importance of thoroughly cleaning food-contact surfaces prior to sanitizer interventions and effective cleaning and sanitization. Data also indicates that damaged/worn equipment and food-contact surfaces are more prone to *Listeria* contamination and could be persistent *Lm* contamination sources.

<u>Objective 2.</u> Examine antimicrobial efficacies of steam against *Listeria* biofilm on different food-contact surfaces.

Heating in the form of hot air, hot water, or steam is a traditional method for microbial reduction. A 6-min of hot water immersion treatment at 60 °C reduced 7-day-old *L. monocytogenes* biofilm on stainless steel (SS) by 3.2 $\log_{10}$ CFU (Tobin et al., 2020). A 15-sec of hot water treatment at 95-100 °C provided ~ 7 log reductions of *L. monocytogenes* attached to the inner surface of the model drainpipes (Berrang et al., 2014). Steam carries latent heat and is more efficient for microbial inactivation than hot air, or water. Steam application (> 93.3 °C for at least five minutes) has been approved by FDA to disinfect water-contact surfaces in bottled drinking water facilities (FDA, 2019). Steam offers various advantages over sanitizers and other intervention methods. It can heat surfaces/target materials quickly and reach into crevices/cracks while leaving no chemical residue on treated surfaces and it is environmentally friendly. Thus, the effectiveness of steam against *Lm* biofilm was further evaluated.

1. Steam and food-contact surface coupon temperatures

The steam temperature was maintained at 100 °C with a minor fluctuation. The temperature of the treated surface coupons rapidly reached 92 °C within 6 sec. The surface temperature of SS coupons at 6-sec of exposure was higher than that of PET, LDPE, PVC, and rubber surfaces. During subsequent steam exposure, the mean surface temperatures of treated surface coupons were similar for a 180-sec steam exposure, which was 98.1 ± 0.3 °C on SS, 97.8 ± 0.4 °C on PET, 96.6 ± 0.3 °C on LDPE, 96.9 ± 0.3 °C on PVC and 96.2 ± 0.3 °C on rubber surfaces (Fig. 8 CD).

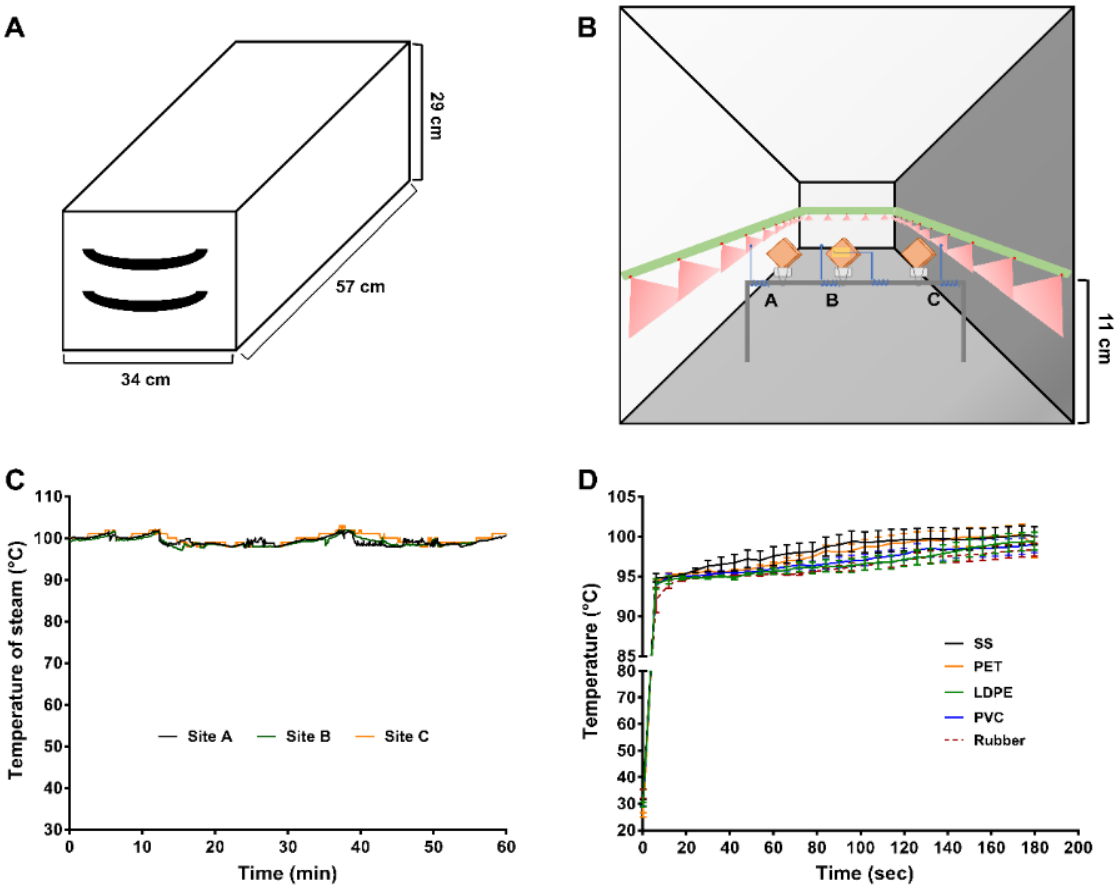


Fig. 8 Steam blancher apparatus and temperature profiles. A. Thaaaaaae dimension. B. Interior view of the steam blancher. Green: steam pipelines; red dots: steam nozzles, 25 in total. C. Temperature profile of steam during 60 min duration. D. Temperature profile of surface coupons during 180-sec treatment.

2. Steam inactivation of *L. innocua* biofilms on different food-contact surfaces

Steam had a quick bactericidal effect against 7-day-old *L. innocua* biofilms on all surfaces. A 6-sec exposure of steam provided 3.2, 2.6, 2.4, 2.5, and 2.6 $\log_{10}$ CFU/coupon reductions of *L. innocua* biofilm on SS, PET, LDPE, PVC, and rubber surface coupons, respectively (Fig. 9A). Fig 9B showed a representative image of Live/Dead staining of *L. innocua* cells in 7-day-old biofilms on SS before and after a 6-sec steam treatment, which further demonstrated the rapid bactericidal effect of steam.

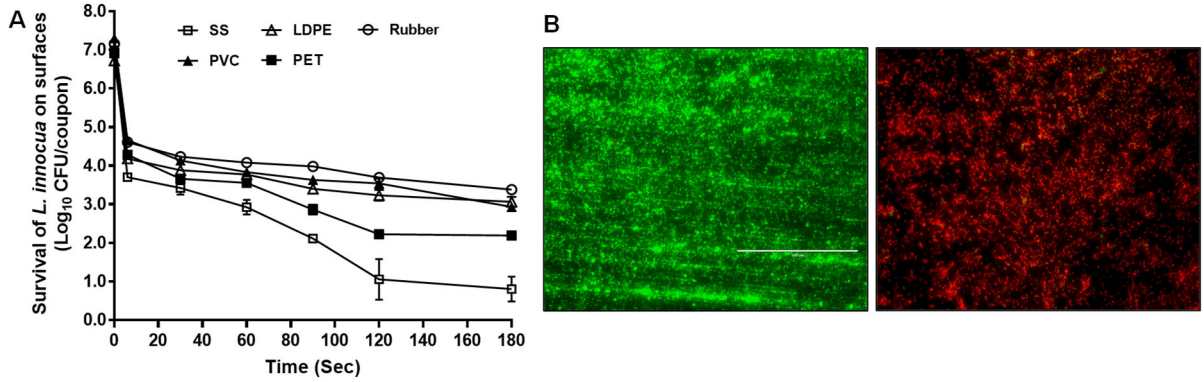
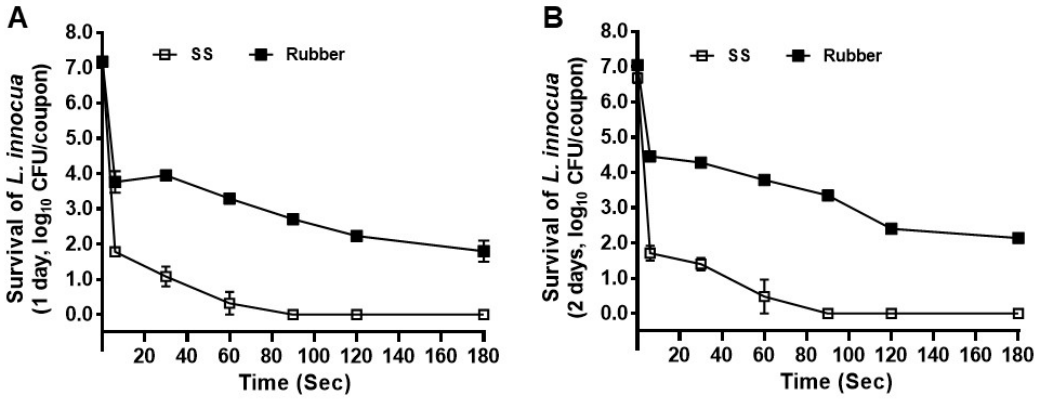


Fig. 9. Steam efficacy against cells in *L. innocua* biofilm on food-contact surfaces. 7-day-old biofilms were subjected to 100 °C steam for 0-180 sec. A. Representative survival of *L. innocua* biofilm on different food-contact surface coupons. B. Live/Dead staining of *L. innocua* cells in 7-day-old biofilm on SS surface. Left; *L. innocua* before steam treatment; right: *L. innocua* cells after a 6-sec steam treatment; Green: live cells; Red: dead cells; bar: 100 µm. Mean ± SEM, n = 9.



C

The reduction of *L. innocua* on surfaces ($\log_{10}$ CFU/coupon)

Time (sec)	1 day		2 days	
	Stainless steel	Rubber	Stainless steel	Rubber
6	5.5 ± 0.1^{aB}	2.7 ± 0.4^{aA}	5.1 ± 0.1^{aB}	2.6 ± 0.2^{aA}
30	6.1 ± 0.2^{abB}	2.9 ± 0.2^{abA}	5.6 ± 0.4^{aB}	2.5 ± 0.3^{aA}
60	6.7 ± 0.2^{bcB}	3.5 ± 0.2^{bA}	6.5 ± 0.2^{bB}	3.0 ± 0.1^{abA}
90	$>6.7^{cC}$	4.3 ± 0.3^{cB}	$>6.6^{bC}$	3.4 ± 0.4^{bA}
120	$>6.7^{cB}$	4.7 ± 0.1^{cA}	$>6.6^{bB}$	4.4 ± 0.2^{cA}
180	$>6.7^{cB}$	5.7 ± 0.4^{dA}	$>6.6^{bB}$	5.6 ± 0.5^{dA}

Mean ± SEM was averaged from three independent studies where three replicates were used per treatment. $^{a-d}$ Mean within a column without a common letter differ significantly ($P < 0.05$). $^{A-C}$ Mean within a row without a common letter differ significantly ($P < 0.05$).

Fig.10. Steam efficacy against *L. innocua* cells on food-contact surfaces. The 1-day/2-day attached *L. innocua* on surface were subjected to 100 °C steam for 0-180 sec. Mean ± SEM was averaged from three independent studies where three replicates were used per treatment. $^{a-d}$ Mean within a column without a common letter differ significantly ($P < 0.05$). $^{A-C}$ Mean within a row without a common letter differ significantly ($P < 0.05$).

The inactivation rate of steam against *L. innocua* biofilm on all surfaces declined with increasing treatment time, especially on rubber surfaces. Among all surfaces treated, steam pasteurization was most effective against *L. innocua* biofilm on SS, followed by PET. A 30-, 60-, 120- and 180- sec steam treatment resulted in 4.0, 4.6, 5.7, and 6.4 $\log_{10}$ CFU/coupon reductions on SS, and 3.1, 3.3, 4.6, and 4.8 $\log_{10}$ CFU/coupon reductions on PET surface coupons, respectively (Fig. 9). Steam at 100 °C had comparable treatment efficacy on both LDPE and PVC surface; a 30-180 sec steam exposure caused 2.8 - 4.2 and 2.7 - 4.5 $\log_{10}$ CFU/coupon reductions on LDPE and PVC coupons, respectively (Fig. 9).

The exact mechanism for the tailing effects on different surfaces is not known. To evaluate the contributions of biofilm structures to the declined killing rate and the surviving tail during subsequent steam exposure, we evaluated the steam efficacy against surface-attached *L. innocua* or *L. innocua* in young biofilms on SS (most effective) and rubber (least effective) surface coupons (Fig. 10). In support of the role of biofilm architecture, steam is more effective against *L. innocua* attached on a surface or in young biofilm. A 90-sec of steam treatment reduced *L. innocua* in 1-day/2-day-old attachment/biofilm on SS to below the detection limit. There was an additional ~3 log reduction of *L. innocua* in 1-day/2-day-old attachment/biofilm on rubber surfaces (Fig. 10). However, there was still an obvious tailing effect of the inactivation curve of *L. innocua* especially on rubber surfaces, indicating factors other than biofilm structure contributed to the reduced effectiveness of steam for inactivating *L. innocua* in biofilms on rubber.

3. Impact of organic matter on the efficacies of steam pasteurization against *L. innocua* biofilm on different food-contact surfaces

Compared to clean surfaces, steam treatments were equally or more effective against 7-day-old biofilms formed on coupons that had been conditioned with diluted apple juice, a source of organic matter (Table 3). Like clean surfaces, steam caused a rapid kill of *L. innocua* biofilms on soiled surfaces with a 6-sec of exposure, reducing cell counts by 2.5 - 4.1 $\log_{10}$ CFU/coupon on all surfaces. Increasing the treatment time from 6 sec to 30 sec enhanced inactivation efficacies on SS and PET surfaces only (Table 3) (Hua et al., 2021).

Table 3 Impacts of organic matter on efficacy of steam

	Steam(sec)	Reduction ($\log_{10}$ CFU/coupon)	
		Clean	Soiled
SS	6	3.2 ± 0.1^{aA}	4.1 ± 0.1^{aB}
	30	3.8 ± 0.2^{bA}	4.4 ± 0.1^{aB}
PET	6	2.5 ± 0.1^{aA}	2.8 ± 0.1^{aA}
	30	2.8 ± 0.1^{aA}	3.5 ± 0.1^{bB}
LDPE	6	2.4 ± 0.1^{aA}	2.9 ± 0.2^{aA}
	30	2.9 ± 0.1^{bA}	3.0 ± 0.1^{aA}
PVC	6	2.5 ± 0.1^{aA}	2.8 ± 0.1^{aA}
	30	2.7 ± 0.1^{aA}	2.8 ± 0.1^{aA}
Rubber	6	2.6 ± 0.1^{aA}	2.5 ± 0.1^{aA}
	30	2.6 ± 0.1^{aA}	2.6 ± 0.1^{aA}

7-day-old biofilms on clean or soiled surfaces were treated with 100°C steam for 6 sec or 30 sec. $^{A-B}$ means within a row without the same letter differ significantly ($P < 0.05$). $^{a-b}$ means within a column without the same letter differ significantly for the same sanitizer treatment ($P < 0.05$). Mean ± SEM, n = 9.

4. Surface properties before and after steam treatments

The hydrophobicity of SS, PET, LDPE, or PVC was smaller than the rubber surface. The PET surface had the smallest R_a value, an indicator of the roughness, followed by LDPE, SS, and PVC, while rubber had the largest R_a value. Repeated steam exposure had no effects on the hydrophobicity

and roughness of SS, PET, and rubber surfaces, but negatively impacted PVC and LDPE surfaces. The detailed data can find in our published paper (Hua et al., 2021).

In summary, steam exhibited a fast killing kinetic against *L. innocua* biofilm on different food-contact surfaces; however, the killing rate of steam decreased dramatically during subsequent steam treatment and exhibited a tailing effect which was more pronounced on rubbers, PVC, and LDPE surfaces. Our data suggested that a short duration of steam exposure alone or in combination with chemical disinfection might be a promising sanitization strategy for removing *Listeria* biofilm or other foodborne pathogens on food contact surfaces, especially for SS, PET, and rubber surfaces.

<u>Objective 3.</u> Evaluate the antimicrobial efficacies of steam in combination with the selected sanitizer against biofilm on the common food-contact surface using optimized parameters.

Objective 1 study indicates that PAA was more effective than chlorine, QAC, and chlorine dioxide in removing *Lm* biofilms from commonly used food-contact surfaces. However, the anti-*Listeria* efficacies of PAA were compromised by the organic soiling and surface defects. Objective 2 study indicates that steam is an effective method for surface decontamination that incurs a quick inactivation of *Listeria* biofilms on stainless steel (SS) surfaces. A 6-sec treatment of steam at 100 °C reduced a 3.1 $\log_{10}$ CFU/coupon *Listeria* in biofilm on the SS coupons; however, the extension of steam treatment time beyond 6 seconds lowered the killing rate of the steam against *Listeria* in biofilms. Data indicated that steam treatment or PAA alone was insufficient to eradicate *Listeria* biofilms from food-contact surfaces. Therefore, the hurdle treatment in combination with short steam and PAA treatment was further evaluated.

1. Effectiveness of hurdle treatments against *L. innocua* biofilms on food-contact surfaces

The hurdle treatment combining saturated steam and PAA exhibited significantly (P<0.05) higher efficacy than PAA or saturated steam treatment alone (Fig. 11). The PAA (80 ppm, 1 min) followed by a 6-sec steam exposure lowered 6.75, 6.96, and 5.54 $\log_{10}$ CFU/coupon of *L. innocua* from SS, PET, and rubber surface coupons, respectively, compared to 2.63 - 3.34 $\log_{10}$ CFU/coupon reductions by saturated steam (100 °C, 6 sec) alone and 2.66 - 2.85 $\log_{10}$ CFU/coupon reductions by PAA (80 ppm, 1 min) treatment alone (Fig. 11).

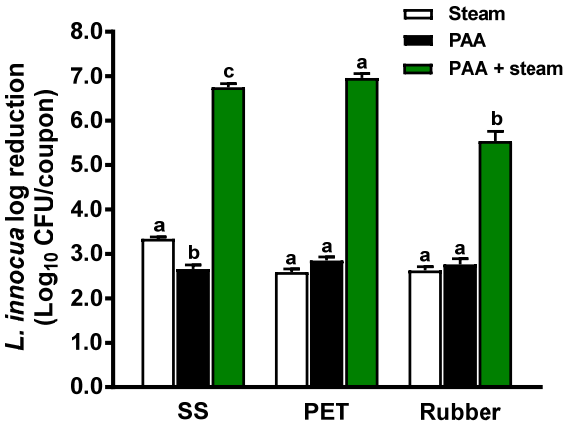


Fig. 11. Efficacy of PAA in the combination of saturation steam in removing *L. innocua* biofilms from the food-contact surfaces. The 7-day-old *L. innocua* biofilms on surface coupons were treated with PAA (80 ppm, 1 min), steam (100 °C, 6 sec), and their combination. Mean ± SEM was averaged from three independent studies, with four replicates per independent study. [a-c] Bars topped with different letters are significantly (P < 0.05) different for each surface type.

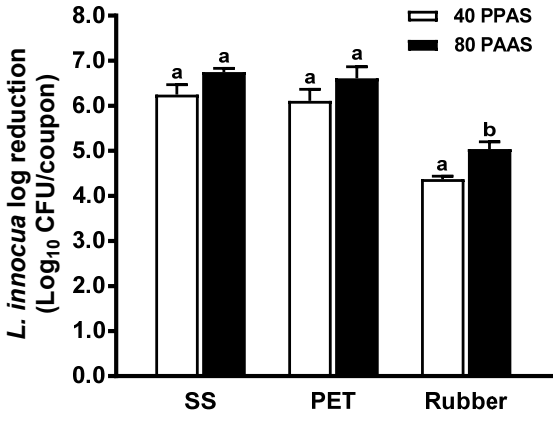


Fig. 12. Effectiveness of saturation steam with different PAA concentrations against *L. innocua* biofilms on food-contact surface. The 7-day-old *L. innocua* biofilms on surfaces treated with steam (100 °C, 6 sec) in combination with 40 ppm or 80 ppm PAA. Mean ± SEM was averaged from three independent studies, with four replicates per independent study. a-b Bars topped with different letters are significantly (P < 0.05) different for each surface type.

The hurdle treatment of 6 sec of steam in combination with 80 ppm or 40 ppm PAA had similar efficacies against *L. innocua* biofilm on SS and PET surfaces, which resulted in > 6 $\log_{10}$ CFU/coupon *L. innocua* within biofilms on both surfaces. However, the efficacy of 40 ppm PAA + steam against *L. innocua* biofilm on rubber surfaces was lower than that of 80 ppm PAA + steam treatment (P<0.05) (Fig. 12). Regardless of PAA levels, PAA + steam treatments had comparable efficacy on SS and PET, which was more effective than that on rubber surfaces. Furthermore, the efficacy of PAA and steam hurdle treatment was not significantly impacted by treatment order, whether treated with steam followed by PAA treatment or firstly treated with PAA followed by steam exposure (data not shown).

2. The impact of organic matter on the effectiveness of PAA + steam treatment against *L. innocua* biofilms on food contact surfaces

Surfaces of SS, PET, and rubber conditioned with apple juice reduced the effectiveness of 40 ppm PAA + steam treatment in removing *L. innocua* biofilm on SS and PET surfaces (P<0.05), but its efficacy on rubber surfaces was not impacted. The PAA at 40 ppm for 1 min treatment followed by 6-sec saturated steam exposure removed 5.56, 5.76, and 4.17 $\log_{10}$ CFU/coupon *L. innocua* on SS, PET, and rubber surfaces, respectively, in the presence of apple juice soiling.

3. The impact of surface condition on the effectiveness of PAA + steam treatment against L. innocua biofilm on surfaces

Compared to the new surface, the efficacy of 40 ppm PAA + steam treatment against *L. innocua* biofilm on worn SS and PET surfaces was significantly decreased (Table 4). Though we observed higher *L. innocua* reductions on worn PET and rubber surfaces compared to new ones after steam treatment alone. Given that the initial *L. innocua* level on worn PET and rubber was ~ 1 $\log_{10}$ CFU/coupon higher than on new PET and rubber, there were higher loads of *L. innocua* on worn PET and rubber after steam treatments than that on new ones. Collectively, the anti-*Listeria* efficacy of steam treatment, with or without 40 ppm PAA treatment, on all surface coupons tested was diminished by surface defects (Table 4).

Table 4 Efficacy of the hurdle treatment against *L. innocua* biofilms on worn surfaces

			Reduction ($\log_{10}$ CFU/coupon)	
Surface	**Conditions**	**Initial levels**	**Steam**	**PAA + steam**
SS	New, clean	6.83 ± 0.05^{a}	3.34 ± 0.04^{aA}	$>6.53^{aB}$
	Worn, clean	7.22 ± 0.04^{a}	2.56 ± 0.04^{bA}	5.91 ± 0.27^{bB}
	Worn, soiled	7.15 ± 0.06^{a}	2.70 ± 0.12^{bA}	5.08 ± 0.12^{cB}
PET	New, clean	7.13 ± 0.09^{a}	2.59 ± 0.07^{aA}	6.61 ± 0.26^{aB}
	Worn, clean	8.28 ± 0.07^{b}	3.50 ± 0.07^{bA}	5.69 ± 0.22^{bB}
	Worn, soiled	8.18 ± 0.07^{b}	3.33 ± 0.05^{bA}	5.18 ± 0.08^{cB}
Rubber	New, clean	7.03 ± 0.09^{a}	2.65 ± 0.09^{aA}	4.37 ± 0.07^{aB}
	Worn, clean	8.00 ± 0.05^{b}	3.23 ± 0.10^{bA}	4.84 ± 0.04^{bB}
	Worn, soiled	7.97 ± 0.07^{b}	2.79 ± 0.13^{aA}	4.49 ± 0.04^{cB}

The 7-day-old *L. innocua* biofilms on were treated with steam (100 °C, 6 sec) with or without 40 ppm PAA. Mean ± SEM, n=12. $^{a-c}$ numbers topped with the same letters did not differ significantly (P < 0.05) within each column for the same surface material. $^{A-B}$ numbers topped with the same letters did not differ significantly (P < 0.05) within each row.

In summary, a low concentration of PAA in the combination with quick steam exposure was a viable sanitization intervention for food contact surfaces. PAA at 40 ppm in combination with 6-sec saturated steam exposure provided > 6 log reduction of *L. innocua* biofilm on SS and PET surfaces. PAA at 80 ppm and 6-sec saturated steam hurdle intervention resulted in ~ 5 log reduction on the rubber surface.

EXECUTIVE SUMMARY

L. monocytogenes forms biofilms on different food-contact surfaces, providing a continuous source of contamination to foods that encounter contaminated surfaces. Considering the caramel apple listeriosis outbreak, multiple food-contact surfaces including the polishing brush, drying brush, conveyor, and wooden bin inner surface was confirmed to be *L. monocytogenes* positive (Angelo et al., 2017). These types of contamination on commonly utilized surfaces highlighted the importance of effectively sanitizing food-contact surfaces. Direct food-contact surfaces have been required to be fully cleaned to prevent contamination/cross-contamination of "covered" produce and packing environments regulated under the Food Safety Modernization Act (FSMA) Produce Safety Rule (FSMA, 2016). The overall goal is to comprehensively evaluate the antimicrobial efficacy of commonly used commercial sanitizers at practical concentration and steam treatment against *Listeria* biofilm on different food contact surfaces. Given the food-contact surfaces are subjected to natural aging and abrasion with usage and time and contamination of food residues, we further evaluate the impacts of surface defects and organic soiling on the effectiveness of sanitization.

Our data indicated that all sanitizers at the concentrations commonly used in the food industry showed a stronger bactericidal effect against young (2-day-old) *L. monocytogenes* biofilm than old (7-day-old) biofilm. *L. monocytogenes* biofilm on stainless steel 2B finish exhibited higher resistance than that on stainless steel 4 finish. The population of *L. monocytogenes* in biofilms on all surface coupons except stainless steel surfaces was significantly higher on the defective surfaces than on new ones. Worn food-contact surfaces reduced the effectiveness of all sanitizer treatments, indicating damaged/worn equipment and food-contact surfaces are more prone to *Listeria* contamination. Food debris/organic soiling of food-contact surfaces reduced the anti-*Listeria* efficacies of all sanitizers against biofilms on both new and worn surfaces regardless of the types of surface coupons. Among all sanitizers, PAA was the most effective sanitizer against *L. monocytogenes* biofilms on different surfaces.

Steam exhibited a fast killing kinetic against *L. innocua* biofilm on different food-contact surfaces; a 6-sec steam treatment attained a 2.4 - 3.1 $\log_{10}$ CFU/coupon reduction depending on surface materials. However, the killing rate of steam decreased dramatically during subsequent steam treatment and exhibited a tailing effect which was more pronounced on rubbers, PVC, and LDPE surfaces, followed by PET and then SS surface. Organic matter soils did not compromise the bactericidal effects of steam against *L. innocua* biofilm on tested surfaces. Data indicated that steam treatment or PAA alone was insufficient to eradicate *Listeria* biofilms from food-contact surfaces. Therefore, the hurdle treatment in combination with short steam and PAA treatment was further evaluated. PAA at 40 ppm in combination with 6-sec saturated steam exposure provided > 6 log reduction of *L. innocua* biofilm on SS and PET surfaces. PAA at 80 ppm and 6-sec saturated steam hurdle intervention resulted in ~ 5 log reduction on the rubber surface. Our data suggested that a short duration of steam exposure alone or in combination with PAA or chemical disinfection might be a promising sanitization strategy for removing *Listeria* biofilm or other foodborne pathogens on food contact surfaces.

Data highlights the importance of surface maintenance and thorough cleaning of food-contact surfaces prior to sanitizer interventions and effective cleaning and sanitization. Results from this study also reflected the significance of the periodical application of sanitizers to avoid the establishment of the aged biofilm, which was much more difficult to be eradicated compared to the fresh one.

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