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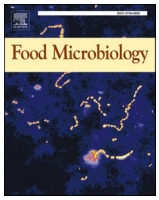

Pseudomonadaceae increased the tolerance of *Listeria monocytogenes* to sanitizers in multi-species biofilms

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ABSTRACT

The persistence of the foodborne pathogen *Listeria monocytogenes* in food processing facilities may be facilitated by the formation of multi-species biofilms by environmental microbiota. This study aimed to determine whether multi-species biofilm formation results in an increased tolerance of *L. monocytogenes* in biofilms to the sanitizers benzalkonium chloride (BAC) and peroxyacetic acid (PAA) at concentrations commonly used in food processing facilities. Biofilms composed of microbiota previously shown to co-occur with *L. monocytogenes* in tree fruit packing facilities (i.e., *Pseudomonadaceae, Xanthomonadaceae, Flavobacteriaceae*, and *Microbacteriaceae*) were formed with *L. monocytogenes* in single- and multi-family assemblages. Multi-family biofilms were exposed to 250 or 500 ppm of PAA, or 200 ppm of BAC to determine the die-off kinetics of *L. monocytogenes*. Furthermore, the ability of a commercial biofilm remover to disrupt biofilms and inhibit bacteria in the formed single- and multi-family assemblage biofilms was assessed. The die-off kinetics of total bacteria and *L. monocytogenes* in biofilm assemblages throughout the exposure to a sanitizer was determined using the aerobic plate count and the most probable number methods, respectively. Biofilm assemblages that included *Pseudomonadaceae* resulted in an increased tolerance of *L. monocytogenes* to BAC and PAA compared to biofilm assemblages without *Pseudomonadaceae*. Further, the use of the biofilm remover significantly disrupted biofilms and reduced the concentration of *L. monocytogenes* in single- and multi-family biofilms by 5 or more logarithmic units. These findings highlight the need to improve the control of biofilm-forming microbiota in food processing facilities to mitigate the persistence of *L. monocytogenes.*

1. Introduction

Foodborne pathogen *Listeria monocytogenes* is of a significant food safety concern due to its high mortality rate of 20–30% among individuals with listeriosis (CDC, 2024a; De Noordhout et al., 2014). In the United States, the Centers for Disease Control and Prevention reported 29 foodborne outbreaks of listeriosis over the last decade (CDC, 2024a), often linked to ready-to-eat foods like deli meats, cheese, raw milk, ice cream, and enoki mushrooms (Buchanan et al., 2017; Mohapatra et al., 2024; Silk et al., 2012). More recently, outbreaks have been associated also with contaminated fresh produce, including tree fruits and leafy greens (Chen et al., 2017). A notable outbreak occurred in 2014–2015, involving caramel-coated apples, highlighting the need for improved monitoring and control measures for *L. monocytogenes* in tree fruit packing and processing environments (Chen et al., 2017; Mohapatra et al., 2024). Using whole genome sequencing, the outbreak investigation determined that the *L. monocytogenes* isolates collected from an apple packing facility were highly related to clinical strains, suggesting the environment was likely the source of contamination (CDC, 2024b). Further surveys of tree fruit packing environments have shown that

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L. monocytogenes can persist on produce processing equipment and within the environment, posing a significant risk for recurrent contamination of food (Chen et al., 2022; Rolon et al., 2024; Ruiz-Llacsahuanga et al., 2021; Simonetti et al., 2021; Sullivan et al., 2022).

The proposed mechanism for *L. monocytogenes'* survival and persistence in food processing environments is by colonizing the environment and forming surface- or non-surface-attached biofilms, or integrating into biofilms formed by other environmental microbiota (Fan et al., 2020; Osek et al., 2022; Puga et al., 2018; Reis-Teixeira et al., 2017; Sauer et al., 2022). These biofilms are heterogenous, diverse, and coordinated communities of microorganisms protected by a matrix of extracellular polymeric substances (EPS) (Sauer et al., 2022). Within biofilms, complex inter- and intra-species relationships can enhance the adherence and colonization of *L. monocytogenes* to food processing surfaces as well as affect its tolerance to sanitizers (Fagerlund et al., 2017; Røder et al., 2015a; Zilelidou and Skandamis, 2018). Microorganisms within biofilms often exhibit reduced susceptibility to sanitizers compared to their planktonic counterparts, which can lead to sanitation failures and the persistence of pathogens. For instance, *L. monocytogenes* in four and ten-day old biofilms showed a significant increase in resistance to benzalkonium chloride (BAC) compared to planktonic cells (Saá Ibusquiza et al., 2011). Furthermore, the presence of environmental microbiota in biofilms can provide additional protection against antimicrobials. A previous study demonstrated that a multispecies biofilm composed of five strains of *Pseudomonas* spp. enhanced the survival of *L. monocytogenes* when exposed to a peroxyacetic acid (PAA)-based commercial sanitizer (Thomassen et al., 2023).

In our previous longitudinal survey of tree fruit packing facilities, we identified biofilm-forming microbiota from the families *Pseudomonadaceae, Xanthomonadaceae, Flavobacteriaceae*, and *Microbacteriacea*, which frequently co-occurred with *L. monocytogenes* (Rolon et al., 2023; Tan et al., 2019). Notably, we observed that *L. monocytogenes* exhibited enhanced growth in multi-family biofilms and an increased tolerance to 12.5 ppm benzalkonium chloride (BAC) when present in these biofilms, compared to when *L. monocytogenes* was alone or in planktonic cultures (Rolon et al., 2024). These findings suggested that certain environmental microbiota in food processing facilities can facilitate biofilm formation and enhance the tolerance of *L. monocytogenes* to sanitizers (Rolon et al., 2024). However, the concentration of BAC used in our previous study was below the recommended levels for sanitizing environmental non-food contact surfaces. In this study, we aimed to assess whether multi-species biofilms, comprised of environmental microbiota, can enhance the tolerance of *L. monocytogenes* to two commonly used sanitizers, BAC and peroxyacetic acid (PAA) at concentrations typically applied during cleaning and sanitizing in food processing facilities. Additionally, we evaluated the efficacy of a commercial biofilm-removing product in disrupting multi-family biofilms, reducing the microbial population within these biofilms, and inactivating *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial cultures

L. monocytogenes (n = 7), *Pseudomonadaceae* (n = 8), *Xanthomonadaceae* (n = 8), *Microbacteriaceae* (n = 6), and *Flavobacteriaceae* (n = 3) strains were previously isolated from environmental samples collected from tree fruit packing facilities, characterized using whole genome sequencing, and preserved in Brain Heart Infusion broth (BD, Franklin Lakes, NJ) supplemented with sterile glycerol (VWR, Radnor, PA) at −80 °C (Table 1) (Rolon et al., 2024). Isolates were resuscitated by streaking onto Reasoner's 2A (R2A) agar (BD, Franklin Lakes, NJ) and incubating for 24–72 h at 30 °C or 20 °C. R2A medium was chosen to mimic the nutrient-restricted conditions on environmental surfaces in tree fruit packing facilities. Strains were further sub-cultured as described in the following sections.

Table 1
Bacterial strains used in this study.

Family	Strain	Species [a]
Flavobacteriaceae	PS02336	*Flavobacterium* spp.
	PS02337	*Flavobacterium* spp.
	PS02338	*Flavobacterium* spp.
Microbacteriaceae	PS01271	*Curtobacterium* spp.
	PS01859	*Gulosibacter massiliensis*
	PS02066	*Microbacterium* spp.
	PS02068	*Microbacterium* spp.
	PS02072	*Microbacterium* spp.
	PS02292	*Agrococcus* spp.
Listeriaceae	PS01272	*Listeria monocytogenes* (lineage I, IVb variant, serotype 4b)
	PS01277	*Listeria monocytogenes* (lineage II, IIa, serotype 1/2a and 3a)
	PS01278	*Listeria monocytogenes* (lineage I, IIb, serotype 1/2b, 3b, and 7)
	PS01281	*Listeria monocytogenes* (lineage II, IIa, serotype 1/2a and 3a)
	PS01291	*Listeria monocytogenes* (lineage I IVb, serotype 4b, 4d, and 4e)
	PS01293	*Listeria monocytogenes* (lineage III)
	PS01295	*Listeria monocytogenes* (lineage III)
Pseudomonadaceae	PS01270	*Pseudomonas atacamensis*
	PS01297	*Pseudomonas helleri*
	PS01301	*Pseudomonas coleopterorum*
	PS01303	*Pseudomonas rustica*
	PS01856	*Pseudomonas paracarnis*
	PS02288	*Pseudomonas kuykendallii*
	PS02302	*Pseudomonas* spp.
	PS02303	*Pseudomonas mandelii*
Xanthomonadaceae	PS02289	*Stenotrophomonas* spp.
	PS02297	*Stenotrophomonas nitritireducens*
	PS02298	*Stenotrophomonas* spp.
	PS02299	*Stenotrophomonas maltophila*
	PS02300	*Stenotrophomonas* spp.
	PS02301	*Stenotrophomonas* spp.
	PS02304	*Xanthomonas* spp.
	PS02340	*Luteimonas* spp.

[a] Isolates were identified using whole genome sequence analyses as reported in Rolon et al. (2024). Lineage and serotype information is provided for each *L. monocytogenes* isolate.

2.2. Antimicrobial susceptibility assay

The minimal inhibitory concentrations (MIC) of PAA and a commercial biofilm removing product were determined for all bacterial isolates using the broth microdilution assay (CLSI, 2009). The PAA and BAC are parent compounds of sanitizer products and were used in this study to mitigate the effects of other compounds added to commercial sanitizers, as those may vary among different commercial products. However, given that the chemical composition of the biofilm remover was proprietary, we used the commercial product as is. MICs of BAC have been reported in our previous study (Rolon et al., 2024). Stock solutions of PAA (4000 ppm; Pfaltz & Bauer, Waterbury, CT) and the biofilm remover (17.2 ppm of active ingredients; Boost 3200 & 3201, Ecolab, Saint Paul, MN) were prepared in Mueller Hinton (MH) broth (BD, Franklin Lakes, NJ) and sterilized by filtration using a 0.2 μm cellulose filter (Whatman Membranes, Cytiva, Marlborough, MA). Stock solutions were then two-fold diluted in MH broth in a 96-well microtiter plate to achieve a range of concentrations from 1000–3.9 ppm for PAA and from 4.3 to 0.2 ppm of active ingredients for the biofilm remover. One isolated colony of each bacterial strain was suspended in MH broth and incubated at 30 °C with shaking at 200 rpm until the turbidity exceeded an optical density at 600 nm (OD_{600}) of 0.2, as measured with an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). Following the incubation, cultures were diluted in MH broth to achieve a final concentration of ~$5 * 10^5$ CFU/mL in the assay plate. Four wells of the microtiter plate containing the bacterial culture in MH broth without antimicrobial were used as positive controls to verify the growth of each

strain. Four additional wells containing only MH broth were used as negative controls to assess potential contamination. Microtiter plates were incubated at 30 °C for 24 h. The temperature of incubation was modified from the CLSI standard method, since some bacterial strains did not grow at 35 °C. The MIC for each antimicrobial was defined as the lowest concentration of an antimicrobial that resulted in no microbial growth (CLSI, 2009) and was determined by visual inspection and measurement of the OD_{600} using a Synergy Neo2 microplate reader (BioTek, Thermo Fisher Scientific, Waltman, NJ). Each test was conducted in at least two independent biological replicates, each with two technical replicates.

2.3. Die-off kinetics of L. monocytogenes in single- and multi-family assemblage biofilms exposed to benzalkonium chloride and peroxyacetic acid

Single- and multi-family assemblage biofilms were prepared as previously described (Rolon et al., 2024). Briefly, one colony of each bacterial strain was suspended in R2A broth (Neogen, Lansing, MI) and incubated at the optimal growth temperature for each strain (i.e., 20 or 30 °C) until the turbidity of the culture exceeded an OD_{600} of 0.2. Each culture was then adjusted to a concentration of ~1 * 10^7 CFU/mL by dilution in R2A broth. The concentration of each culture was confirmed by spiral plating (easySpiral, Interscience, Saint Nom la Brétèche, France) onto R2A agar plates and incubation at 30 °C for 72 h. Bacterial cultures were then combined in equal volumes into single- and multi-family assemblages in a full factorial design, resulting in 16 treatments (Table 2). Five wells of a Minimal Biofilm Eradication Concentration (MBEC) assay plates (Innovotech, Edmonton, Canada) were filled with 150 µl of each assemblage. Additionally, 16 wells containing 150 µl of sterile R2A broth were used as negative controls. Inoculated MBEC assay plates were incubated for 3 days at 15 °C, with medium replacement every 24 h. This incubation temperature for biofilm growth was selected as it was the mean temperature measured in tree fruit packing facilities (Rolon et al., 2023; Tan et al., 2019).

Table 2
Single- and multi-family assemblages tested for tolerance to sanitizers.

Treatment[a]	Assemblage	Assemblage Complexity
L	*L. monocytogenes*	Single family
L + P	*L. monocytogenes* + *Pseudomonadaceae*	Two-family
L + X	*L. monocytogenes* + *Xanthomonadaceae*	
L + M	*L. monocytogenes* + *Microbacteriaceae*	
L + F	*L. monocytogenes* + *Flavobacteriaceae*	
L + P + X	*L. monocytogenes* + *Pseudomonadaceae* + *Xanthomonadaceae*	Three-family
L + P + M	*L. monocytogenes* + *Pseudomonadaceae* + *Microbacteriaceae*	
L + P + F	*L. monocytogenes* + *Pseudomonadaceae* + *Flavobacteriaceae*	
L + X + M	*L. monocytogenes* + *Xanthomonadaceae* + *Microbacteriaceae*	
L + X + F	*L. monocytogenes* + *Xanthomonadaceae* + *Flavobacteriaceae*	
L + M + F	*L. monocytogenes* + *Microbacteriaceae* + *Flavobacteriaceae*	
L + P + X + M	*L. monocytogenes* + *Pseudomonadaceae* + *Xanthomonadaceae* + *Microbacteriaceae*	Four-family
L + P + X + F	*L. monocytogenes* + *Pseudomonadaceae* + *Xanthomonadaceae* + *Flavobacteriaceae*	
L + P + M + F	*L. monocytogenes* + *Pseudomonadaceae* + *Microbacteriaceae* + *Flavobacteriaceae*	
L + X + M + F	*L. monocytogenes* + *Xanthomonadaceae* + *Microbacteriaceae* + *Flavobacteriaceae*	
L + P + X + M + F	*L. monocytogenes* + *Pseudomonadaceae* + *Xanthomonadaceae* + *Microbacteriaceae* + *Flavobacteriaceae*	Five-family

[a] Microbial assemblage abbreviations: F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*.

To assess the tolerance of *L. monocytogenes* to sanitizers, single- and multi-family assemblage biofilms were exposed to 250 ppm PAA, 500 ppm PAA, or 200 ppm BAC. Experiments were conducted in at least 2 independent biological replicates, each with 5 technical replicates. PAA and BAC (Spectrum, New Brunswick, NJ) solutions were prepared in sterile 0.85% NaCl solution to mitigate bacterial osmotic stress and sterile filtered using a 0.2 µm cellulose filter (Whatman Membranes, Cytiva, Marlborough, MA). MBEC peg lids with grown biofilms were rinsed by submerging a lid in sterile 0.85% NaCl for 10 s. The rinsed peg lids were then transferred to a 96-well plate pre-filled with 200 µl PAA (250 or 500 ppm) or BAC solution (200 ppm) per well and incubated at 15 °C for 0, 15, 30, or 60 min for 250 ppm PAA, for 0, 2, 15, and 30 min for 200 ppm BAC, and for 0, 2, 10, and 15 min for 500 ppm PAA. Given the observed tolerance of *L. monocytogenes* in biofilms to 250 ppm PAA and 12.5 ppm BAC (Rolon et al., 2024) compared to planktonic cultures, we tested whether increasing the concentrations to 500 ppm PAA and 200 ppm BAC would reduce *L. monocytogenes'* tolerance in biofilms. Preliminary experiments indicated a faster die-off at these higher concentrations, prompting us to include additional sampling time points before 15 min and to conclude the experiments earlier.

The time points for exposure to different concentrations of sanitizers were informed by preliminary experiments (data not shown). After the PAA or BAC exposure, biofilms were rinsed by submersion in sterile 0.85% NaCl for 10 s, transferred to Dey Engley (DE) neutralizing broth (BD, Franklin Lakes, NJ), and sonicated for 30 min (VWR, Radnor, PA) to release biofilm biomass for further quantification of aerobic mesophilic microbiota and *L. monocytogenes*. The suspensions of five technical replicates per treatment were pooled prior to quantification of microbial concentration. To quantify the total concentration of aerobic bacteria, the released biomass was ten-fold diluted in 0.85% NaCl solution, spread plated onto R2A agar, and incubated for 72 h at 30 °C. A miniaturized most probable number (MPN) assay was used to quantify *L. monocytogenes* (Rolon et al., 2024). The MPN method was used to mitigate the interference of other microbiota with the quantification of *L. monocytogenes* and to promote the recovery of sub-lethally injured or dormant cells that may not be detected by direct plating. Briefly, the released biomass was 10-fold diluted 8 times in 900 µl of Buffered *Listeria* Enrichment broth (BLEB) (Criterion, Santa Maria, CA) in triplicate in 96-deep well plates (VWR, Radnor, PA) and incubated for 4 h at 30 °C. Subsequently, each well was supplemented with 4 µl of *Listeria* Selective Enrichment Supplement (90 mg acriflavine, 450 mg cycloheximide, and 360 mg sodium nalidixic acid in 40 mL of sterile deionized water) (Sigma Aldrich, St. Lois, MO) and incubated at 30 °C for an additional 44 h. After the incubation, 2.5 µl of enrichments were spot-inoculated onto agar *Listeria* according to Ottavani & Agosti (ALOA) (Biomerieux, Marcy-l'Étoile, France) and incubated for 24 h at 37 °C. The number of positive dilutions for *L. monocytogenes* were counted and used to calculate MPN/peg using the MPN calculator available on the FDA Bacteriological Analytical Manual (BAM) (Blodgett, 2020).

The USDA Integrated Pathogen Modelling Program (v. 2013) (Huang, 2014) was used to fit the log-linear with tail and the Weibull models to the *L. monocytogenes* die-off kinetic data. The goodness-of-fit of the models was evaluated with the Root Mean Square Error (RMSE) and the Akaike's Information Criterium (AIC), which allowed for the selection of the best model. The tolerance of *L. monocytogenes* to PAA and BAC in multi-family assemblage biofilms was defined as the time required to achieve a 2-log reduction in *L. monocytogenes* concentration (Brauner et al., 2016), also known as the minimum duration for killing (MDK99). MDK99 was calculated from the best fitting modeled curves.

2.4. Die-off kinetics of L. monocytogenes in planktonic cultures of single- and multi-family assemblages exposed to peroxyacetic acid

Planktonic cultures of single- and multi-family assemblages were prepared to test their effect on the tolerance of *L. monocytogenes* to PAA.

Each bacterial strain was grown in R2A broth and incubated at 20 or 30 °C until the turbidity of the culture reached an OD_{600} of >0.2. The cultures were centrifuged for 5 min at 16,000 g (Avanti J-26 XPI, Beckman Coulter, USA) and the pellets were resuspended and adjusted in sterile 0.85% NaCl to ~1 * 10^7 CFU/mL. Saline solution was used for inoculum preparation instead of R2A broth to mitigate the potential neutralizing effect of R2A broth on the antimicrobial activity of PAA, which was not required in the biofilm assay due to biofilm rinsing. The concentration of each bacterial suspension was verified by spiral plating onto R2A agar plates (easySpiral automatic plater, Interscience, Saint Nom la Brétèche, France) followed by incubation at 30 °C for 72 h. Single- and multi-family assemblages were prepared by mixing equal volumes of each strain. Planktonic cultures of single- and multi-family assemblages (i.e., 2 mL) were exposed to PAA (250 ppm) for 0, 15, 30, or 60 min at 15 °C, followed by the addition of 2 mL double-strength DE neutralizing buffer. BAC was not tested on planktonic cultures as it has been tested and reported in our previous work (Rolon et al., 2024). The concentration of total aerobic bacteria and *L. monocytogenes* present in biofilms and planktonic cultures was quantified as described in the previous section. The experiment was performed in 3 independent experiments, each with 1 biological replicate.

2.5. The effect of a biofilm remover on biofilm disruption and *L. monocytogenes* reduction in single- and multi-family assemblage biofilms

Single- and multi-family assemblages of biofilms grown as outlined above (section 2.3) were exposed to the biofilm remover prepared in the ratio of 1:1:8 disinfectant:activator:water (as recommended by the product label), which was equivalent to 137 ppm of active ingredients. The biofilm remover was prepared in sterile 0.85% NaCl and sterilized by filtration. Biofilms were rinsed by submersion in 0.85% NaCl for 10 s and were transferred to a 96-well plate pre-filled with 200 µl per well of the prepared biofilm remover. Biofilms were incubated in the biofilm remover solution at 15 °C for 0 and 10 min, equivalent to the contact time recommended by the product label. The amount of biofilms on pegs before and after exposure to the biofilm remover was quantified using the crystal violet assay as previously described (Rolon et al., 2024). Briefly, the MBEC pegs lids containing the biofilms were rinsed for 10 s with sterile 0.85 % NaCl solution, transferred to a sterile 96-well plate base, and heat fixed for 40 min at 65 °C in a Multi-Therm microplate shaker (Benchmark Scientific, Sayreville, NJ). A 1% w/v solution of crystal violet (200 µl; Ward's Science, Rochester, NY) was added to each well and incubated at room temperature for 45 min, followed by two 10 s rinses with sterile 0.85 % NaCl solution and de-staining with 95 % ethanol for 15 min. The amount of crystal violet retained by the biofilms was quantified by measuring the absorbance of the crystal violet-ethanol solution at 570 nm on a microplate reader (BioTek Synergy Neo2 Hybrid Multi-Mode Microplate Reader, Thermo Fisher Scientific, Waltman, NJ). The experiment was conducted in three independent biological replicates. The total aerobic mesophilic bacteria and *L. monocytogenes* present in the biofilms before and after exposure to the biofilm remover were quantified as described in a previous section. Furthermore, the biofilm was quantified using the crystal violet assay before and after exposure to the biofilm remover, as described previously.

2.6. Statistical analysis

All statistical analyses were conducted in R v.4.3.2 (R Core Team, 2023). Given that the datasets did not meet the ANOVA assumptions ($p < 0.05$), a non-parametric Kruskal-Wallis test was applied to assess the effect of BAC and PAA on the total aerobic mesophilic bacteria and *L. monocytogenes* in a biofilm and a planktonic culture using the R package rstatix, followed by the Dunn's multiple comparison post-hoc test using the R package FSA v.0.9.5 (Ogle et al., 2023). A two-tailed *t*-test was used to assess whether the presence of *Pseudomonadaceae* in multi-family assemblages affected the tolerance of *L. monocytogenes* to the BAC and PAA, and to assess the effect of a biofilm remover on the total biofilm quantity, aerobic mesophilic bacteria, and *L. monocytogenes* concentration using the R package stats v.4.4.0 (R Core Team, 2023). All results are presented as mean ± standard error of the mean.

3. Results

3.1. *Flavobacteriaceae* were most susceptible to peroxyacetic acid and *Pseudomonadaceae* were least susceptible to a biofilm remover

Minimum inhibitory concentrations of PAA for *L. monocytogenes* and environmental microbiota isolates ranged between 7.8 and 250 ppm and varied among taxonomic families. The MIC of PAA for all seven tested *L. monocytogenes* strains (PS01272, PS01277, PS01278, PS01281, PS01291, PS01293, PS01295), five strains of *Pseudomonadaceae* (PS01270, PS01297, PS01301, PS01303, PS01856), and four strains of *Microbacteriaceae* (PS01271, PS01859, PS02066, PS02072) was 250 ppm (Table 3). The PAA MIC for the rest of *Microbacteriaceae* (n = 2) and *Pseudomonadaceae* (n = 3) strains, as well as three strains of *Xanthomonadaceae* (PS02297, PS02299, and PS02301) was 125 ppm (Table 3). The remaining *Xanthomonadaceae* (n = 5) strains had a PAA MIC of 62.5 ppm (Table 3). The PAA MIC for *Flavobacteriaceae* strains was the lowest among all the tested bacteria, ranging between 31.3 ppm (PS02336, PS02338) and 7.8 ppm (PS02337) (Table 3). The MICs for BAC were reported in our previous study (Rolon et al., 2024). Briefly, BAC MICs for *L. monocytogenes* ranged from 1.56 to 3.12 ppm, for *Flavobacteriaceae*

Table 3
Minimal inhibitory concentrations of peroxyacetic acid (PAA) and the commercial biofilm remover for *L. monocytogenes* and environmental strains.

Family	Strain	Species	MIC (ppm)	
			PAA	Biofilm remover
Flavobacteriaceae	PS02336	*Flavobacterium* spp.	31.3	0.27
	PS02337	*Flavobacterium* spp.	7.8	0.27
	PS02338	*Flavobacterium* spp.	31.2	0.07
Microbacteriaceae	PS01271	*Curtobacterium* spp.	250	0.13
	PS01859	*Gulosibacter massiliensis*	250	0.07
	PS02066	*Microbacterium* spp.	250	0.07
	PS02068	*Microbacterium* spp.	125	0.27
	PS02072	*Microbacterium* spp.	250	0.13
	PS02292	*Agrococcus* spp.	125	0.13
Listeriaceae	PS01272	*Listeria monocytogenes*	250	0.27
	PS01277	*Listeria monocytogenes*	250	0.27
	PS01278	*Listeria monocytogenes*	250	0.07
	PS01281	*Listeria monocytogenes*	250	0.13
	PS01291	*Listeria monocytogenes*	250	0.07
	PS01293	*Listeria monocytogenes*	250	0.27
	PS01295	*Listeria monocytogenes*	250	0.07
Pseudomonadaceae	PS01270	*Pseudomonas atacamensis*	250	0.53
	PS01297	*Pseudomonas helleri*	250	0.53
	PS01301	*Pseudomonas coleopterorum*	250	0.53
	PS01303	*Pseudomonas rustica*	250	0.53
	PS01856	*Pseudomonas paracarnis*	250	2.1
	PS02288	*Pseudomonas kuykendallii*	125	0.27
	PS02302	*Pseudomonas* spp.	125	0.53
	PS02303	*Pseudomonas mandelii*	125	0.27
Xanthomonadaceae	PS02289	*Stenotrophomonas* spp.	62.5	0.13
	PS02297	*Stenotrophomonas nitritireducens*	125	0.07
	PS02298	*Stenotrophomonas* spp.	62.5	0.13
	PS02299	*Stenotrophomonas maltophila*	125	0.27
	PS02300	*Stenotrophomonas* spp.	62.5	0.13
	PS02301	*Stenotrophomonas* spp.	125	0.13
	PS02304	*Xanthomonas* spp.	62.5	0.07
	PS02340	*Luteimonas* spp.	62.5	0.13

between 6.25 and 12.5 ppm, for *Microbacteriaceae* from 1.25 to 12.5 ppm, for *Xanthomonadaceae* from 3.12 to 25 ppm, and for *Pseudomonadaceae* from 12.5 to 100 ppm (Rolon et al., 2024).

MICs of the commercial biofilm remover for the tested bacteria ranged between 0.07 and 2.10 ppm of active ingredients. *Pseudomonadaceae* strains generally had higher MICs compared to the other tested isolates, with MIC values of 2.10 (n = 1), 0.53 (n = 5), and 0.27 ppm (n = 2) (Table 3). The MICs of the biofilm remover for the tested *L. monocytogenes* ranged from 0.27 (n = 1), 0.13 ppm (n = 3), to 0.07 ppm (n = 3) (Fig. 1). MICs for *Flavobacteriaceae* strains were 0.27 (n = 2) or 0.07 (n = 1) ppm (Table 3), and for strains of *Microbacteriaceae* 0.07 (n = 3), 0.13 (n = 2) ppm, or 0.27 (n = 1) ppm. The biofilm remover MICs for *Xanthomonadaceae* strains ranged from 0.07 (n = 2), 0.13 (n = 5) to 0.27 (n = 1) ppm (Table 3).

3.2. Multifamily biofilms containing Pseudomonadaceae increased the tolerance of L. monocytogenes to 250 and 500 ppm peroxyacetic acid

To assess whether the presence of environmental microbiota attenuates the die-off kinetics of *L. monocytogenes* due to exposure to PAA, we exposed single- and multi-family assemblage biofilms and planktonic cultures to 250 ppm PAA and quantified *L. monocytogenes* and total aerobic bacteria during a 1-h exposure. In planktonic single- and multi-family assemblages, the concentration of total bacteria (Fig. 1A) and *L. monocytogenes* (Fig. 1B) declined below the limit of quantification within 15 min of exposure to PAA. Similarly, in *L. monocytogenes* biofilm and in multi-family biofilms without *Pseudomonadaceae*, the concentration of total aerobic mesophilic bacteria (Fig. 1A) and *L. monocytogenes* (Fig. 1B) declined below the limit of quantification within 15 min of exposure to PAA (Fig. 1A). In contrast, biofilms formed by multi-family assemblages that contained *Pseudomonadaceae* exhibited an increased tolerance to PAA; a 1-h exposure to PAA was required to reduce the total aerobic mesophilic bacteria (Fig. 1A) and *L. monocytogenes* (Fig. 1B) below the limit of quantification. To assess the tolerance of *L. monocytogenes* to PAA, the die-off curves in multi-family biofilm assemblages were modeled using the log linear model with tail or the Weibull model, and the time required to reduce the concentration of *L. monocytogenes* by two logarithmic units (i.e., MDK99) was calculated based on the best fitting model (Tables S1, S2, and S3). Overall, when PAA was applied at 250 ppm, the time necessary to reduce the population of *L. monocytogenes* by two logs in the biofilms containing *Pseudomonadaceae* ranged between 12 and 22 min, whereas in the assemblages without *Pseudomonadaceae* the MDK99 was less than 1 min or below the limit of quantification within 15 min of exposure (Table 4).

To determine whether *L. monocytogenes* in multi-family biofilm assemblages can tolerate higher concentrations of PAA, we repeated the experiment by exposing biofilms to 500 ppm of PAA (Fig. 3A). Indeed, biofilm assemblages containing *Pseudomonadaceae* required a longer exposure to 500 ppm PAA to reduce the concentration of *L. monocytogenes* below the limit of quantification, compared to all other tested biofilm assemblages (Fig. 2A). In contrast, *L. monocytogenes* was reduced below the limit of quantification within 2 min of exposure to 500 ppm of PAA in biofilm assemblages without *Pseudomonadaceae* (Fig. 2A). The log linear model with tail or the Weibull model were fitted to the die-off data to determine the MDK99 for *L. monocytogenes* upon exposure to 500 ppm of PAA. Between 2 and 4 min of exposure to 500 ppm of PAA was required to reduce the concentration of *L. monocytogenes* by two logarithmic units in biofilms containing *Pseudomonadaceae* (Table 4).

3.3. Multi-family biofilms containing Pseudomonadaceae significantly increased the tolerance of L. monocytogenes to 200 ppm benzalkonium chloride

We previously reported the effect of 12.5 ppm of BAC on the die-off kinetics of *L. monocytogenes* in single and multi-family assemblages (Rolon et al., 2024). Here, we assessed the effect of higher concentrations of BAC (200 ppm), which is similar to the recommended concentrations for sanitizing food processing facilities (Merchel Piovesan Pereira and Tagkopoulos, 2019; Møretrø et al., 2017; Poimenidou et al., 2016). In single-family biofilms composed of *L. monocytogenes* strains (i.e., labeled as L) and in 2-family biofilms composed of *L. monocytogenes* and *Flavobacteriaceae* (i.e., L + F), the concentration of *L. monocytogenes* declined below the limit of quantification within 2 min of exposure to 200 ppm BAC and MDK99 was not possible to calculate (Fig. 2B). In contrast, in all other multi-family assemblage biofilms, *L. monocytogenes* exhibited an increased tolerance to 200 ppm BAC compared to the biofilm comprised of just *L. monocytogenes* (Fig. 2B). Specifically, the time required to reduce *L. monocytogenes* population by two logs (i.e., MDK99) in multi-family biofilms ranged from less than 1 min to 21 min, depending on the microbial composition of the biofilm (Table 4). *L. monocytogenes* had a significantly higher tolerance to 200 ppm BAC in all biofilm assemblages that contained *Pseudomonadaceae* (i.e., MDK99 ranging from 9 to over 21 min), compared to the biofilm assemblages without *Pseudomonadaceae* (i.e., MDK99 of less than 1–7 min) ($p = 0.0001$).

3.4. A commercial biofilm remover significantly disrupted all biofilms containing Pseudomonadaceae, 2-family biofilm of L. monocytogenes and Xanthomonadaceae, and 4-family biofilms of L. monocytogenes, Xanthomonadaceae, Microbacteriaceae, and Flavobacteriaceae

To assess the disruptive effect of a biofilm removing product, we exposed single- and multi-family biofilms to a biofilm remover for 10 min at the concentration recommended by the manufacturer. The amount of biofilm disruption due to the application of the biofilm remover was quantified with the crystal violet assay, which stains both cells and biofilm matrix components. A 10-min application of the biofilm remover resulted in a significant reduction of biofilm assemblages containing *Pseudomonadaceae*, the biofilm composed of *L. monocytogenes* and *Xanthomonadaceae* (i.e., L + X), and the biofilm composed of *L. monocytogenes*, *Xanthomonadaceae*, *Microbacteriaceae*, and *Flavobacteriaceae* (i.e., L + X + M + F) ($p < 0.04$) (Fig. 3A). Furthermore, the biofilm remover reduced total aerobic bacteria by 4.9–6.5 $\log_{10}$ CFU/peg (Fig. 3B) and *L. monocytogenes* by 5.0–6.2 $\log_{10}$ MPN/peg (Fig. 3C). However, a 10-min treatment with the biofilm remover did not reduce *L. monocytogenes* below the limit of quantification in 4 biofilm assemblages containing *Pseudomonadaceae* (i.e., L + P, L + P + F, L + P + X + M, and L + P + M + F) (Fig. 3C), suggesting a protective effect of *Pseudomonadaceae.*

4. Discussion

4.1. Environmental microbiota from tree fruit packing facilities differed in their susceptibility to peroxyacetic acid and a commercial biofilm remover

The efficacy of sanitizers is critically important for the control of *L. monocytogenes* in food processing facilities. Antimicrobial susceptibility is typically assessed using a broth microdilution assay that provides information about the minimal inhibitory concentration (MIC) of an antimicrobial (Bland et al., 2022; Rodríguez-Melcón et al., 2021). We found that the MICs of PAA were similar among *L. monocytogenes, Microbacteriaceae,* and *Pseudomonadaceae,* but were lower for *Flavobacteriaceae* and *Xanthomonadaceae.* Previous studies reported PAA MICs for *L. monocytogenes* ranging from 100 to 2000 ppm (Alonso-Hernando et al., 2009; Rodríguez-Melcón et al., 2021), whereas we found that all tested *L. monocytogenes* strains had an MIC of 250 ppm. Since the maximum recommended concentration of PAA in food processing environmental surfaces is twice the reported MIC for *L. monocytogenes* strains in this study (i.e., 500 ppm) (CFR 21§173, 2023), our results highlight an increased risk for sanitation failure when controlling *L. monocytogenes*, since sanitizers may be inadvertently

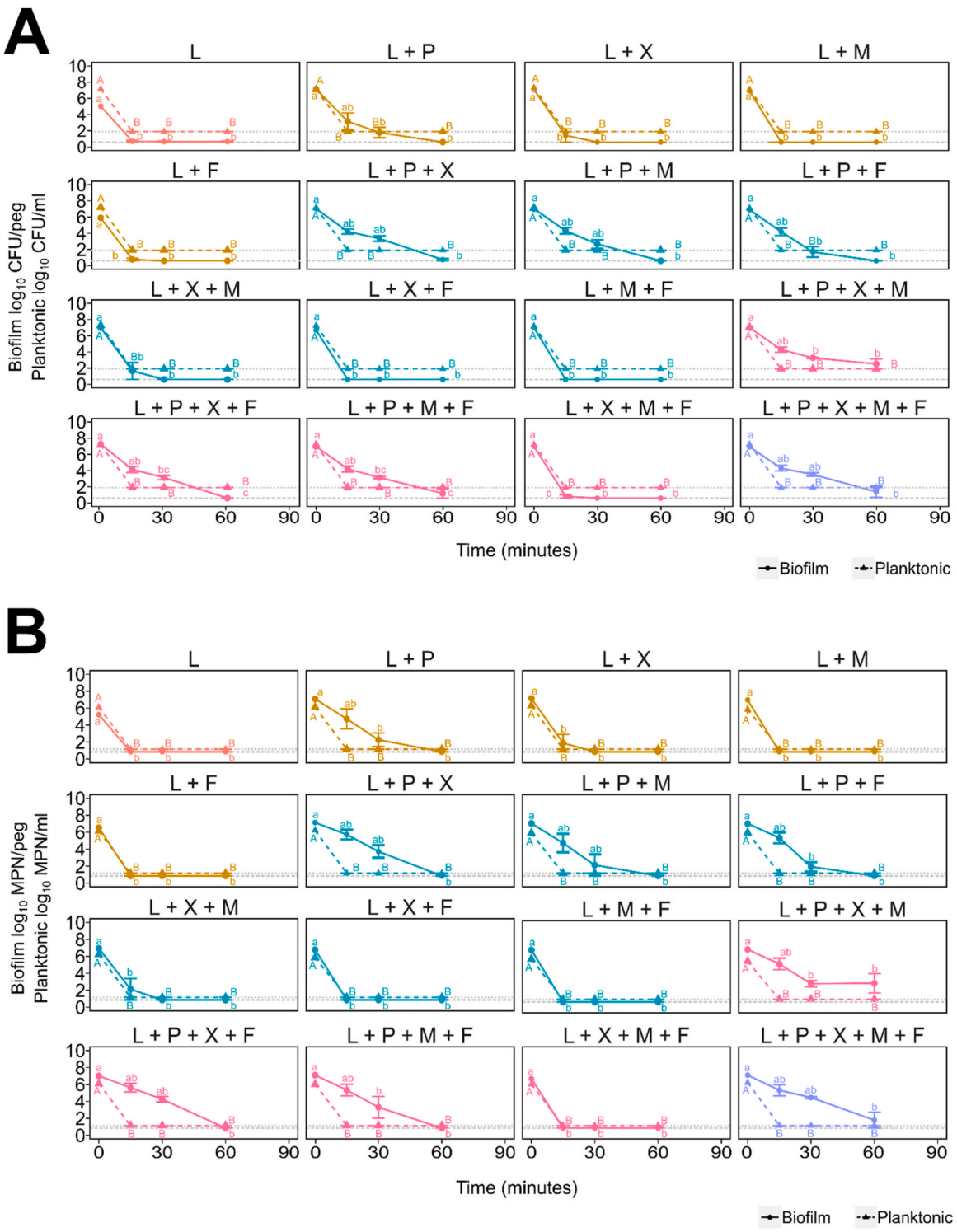

Fig. 1. Microbial die-off kinetics in multi-family assemblages due to exposure to peroxyacetic acid. Reduction in total aerobic mesophilic bacteria (A) and *L. monocytogenes* (B) concentration in biofilms (circles with full lines) and planktonic cultures (triangles with dashed lines) exposed to 250 ppm of peroxyacetic acid (PAA). Data points represent the mean ± standard error of data collected in three independent biological replicates. Data for each microbial assemblage is presented in a separate panel and is color coded based on the number of families in an assemblage. The significance of the reduction in microbial concentration was assessed separately for biofilms and planktonic cultures. In each panel, different uppercase or lowercase letters indicate statistical significance ($\alpha = 0.05$) of differences in the bacterial concentration of planktonic or biofilm assemblages, respectively, as determined using the Kruskal-Wallis test followed by *post-hoc* Dunn's test. Dashed and dotted grey line represents the limit of quantification of the aerobic plate count method (0.6 $\log_{10}$ CFU/peg or 1.9 $\log_{10}$ CFU/mL) and of the most probable number method (0.9 $\log_{10}$ MPN/peg or 1.1 $\log_{10}$ MPN/mL) for biofilms and planktonic cultures, respectively. Microbial assemblages are labeled as follows: F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Tolerance of *L. monocytogenes* in multi-family assemblage biofilms after exposure to 250 and 500 ppm of peroxyacetic acid (PAA) and 200 ppm of benzalkonium chloride (BAC).

Assemblage[a]	PAA 250 ppm		PPA 500 ppm		BAC 200 ppm	
	Best model[b]	MDK99 (min)[c]	Best model[b]	MDK99 (min)[c]	Best model[b]	MDK99 (min)[c]
L	–	–	–	–	–	–
L + P	Log linear	13	Log linear	2	Log linear	9
L + X	Weibull	<1	–	–	Log linear	7
L + M	–	–	–	–	Weibull	<1
L + F	–	–	–	–	–	–
L + P + X	Log linear	18	Log linear	2	Log linear	9
L + P + M	Log linear	12	Log linear	2	Weibull	18
L + P + F	Log linear	12	Log linear	2	Log linear	21
L + X + M	–	–	–	–	Weibull	3
L + X + F	–	–	–	–	Log linear	2
L + M + F	–	–	–	–	Weibull	<1
L + P + X + M	Log linear	15	Log linear	2	Weibull	14
L + P + X + F	Weibull	22	Log linear	4	Weibull	19
L + P + M + F	Log linear	16	Log linear	2	Log linear	17
L + X + M + F	–	–	–	–	Weibull	2
L + P + X + M + F	Log linear	20	Log linear	2	Weibull	17

[a] Labels for microbial assemblage treatments: F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*.
[b] Indicates whether the log linear model with tail or the Weibull model was the best fit for the inactivation data, as assessed using the Akaike's Information Criteria (Table S1, Table S2, Table S3).
[c] MDK99, the time required to achieve a 2-log reduction in *L. monocytogenes* concentration, also known as the minimum duration for killing 99% of the population.

diluted after their application in wet food processing environments. The susceptibility of the *Pseudomonadaceae* strains tested in our study was lower compared to MICs of 600 to 20,000 ppm reported for *P. fluorescens, P. lundensis, P. putida, P. libanensis,* and *P. veronii* isolated from a salmon processing facility (Thomassen et al., 2023).

In contrast to relatively high MICs of tested strains to PAA, our work shows that MICs of the commercial biofilm remover for the tested strains (i.e., 0.07–2.10 ppm of active ingredients) were substantially below the manufacturer recommended application concentration (i.e., 137 ppm of active ingredients) for sanitation of non-food contact surfaces (ECOLAB, 2014). In general, isolates of *Flavobacteriaceae*, *Microbacteriaceae*, *Xanthomonadaceae*, and *L. monocytogenes* were more sensitive to the biofilm remover (MICs from 0.07 ppm to 0.27 ppm) compared to the *Pseudomonadaceae* strains (MICs from 0.27 ppm to 2.1 ppm). The biofilm remover used in this study is a new-generation sanitizer based on PerQuat technology containing quaternary ammonium compounds (QAC) and hydrogen peroxide as active antimicrobial ingredients (Sterilex, 2024). The MICs for QAC based-products are relatively high for *Pseudomonas* spp., typically displaying MICs above 50 ppm (Merchel Piovesan Pereira and Tagkopoulos, 2019). In our previous study, we determined that the *Pseudomonadaceae* strains had MICs to BAC ranging from 12.5 to 100 ppm (Rolon et al., 2024). Thus, we hypothesize that the resistance of *Pseudomonas* bacteria to QAC sanitizers may be the cause of the increased tolerance to the tested biofilm remover.

4.2. The presence of Pseudomonadaceae in multi-species biofilms increased the tolerance of L. monocytogenes to peroxyacetic acid and benzalkonium chloride

Formation of biofilms by environmental microbiota can enhance the survival and persistence of *L. monocytogenes* in food processing facilities by shielding bacteria from the antimicrobial action of sanitizers (Fagerlund et al., 2017; Li et al., 2021; Pang and Yuk, 2019; Sanchez-Vizuete et al., 2015). We observed that the presence of *Pseudomonadaceae* in multi-family assemblage biofilms increased the tolerance (i.e., reduced the die-off kinetics) of *L. monocytogenes* upon exposure to 250 ppm PAA, 500 ppm PAA, and 200 ppm BAC, representing concentrations typically applied in food processing facilities. Similarly, multi-species biofilms containing five strains of *Pseudomonas* spp. reduced the die-off kinetics of *L. monocytogenes* when exposed to 500 ppm PAA for 15 min, compared to *L. monocytogenes*-only biofilm (Thomassen et al., 2023). Previous studies have focused on investigating changes in the survival of *L. monocytogenes* in multi-species biofilms, without quantifying potential changes in the tolerance to sanitizer exposure (Fagerlund et al., 2017; Saá Ibusquiza et al., 2012). For example, biofilms made of *P. putida* and *L. monocytogenes* displayed a significant increase in the concentration of BAC required to reduce the population of *L. monocytogenes* by 90% (Saá Ibusquiza et al., 2012), and the minimum biofilm eradication concentration (MBEC) of BAC against *Listeria monocytogenes* has been reported to increase from 6.2 ± 1.4 to 58.3 ± 7.5 ppm when co-cultured with *Pseudomonas fluorescens* (Haddad et al., 2021).

Pseudomonas spp. strains are efficient biofilm producers (Mann and Wozniak, 2012; Sanchez-Vizuete et al., 2015; Wagner et al., 2020), and *L. monocytogenes,* as a relatively poor biofilm producer, can leverage the protection provided by a *Pseudomonas* biofilm matrix in stressful conditions, such as under antimicrobial pressure (Rodríguez-Melcón et al., 2021). The tolerance of multi-species biofilms to sanitizers can be attributed to multiple factors including matrix structure and interspecies interactions, along with transition to persister cell physiology (Knudsen et al., 2013). However, the specific mechanisms underlying this tolerance are still poorly understood, due to the complexity and heterogeneity of biofilm structures. Generally, species residing within a biofilm can interact through competition, coaggregation, and metabolic cross-feeding (Burmølle et al., 2014; Ren et al., 2015; Røder et al., 2015b; Tuytschaever et al., 2023). For example, coaggregation of bacteria of different species may promote or enhance EPS matrix synthesis and increase the biofilm population. Furthermore, the high cell density in biofilms can promote an accumulation of the quorum sensing molecules necessary for the coordination of biofilm development, self-organization, and cell cooperation for the production of EPS matrix and extracellular enzymes (Irie et al., 2012; Karygianni et al., 2020; Puga et al., 2018). Given that each species in the biofilm may produce different EPS molecules, the resulting biofilm EPS matrix may become more viscous, reducing diffusion rates for the antimicrobial substances used during sanitizing operations (Mann and Wozniak, 2012). Furthermore, the enzymes produced by different species may act synergistically against antimicrobials benefiting the non-producing species (Aqel et al., 2023; Bridier and Briandet, 2022). Community-level protection in mixed-species biofilm has been shown to be affected by the spatial organization of the community members. For example, *L. monocytogenes* was able to penetrate into preestablished *P. fluorescens* biofilms which

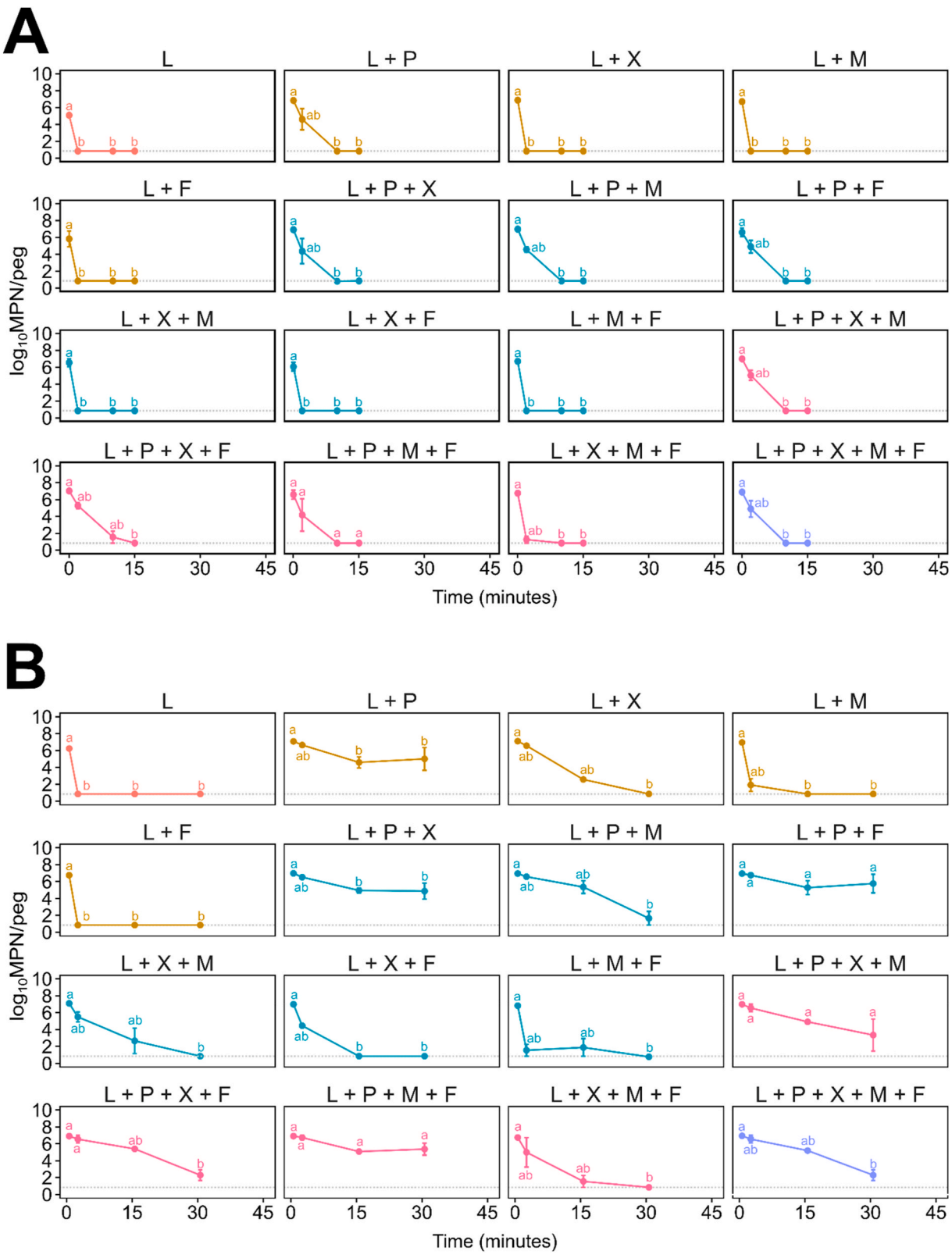

Fig. 2. Tolerance of *L. monocytogenes* to high concentrations of sanitizers in single- and multi-family biofilm assemblages. Reduction in the concentration of *L. monocytogenes* biofilms exposed to 500 ppm of peroxyacetic acid (PAA) (A) or 200 ppm of benzalkonium chloride (BAC) (B). Data points represent the mean ± standard error of two independent biological replicates. Data for each microbial assemblage is presented in an independent panel and is color coded based on the number of families in an assemblage. In each panel, different lowercase letters indicate statistical differences ($\alpha = 0.05$) in the bacterial concentration as determined using the Kruskal-Wallis test followed by *post-hoc* Dunn's test. The dotted grey line represents the limit of quantification of the most probable number method (0.9 $\log_{10}$ MPN/peg). Labels of microbial assemblage treatments: F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

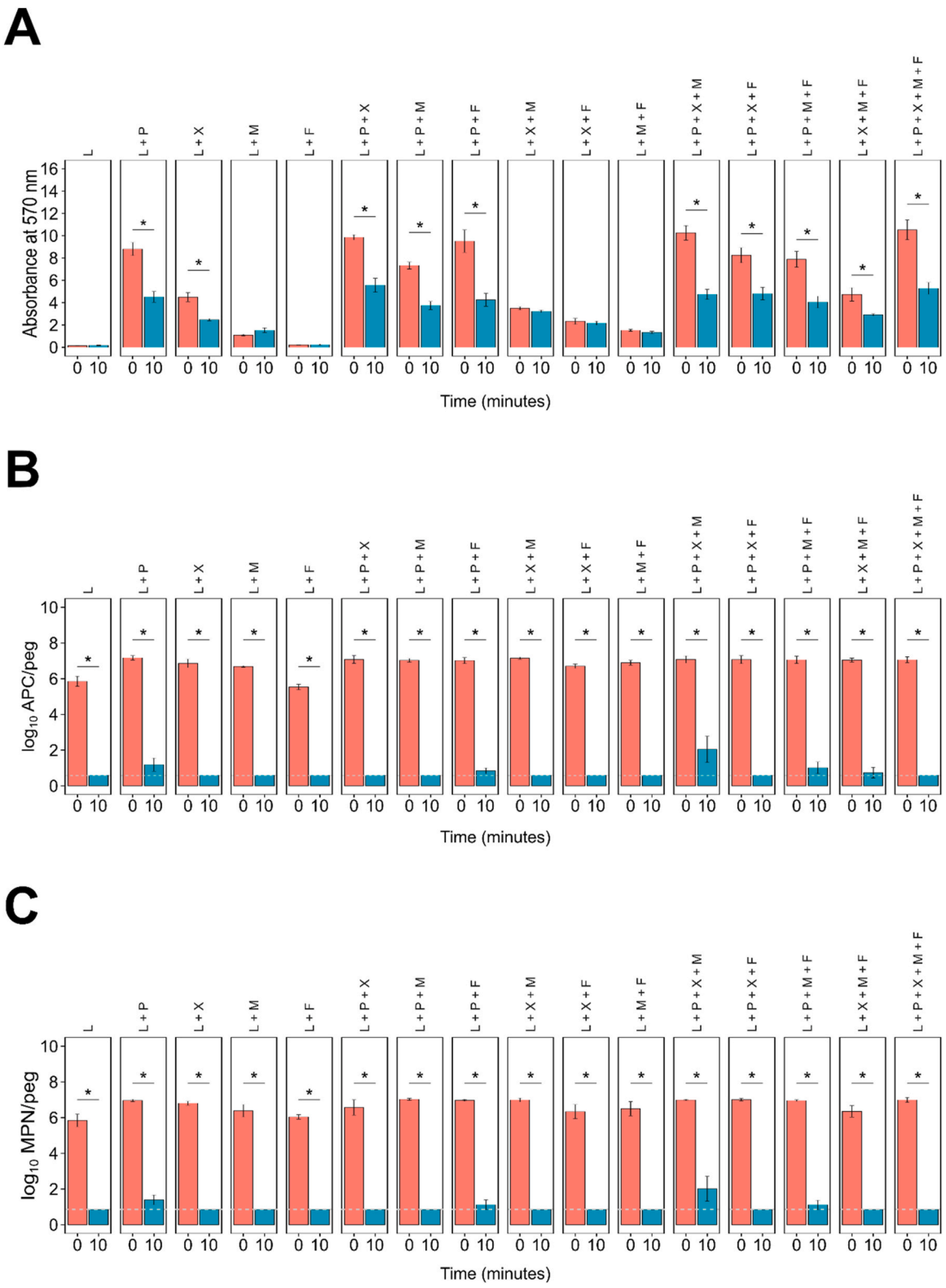

Fig. 3. Microbial reduction in and disruption of single- and multi-family biofilm assemblages treated with a biofilm remover. Biofilm disruption as measured by the crystal violet assay (A), reduction in total aerobic mesophilic bacteria (B) and *L. monocytogenes* (C) concentration in biofilms exposed to 137 ppm (active ingredients) of a commercial biofilm remover. Data points represent the mean ± standard error of three independent biological replicates, each conducted with 5 technical replicates. An asterisk indicates statistically significant differences ($\alpha = 0.05$) in the bacterial concentration, as determined using a *t*-test. The dotted grey line represents the limit of quantification of the aerobic plate count method (0.6 $\log_{10}$ CFU/peg) and the most probable number method (0.9 $\log_{10}$ MPN/peg). The composition of microbial assemblages is labeled as follows: F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

led to matrix over-production and accumulation of *Listeria* cells in the bottom layers of the biofilms (Puga et al., 2018). Due to significant gaps in the understanding of biofilm-mediated tolerance, further research is needed to understand the mechanisms by which the tolerance of *L. monocytogenes* to sanitizers is enhanced in multi-species biofilms containing *Pseudomonadaceae*.

4.3. A commercial biofilm remover significantly disrupted all biofilms containing Pseudomonadaceae, 2-family biofilm of L. monocytogenes and Xanthomonadaceae, and 4-family biofilms of L. monocytogenes, Xanthomonadaceae, Microbacteriaceae, and Flavobacteriaceae

Due to the importance of controlling biofilms in food processing facilities, we tested the ability of a commercial biofilm remover to disrupt single- and multi-family biofilm assemblages. The biofilm remover significantly disrupted biofilms formed by assemblages containing *Pseudomonadaceae* but did not completely eliminate them. Given that the crystal violet assay used to quantify total biofilm biomass stains cells and EPS matrix components (Azeredo et al., 2017), it is likely that some residual EPS components were not completely removed from the surfaces of the pegs. Furthermore, while the biofilm remover significantly reduced *L. monocytogenes* by more than five logarithmic units in all multi-family assemblage biofilms, the pathogen was not reduced below the limit of detection in some biofilm assemblages containing *Pseudomonadaceae* after the recommended 10-min exposure time. Nonetheless, the reduction in *L. monocytogenes* achieved by the biofilm remover should be sufficient since it is unlikely that a food processing facility environment would harbor over five logs of *L. monocytogenes*. A previous study tested the effect of the same biofilm remover product on a 7-day *L. monocytogenes*-only biofilm and showed that the use of 5% and 10% biofilm remover (equivalent to 0.62–1.26% active ingredients) reduced a *L. monocytogenes* biofilm below the limit of detection, (>6 log reduction) in 2.5 and 1-min exposure, respectively (Aryal and Muriana, 2019). However, they did not measure the effect of other microbiota commonly present in food processing environments along with *L. monocytogenes*.

4.4. Limitations

All experiments reported here were conducted under laboratory conditions, using microbiological media that may not resemble the conditions in tree fruit packing facilities. Further, use of the MBEC device to grow multi-family biofilms does not represent the diversity of materials or the dynamic conditions in tree fruit processing facilities that contribute to biofilm formation. Additional research is therefore needed to develop a more realistic biofilm model to further characterize the effect of environmental conditions on the tolerance of *L. monocytogenes* to BAC and PAA. Furthermore, our findings need to be validated using commercial sanitizing products.

4.5. Conclusions

Understanding the effect of environmental microbiota on the efficacy of sanitizers is critically important for establishing effective cleaning and sanitizing practices for the control of *L. monocytogenes*. Here, we demonstrated an increased tolerance of *L. monocytogenes* to benzalkonium chloride and peroxyacetic acid at concentrations commonly used in food processing facilities when integrated in multi-family biofilms containing *Pseudomonas* spp. While it is important to assess the efficacy of sanitizers against target foodborne pathogens, our findings highlight the need to assess sanitizer efficacy in the context of the microbiota that exists in food processing environments. Given the common presence of *Pseudomonas* spp. in food processing environments, it is recommended to assess sanitizer efficacy on both *L. monocytogenes* and *Pseudomonas*. Lastly, the mechanisms underlying the observed increase in the tolerance of *L. monocytogenes* to sanitizers in the presence of *Pseudomonas* spp. in biofilms warrant further investigation to inform the design of targeted sanitation approaches.

CRediT authorship contribution statement

Olena Voloshchuk: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **M. Laura Rolon:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Katelyn V. Bartlett:** Writing – review & editing, Investigation. **Marysabel Mendez Acevedo:** Writing – review & editing, Investigation. **Luke F. LaBorde:** Writing – review & editing, Funding acquisition. **Jasna Kovac:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Data and code availability

Supplementary Tables S1, S2, and S3 and scripts used for data analysis and bioinformatics are available on GitHub: https://github.com/LauRolon/BiofilmsAndTolerance_PAA.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2024.104687.

References

Alonso-Hernando, A., Capita, R., Prieto, M., Alonso-Calleja, C., 2009. Adaptation and cross-adaptation of Listeria monocytogenes and Salmonella enterica to poultry decontaminants. J. Microbiol. 47 (2), 142–146. https://doi.org/10.1007/s12275-008-0237-5.

Aqel, H., Sannan, N., Foudah, R., Al-Hunaiti, A., 2023. Enzyme production and inhibitory potential of Pseudomonas aeruginosa: contrasting clinical and environmental isolates. Antibiotics 12 (9), 1354. https://doi.org/10.3390/antibiotics12091354.

Aryal, M., Muriana, P.M., 2019. Efficacy of commercial sanitizers used in food processing facilities for inactivation of Listeria monocytogenes, E. Coli O157:H7, and Salmonella biofilms. Foods 8 (12), 639. https://doi.org/10.3390/foods8120639.

Azeredo, J., Azevedo, N.F., Briandet, R., Cerca, N., Coenye, T., Costa, A.R., Desvaux, M., Di Bonaventura, G., Hébraud, M., Jaglic, Z., Kačániová, M., Knøchel, S., Lourenço, A., Mergulhão, F., Meyer, R.L., Nychas, G., Simões, M., Tresse, O., Sternberg, C., 2017. Critical review on biofilm methods. Crit. Rev. Microbiol. 43 (3), 313–351. https://doi.org/10.1080/1040841X.2016.1208146.

Bland, R., Brown, S.R.B., Waite-Cusic, J., Kovacevic, J., 2022. Probing antimicrobial resistance and sanitizer tolerance themes and their implications for the food industry through the *Listeria monocytogenes* lens. Compr. Rev. Food Sci. Food Saf. 21 (2), 1777–1802. https://doi.org/10.1111/1541-4337.12910.

Blodgett, R., 2020. BAM appendix 2: most probable number from serial dilutions. Bacteriological Analytical Manual. https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions.

Brauner, A., Fridman, O., Gefen, O., Balaban, N.Q., 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat. Rev. Microbiol. 14 (5), 320–330.

Bridier, A., Briandet, R., 2022. Microbial biofilms: structural plasticity and emerging properties. Microorganisms 10 (1), 138. https://doi.org/10.3390/microorganisms10010138.

Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., Jackson, T.C., Whiting, R.C., 2017. A review of Listeria monocytogenes: an update on outbreaks, virulence, dose-response, ecology, and risk assessments. Food Control 75, 1–13. https://doi.org/10.1016/j.foodcont.2016.12.016.

Burmølle, M., Ren, D., Bjarnsholt, T., Sørensen, S.J., 2014. Interactions in multispecies biofilms: do they actually matter? Trends Microbiol. 22 (2), 84–91. https://doi.org/10.1016/j.tim.2013.12.004.

CDC, 2024a. Multistate foodborne outbreak notices. Multistate Foodborne Outbreak Notices. https://www.cdc.gov/foodborne-outbreaks/active-investigations/all-foodborne-outbreak-notices.html.

CDC, 2024b. 2014 Outbreak of Listeria Infections Linked to Commercially Produced, Prepackaged Caramel Apples Made from Bidart Bros. Apples. https://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html.

CFR 21§173.315, 2023. 21CFR173 315. https://www.govinfo.gov/content/pkg/CFR-2002-title21-vol3/xml/CFR-2002-title21-vol3-sec173-315.xml.

Chen, Y., Luo, Y., Pettengill, J., Timme, R., Melka, D., Doyle, M., Jackson, A., Parish, M., Hammack, T.S., Allard, M.W., Brown, E.W., Strain, E.A., 2017. Singleton sequence type 382, an emerging clonal group of Listeria monocytogenes associated with three multistate outbreaks linked to contaminated stone fruit, caramel apples, and leafy green salad. J. Clin. Microbiol. 55 (3), 931–941. https://doi.org/10.1128/JCM.02140-16.

Chen, Y., Simonetti, T., Peter, K., Jin, Q., Brown, E., LaBorde, L.F., Macarisin, D., 2022. Genetic diversity of Listeria monocytogenes isolated from three commercial tree fruit packinghouses and evidence of persistent and transient contamination. Front. Microbiol. 12, 756688. https://doi.org/10.3389/fmicb.2021.756688.

CLSI, 2009. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically100-Ed34:2024 Performance Standards for Antimicrobial Susceptibility Testing, 34th Edition, pp. M07–A8, Version 29.2.

De Noordhout, Devleesschauwer, B., Angulo, F.J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., Speybroeck, N., 2014. The global burden of listeriosis: A systematic review and meta-analysis. The Lancet Infectious Diseases 14 (11), 1073–1082. https://doi.org/10.1016/S1473-3099(14)70870-9.

ECOLAB, 2014. Boost 3200. https://en-mx.ecolab.com/offerings/boost-3200-3201.

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 (17), e01046. https://doi.org/10.1128/AEM.01046-17, 17.

Fan, Y., Qiao, J., Lu, Z., Fen, Z., Tao, Y., Lv, F., Zhao, H., Zhang, C., Bie, X., 2020. Influence of different factors on biofilm formation of Listeria monocytogenes and the regulation of cheY gene. Food Res. Int. 137, 109405. https://doi.org/10.1016/j.foodres.2020.109405.

Haddad, S., Elliot, M., Savard, T., Deschênes, L., Smith, T., Ells, T., 2021. Variations in biofilms harbouring Listeria monocytogenes in dual and triplex cultures with Pseudomonas fluorescens and Lactobacillus plantarum produced under a model system of simulated meat processing conditions, and their resistance to benzalkonium chloride. Food Control 123, 107720. https://doi.org/10.1016/j.foodcont.2020.107720.

Huang, L., 2014. Ipmp 2013—a comprehensive data analysis tool for predictive microbiology. Int. J. Food Microbiol. 171, 100–107.

Irie, Y., Borlee, B.R., O'Connor, J.R., Hill, P.J., Harwood, C.S., Wozniak, D.J., Parsek, M. R., 2012. Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 109 (50), 20632–20636. https://doi.org/10.1073/pnas.1217993109.

Karygianni, L., Ren, Z., Koo, H., Thurnheer, T., 2020. Biofilm matrixome: extracellular components in structured microbial communities. Trends Microbiol. 28 (8), 668–681. https://doi.org/10.1016/j.tim.2020.03.016.

Knudsen, G.M., Ng, Y., Gram, L., 2013. Survival of bactericidal antibiotic treatment by a persister subpopulation of Listeria monocytogenes. Appl. Environ. Microbiol. 79 (23), 7390–7397. https://doi.org/10.1128/AEM.02184-13.

Li, Q., Liu, L., Guo, A., Zhang, X., Liu, W., Ruan, Y., 2021. Formation of multispecies biofilms and their resistance to disinfectants in food processing environments: a review. J. Food Protect. 84 (12), 2071–2083. https://doi.org/10.4315/JFP-21-071.

Mann, E.E., Wozniak, D.J., 2012. *Pseudomonas* biofilm matrix composition and niche biology. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 36 (4), 893–916. https://doi.org/10.1111/j.1574-6976.2011.00322.x.

Merchel Piovesan Pereira, B., Tagkopoulos, I., 2019. Benzalkonium chlorides: uses, regulatory status, and microbial resistance. Appl. Environ. Microbiol. 85 (13), e00377. https://doi.org/10.1128/AEM.00377-19, 19.

Mohapatra, R.K., Mishra, S., Tuglo, L.S., Sarangi, A.K., Kandi, V., Al Ibrahim, A.A., Alsaif, H.A., Rabaan, A.A., Zahan, Md K., 2024. Recurring food source-based *Listeria* outbreaks in the United States: an unsolved puzzle of concern? Health Science Reports 7 (2), e1863. https://doi.org/10.1002/hsr2.1863.

Møretrø, T., Schirmer, B.C.T., Heir, E., Fagerlund, A., Hjemli, P., Langsrud, S., 2017. Tolerance to quaternary ammonium compound disinfectants may enhance growth of Listeria monocytogenes in the food industry. Int. J. Food Microbiol. 241, 215–224. https://doi.org/10.1016/j.ijfoodmicro.2016.10.025.

Ogle, D.H., Doll, J.C., Wheeler, A.P., Dinno, A., 2023. FSA: Simple Fisheries Stock Assessment Methods [R], Version 0.9.5.

Osek, J., Lachtara, B., Wieczorek, K., 2022. Listeria monocytogenes – how this pathogen survives in food-production environments? Front. Microbiol. 13, 866462. https://doi.org/10.3389/fmicb.2022.866462.

Pang, X., Yuk, H.-G., 2019. Effects of the colonization sequence of Listeria monocytogenes and Pseudomonas fluorescens on survival of biofilm cells under food-related stresses and transfer to salmon. Food Microbiol. 82, 142–150. https://doi.org/10.1016/j.fm.2019.02.002.

Poimenidou, S.V., Chrysadakou, M., Tzakoniati, A., Bikouli, V.C., Nychas, G.-J., Skandamis, P.N., 2016. Variability of Listeria monocytogenes strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. Int. J. Food Microbiol. 237, 164–171. https://doi.org/10.1016/j.ijfoodmicro.2016.08.029.

Puga, C.H., Dahdouh, E., SanJose, C., Orgaz, B., 2018. Listeria monocytogenes colonizes Pseudomonas fluorescens biofilms and induces matrix over-production. Front. Microbiol. 9, 1706. https://doi.org/10.3389/fmicb.2018.01706.

R Core Team, 2023. R: A Language and Environment for Statistical Computing [R], Version 4.3.2.

Reis-Teixeira, F.B.D., Alves, V.F., De Martinis, E.C.P., 2017. Growth, viability and architecture of biofilms of Listeria monocytogenes formed on abiotic surfaces. Braz. J. Microbiol. 48 (3), 587–591. https://doi.org/10.1016/j.bjm.2017.01.004.

Ren, D., Madsen, J.S., Sørensen, S.J., Burmølle, M., 2015. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. ISME J. 9 (1), 81–89. https://doi.org/10.1038/ismej.2014.96.

Røder, H.L., Raghupathi, P.K., Herschend, J., Brejnrod, A., Knøchel, S., Sørensen, S.J., Burmølle, M., 2015a. Interspecies interactions result in enhanced biofilm formation by co-cultures of bacteria isolated from a food processing environment. Food Microbiol. 51, 18–24. https://doi.org/10.1016/j.fm.2015.04.008.

Røder, H.L., Raghupathi, P.K., Herschend, J., Brejnrod, A., Knøchel, S., Sørensen, S.J., Burmølle, M., 2015b. Interspecies interactions result in enhanced biofilm formation by co-cultures of bacteria isolated from a food processing environment. Food Microbiol. 51, 18–24. https://doi.org/10.1016/j.fm.2015.04.008.

Rodríguez-Melcón, C., Alonso-Calleja, C., García-Fernández, C., Carballo, J., Capita, R., 2021. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for twelve antimicrobials (biocides and antibiotics) in eight strains of Listeria monocytogenes. Biology 11 (1), 46. https://doi.org/10.3390/biology11010046.

Rolon, M.L., Tan, X., Chung, T., Gonzalez-Escalona, N., Chen, Y., Macarisin, D., LaBorde, L.F., Kovac, J., 2023. The composition of environmental microbiota in three tree fruit packing facilities changed over seasons and contained taxa indicative of L. monocytogenes contamination. Microbiome 11 (1), 128. https://doi.org/10.1186/s40168-023-01544-8.

Rolon, M.L., Voloshchuk, O., Bartlett, K.V., LaBorde, L.F., Kovac, J., 2024. Multi-species biofilms of environmental microbiota isolated from fruit packing facilities promoted tolerance of Listeria monocytogenes to benzalkonium chloride. Biofilm 7, 100177. https://doi.org/10.1016/j.bioflm.2024.100177.

Ruiz-Llacsahuanga, B., Hamilton, A., Zaches, R., Hanrahan, I., Critzer, F., 2021. Prevalence of *Listeria* species on food contact surfaces in Washington state apple packinghouses. Appl. Environ. Microbiol. 87 (9), e02932. https://doi.org/10.1128/AEM.02932-20, 20.

Saá Ibusquiza, P., Herrera, J.J.R., Cabo, M.L., 2011. Resistance to benzalkonium chloride, peracetic acid and nisin during formation of mature biofilms by Listeria monocytogenes. Food Microbiol. 28 (3), 418–425. https://doi.org/10.1016/j.fm.2010.09.014.

Saá Ibusquiza, P., Herrera, J.J.R., Vázquez-Sánchez, D., Cabo, M.L., 2012. Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by Listeria monocytogenes and Pseudomonas putida. Food Control 25 (1), 202–210. https://doi.org/10.1016/j.foodcont.2011.10.002.

Sanchez-Vizuete, P., Orgaz, B., Aymerich, S., Le Coq, D., Briandet, R., 2015. Pathogens protection against the action of disinfectants in multispecies biofilms. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00705.

Sauer, K., Stoodley, P., Goeres, D.M., Hall-Stoodley, L., Burmølle, M., Stewart, P.S., Bjarnsholt, T., 2022. The biofilm life cycle: expanding the conceptual model of biofilm formation. Nat. Rev. Microbiol. 20 (10), 608–620. https://doi.org/10.1038/s41579-022-00767-0.

Silk, B.J., Date, K.A., Jackson, K.A., Pouillot, R., Holt, K.G., Graves, L.M., Ong, K.L., Hurd, S., Meyer, R., Marcus, R., Shiferaw, B., Norton, D.M., Medus, C., Zansky, S.M., Cronquist, A.B., Henao, O.L., Jones, T.F., Vugia, D.J., Farley, M.M., Mahon, B.E., 2012. Invasive listeriosis in the foodborne diseases active surveillance Network (FoodNet), 2004–2009: further targeted prevention needed for higher-risk groups. Clin. Infect. Dis. 54 (Suppl. l_5), S396–S404. https://doi.org/10.1093/cid/cis268.

Simonetti, T., Peter, K., Chen, Y., Zhang, G., LaBorde, L., Macarisin, D., 2021. Prevalence and distribution of Listeria monocytogenes in three commercial tree fruit packinghouses. Front. Microbiol. 12.

Sterilex, 2024. *PerQuat® technology*. Sterilex. https://www.sterilex.com/solution/perquat-technology/.

Sullivan, G., Orsi, R.H., Estrada, E., Strawn, L., Wiedmann, M., 2022. Whole-genome sequencing-based characterization of *Listeria* isolates from produce packinghouses and fresh-cut facilities suggests both persistence and reintroduction of fully virulent L. Monocytogenes. Appl. Environ. Microbiol. 88 (22), e01177. https://doi.org/10.1128/aem.01177-22, 22.

Tan, X., Chung, T., Chen, Y., Macarisin, D., LaBorde, L., Kovac, J., 2019. The occurrence of Listeria monocytogenes is associated with built environment microbiota in three tree fruit processing facilities. Microbiome 7 (1), 115. https://doi.org/10.1186/s40168-019-0726-2.

Thomassen, G.M.B., Reiche, T., Hjørungnes, M., Mehli, L., 2023. High disinfectant tolerance in Pseudomonas spp. biofilm aids the survival of Listeria monocytogenes. Microorganisms 11 (6), 1414. https://doi.org/10.3390/microorganisms11061414.

Tuytschaever, T., Raes, K., Sampers, I., 2023. *Listeria monocytogenes* in food businesses: from persistence strategies to intervention/prevention strategies—a review. Compr.

Rev. Food Sci. Food Saf. 22 (5), 3910–3950. https://doi.org/10.1111/1541-4337.13219.

Wagner, E.M., Pracser, N., Thalguter, S., Fischel, K., Rammer, N., Pospíšilová, L., Alispahic, M., Wagner, M., Rychli, K., 2020. Identification of biofilm hotspots in a meat processing environment: detection of spoilage bacteria in multi-species biofilms. Int. J. Food Microbiol. 328, 108668. https://doi.org/10.1016/j.ijfoodmicro.2020.108668.

Zilelidou, E.A., Skandamis, P.N., 2018. Growth, detection and virulence of Listeria monocytogenes in the presence of other microorganisms: microbial interactions from species to strain level. Int. J. Food Microbiol. 277, 10–25. https://doi.org/10.1016/j.ijfoodmicro.2018.04.011.