Biofilm 7 (2024) 100177

Contents lists available at ScienceDirect

Biofilm

journal homepage: www.sciencedirect.com/journal/biofilm

Multi-species biofilms of environmental microbiota isolated from fruit packing facilities promoted tolerance of *Listeria monocytogenes* to benzalkonium chloride

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ARTICLE INFO

Keywords:
Listeria monocytogenes
Biofilms
Environmental microbiomes
Microbiota
Antimicrobial tolerance
Sanitizers
Benzalkonium chloride
Pseudomonadaceae
Microbacteriaceae
Xanthomonadaceae
Flavobacteriaceae

ABSTRACT

Listeria monocytogenes may survive and persist in food processing environments due to formation of complex multi-species biofilms of environmental microbiota that co-exists in these environments. This study aimed to determine the effect of selected environmental microbiota on biofilm formation and tolerance of *L. monocytogenes* to benzalkonium chloride in formed biofilms. The studied microbiota included bacterial families previously shown to co-occur with *L. monocytogenes* in tree fruit packing facilities, including *Pseudomonadaceae, Xanthomonadaceae, Microbacteriaceae*, and *Flavobacteriaceae*. Biofilm formation ability and the effect of formed biofilms on the tolerance of *L. monocytogenes* to benzalkonium chloride was measured in single- and multi-family assemblages. Biofilms were grown statically on polystyrene pegs submerged in a R2A broth. Biofilm formation was quantified using a crystal violet assay, spread-plating, confocal laser scanning microscopy, and its composition was assessed using amplicon sequencing. The concentration of *L. monocytogenes* in biofilms was determined using the most probable number method. Biofilms were exposed to the sanitizer benzalkonium chloride, and the death kinetics of *L. monocytogenes* were quantified using a most probable number method. A total of 8, 8, 6, and 3 strains of *Pseudomonadaceae, Xanthomonadaceae, Microbacteriaceae*, and *Flavobacteriaceae*, respectively, were isolated from the environmental microbiota of tree fruit packing facilities and were used in this study. Biofilms formed by *Pseudomonadaceae, Xanthomonadaceae*, and all multi-family assemblages had significantly higher concentration of bacteria, as well as *L. monocytogenes,* compared to biofilms formed by *L. monocytogenes* alone. Furthermore, multi-family assemblage biofilms increased the tolerance of *L. monocytogenes* to benzalkonium chloride compared to *L. monocytogenes* mono-species biofilms and planktonic multi-family assemblages.

These findings suggest that *L. monocytogenes* control strategies should focus not only on assessing the efficacy of sanitizers against *L. monocytogenes,* but also against biofilm-forming microorganisms that reside in the food processing built environment, such as *Pseudomonadaceae* or *Xanthomonadaceae*.

1. Introduction

Listeria monocytogenes is a bacterial pathogen that causes listeriosis, a deadly foodborne disease [1]. In the United States, approximately 1,600 listeriosis cases are reported annually, resulting in 260 deaths [2]. In recent years, tree fruits and other produce have emerged as potential vehicles for *L. monocytogenes*. For example, in 2015, a listeriosis outbreak investigation traced *L. monocytogenes* clinical isolates to the consumption of caramel-coated apples with outbreak strain present in the fruit packing environment [3,4]. Previous research has shown that *L. monocytogenes* can persist in food processing environment for years, resulting in the potential for recurrent contamination of food [5]. One of the mechanisms thought to enhance the survival and persistence of *L. monocytogenes* in food processing environments is through formation of biofilms [6]. While planktonic cells of *L. monocytogenes* are typically susceptible to the cleaning and sanitizing protocols applied in food processing facilities, *L. monocytogenes* may survive sanitizer treatment when enclosed in biofilms. The latter may occur due to the slower sanitizer diffusion into the biofilm and the potential transition of cells to a more antimicrobial-tolerant physiological state [6,7]. It has been

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https://doi.org/10.1016/j.bioflm.2024.100177
Received 7 November 2023; Received in revised form 6 January 2024; Accepted 8 January 2024
Available online 14 January 2024

previously shown that *L. monocytogenes*' monocultures can form biofilms on glass, stainless steel, and polystyrene; however, these structures were thin and weak [6,8]. Nevertheless, *L. monocytogenes* is typically surrounded by other environmental microorganisms in food processing-built environments. The presence of other microorganisms can potentially facilitate formation of robust multi-species biofilms that could increase the tolerance of *L. monocytogenes* to sanitizers, enhancing its survival and persistence [6]. Past studies have shown that when *L. monocytogenes* is cultivated in mixed cultures with *Pseudomonas* spp., biofilm production is enhanced compared to *L. monocytogenes* grown in a monoculture [9,10].

Formation of multi-species biofilms may enhance the survival of *L. monocytogenes* to the action of sanitizers used during the cleaning and sanitizing of food processing facilities [6]. Microbial survival under antimicrobial pressure can be facilitated by two complementary mechanisms: (i) antimicrobial resistance provided by the products of antimicrobial resistance genes or mutations; and (ii) antimicrobial tolerance, which increases microbial survival to an antimicrobial treatment in a transient manner, primarily due to slow growth or reduced metabolic activity [11–15]. Antimicrobial resistance is determined by measuring the minimum inhibitory concentration (MIC), while antimicrobial tolerance is determined by measuring the time required to achieve a 2-log reduction in a microbial population [11]. While an isolate grown in a planktonic form can have the same MIC as an isolate grown in a biofilm, the latter may have increased tolerance to antimicrobials, requiring a longer exposure to achieve a 2-log reduction of the microbial population.

Antimicrobial tolerance in biofilms can occur through multiple mechanisms, including (i) a reduced diffusion rate of antimicrobials into the biofilm structure [12,15], (ii) a breakdown of antimicrobial chemicals into non-inhibitory compounds [16] and (iii) a transition to a persister cell physiology [17], a nearly dormant state in which bacterial cells are more tolerant to antimicrobial treatments [18,19]. Multispecies biofilms have been shown to have an increased tolerance to antimicrobial agents, such as sanitizers, when compared to monoculture biofilms [20–23]. These findings highlight a potentially important yet understudied role of environmental microbiota in the survival and persistence of *L. monocytogenes*. In a previous longitudinal study on the occurrence of *L. monocytogenes* in tree fruit packing facilities, we found that microbiota belonging to taxonomic families *Pseudomonadaceae, Microbacteriaceae, Xanthomonadaceae*, and *Flavobacteriaceae* co-occurred with *L. monocytogenes*. This suggested a potential role of these microbiota in facilitating the persistence of *L. monocytogenes* in the studied food processing facilities [24]. Given that the identified taxa are known biofilms formers [25–27], we hypothesized that formation of multispecies biofilms would facilitate tolerance of *L. monocytogenes* to commonly used sanitizers, such as benzalkonium chloride (BAC). Therefore, the goal of this study was to assess the effect of muti-family assemblage biofilms on the tolerance of *L. monocytogenes* to BAC.

2. Results

2.1. Isolation, identification, and selection of environmental microbiota

Environmental microbiome samples were collected from non-food-contact surfaces of three tree fruit packing facilities located in the Northeastern U.S. A total of 901 bacterial strains were isolated, and of these, 510 isolates were randomly selected for identification using Sanger sequencing of the PCR-amplified 16S rRNA gene region. Isolation with the massively parallel isolation system Prospector yielded 248 identified isolates from taxonomic families *Enterobacteriaceae* (n = 100), *Erwiniaceae* (n = 45), *Microbacteriaceae* (n = 2), and *Pseudomonadaceae* (n = 101) (Fig. S1). Isolation of slow-growing bacteria on Reasoner's 2A (R2A) agar plates yielded an additional 95 identified isolates from 21 distinct taxonomic families, including *Microbacteriaceae* (n = 45), *Pseudomonadaceae* (n = 6), and *Xanthomonadaceae* (n = 7) (Fig. S1). Further identification of yellow colonies (typical for *Flavobacteriaceae*) grown on R2A yielded 109 isolates from 15 distinct taxonomic families, including *Microbacteriaceae* (n = 46), *Pseudomonadaceae* (n = 1), *Flavobacteriaceae* (n = 6), and *Xanthomonadaceae* (n = 10) (Fig. S1). Selective isolation using *Xanthomonas* agar and *Stenotrophomonas* agar yielded additional 28 and 30 bacterial isolates, respectively, from 12 distinct taxonomic families, including the target families *Microbacteriaceae* (n = 1), *Pseudomonadaceae* (n = 7), and *Xanthomonadaceae* (n = 22) (Fig. S1).

Preliminary taxonomic identification at the genus level was carried out by constructing maximum likelihood trees based on the alignments of isolates' Sanger sequences with the 16S rRNA gene sequences of all type-strains of target families available in the Ribosomal Database Project (RDP) database [28]. We detected 1, 13, 4, and 2 genera of *Pseudomonadaceae, Microbacteriaceae, Xanthomonadaceae*, and *Flavobacteriaceae*, respectively. Among these isolates, 8 strains of *Pseudomonadaceae*, 6 strains of *Microbacteriaceae*, 8 strains of *Xanthomonadaceae*, and 3 strains of *Flavobacteriaceae* differed among each other in 2 or more single nucleotide polymorphisms (SNPs) within the V4 region of the 16S rRNA gene sequence, which was required for strain tracking in biofilm experiments. These strains were whole genome sequenced to identify them at a taxonomic species level by using Type (Strain) Genome Server [29] (Table 1). Given that genome-based taxonomic databases lack the genomes of many environmental species, isolates that were not a confident match with a species in a database were assigned a genus name of the closest organism in the database.

Among *Pseudomonadaceae*, 7 out of 8 strains were confidently identified as distinct species of *Pseudomonas,* whereas strain PS02302 was not identified beyond the genus level (Table 1). In the *Microbacteriaceae* family, only 1 out of 6 isolates (PS01859, *Gulosibacter massiliensis*) was confidently identified at the species level. Among the remaining 5 isolates, 3 were identified as *Microbacterium* spp., 1 as *Agrococcus* spp., and 1 as *Curtobacterium* spp. (Table 1). In the *Xanthomonadaceae* family, 2 out of 8 isolates were confidently identified as distinct species of *Stenotrophomonas* (Table 1). Of the remaining isolates, 4 were identified as *Stenotrophomonas* spp., 1 as *Xanthomonas* spp., and one as *Luteimonas* spp. (Table 1). Lastly, all three *Flavobacteriaceae* isolates were identified as *Flavobacterium* spp. (Table 1).

2.2. Environmental microbiota from tree fruit packing facilities differed in their antimicrobial resistance to benzalkonium chloride

The MIC of BAC for the tested environmental strains (Table 1), including *L. monocytogenes* strains (Table 2) was quantified using a broth microdilution assay. *L. monocytogenes* strains had an MIC of 1.56 (n = 5) or 3.12 (n = 1) ppm, except for PS01278, which had a higher MIC of 6.25 ppm (Fig. 1a). Strains of *Flavobacteriaceae* had an MIC of 6.25 (n = 2) or 12.5 (n = 1) ppm, strains of *Microbacteriaceae* had an MIC of 1.25 (n = 1), 6.25 (n = 3) or 12.5 (n = 2) ppm., and strains of *Xanthomonadaceae* had an MIC of 3.12 (n = 2), 6.25 (n = 4), or 25 (n = 2) ppm. In contrast, *Pseudomonadaceae* strains generally had a substantially higher MICs to BAC, ranging from 12.5 (n = 1), 50 (n = 4), to 100 (n = 3) ppm (Fig. 1a).

To determine the genomic potential of isolates to resist BAC, the presence of previously reported genes that may confer resistance to BAC was bioinformatically assessed by analyzing their draft genomes (Fig. 1b). Only one *L. monocytogenes* strain (PS01278) possessed a *qac*C gene, which encodes a small multidrug resistance (SMR) efflux pump. Other strains of *L. monocytogenes* did not possess any genes known to confer resistance to BAC. Three *Flavobacteriaceae* and three *Xanthomonadaceae* (PS02289, PS02299, and PS02301) isolates carried the *emrE* gene, which encodes a multidrug transporter that has been shown to confer resistance to BAC in *L. monocytogenes* [30–32]. The *qac*A gene, a major facilitator super family (MFS) efflux transporter was detected in 4 *Microbacteriaceae* (PS01271, PS02066, PS02068, and PS02072), in 2 *Pseudomonadaceae* (PS01301 and PS02302), and in 3 *Xanthomonadaceae*

Table 1
Selected environmental isolates collected from tree fruit packing facilities.

Taxonomic family	Strain	Species identification	dDDH d_4[a]	NCBI biosample accession number	NCBI genome accession number
Flavobacteriaceae	PS02336	*Flavobacterium* spp.	32.2	SAMN31456487	JASCOF000000000
	PS02337	*Flavobacterium* spp.	33.6	SAMN31456488	JASCOE000000000
	PS02338	*Flavobacterium* spp.	37.2	SAMN31456489	JASCOD000000000
Microbacteriaceae	PS01271	*Curtobacterium* spp.	34.3	SAMN31456439	JASCPS000000000
	PS01859	*Gulosibacter massiliensis*	76.7	SAMN31456451	JASCYH000000000
	PS02066	*Microbacterium* spp.	21.1	SAMN31456453	JASCPH000000000
	PS02068	*Microbacterium* spp.	25.2	SAMN31456455	JASCPF000000000
	PS02072	*Microbacterium* spp.	68.7	SAMN31456459	JASCYF000000000
	PS02292	*Agrococcus* spp.	44.2	SAMN31456469	JASCOW000000000
Pseudomonadaceae	PS01270	*Pseudomonas atacamensis*	71.8	SAMN31456438	JASCPT000000000
	PS01297	*Pseudomonas helleri*	70.8	SAMN31456441	JASCPQ000000000
	PS01301	*Pseudomonas coleopterorum*	70.8	SAMN31456445	JASCPM000000000
	PS01303	*Pseudomonas rustica*	75.0	SAMN31456447	JASCPK000000000
	PS01856	*Pseudomonas paracarnis*	94.1	SAMN31456448	JASCPJ000000000
	PS02288	*Pseudomonas kuykendallii*	71.3	SAMN31456465	JASCPA000000000
	PS02302	*Pseudomonas* spp.	52.1	SAMN31456479	JASCON000000000
	PS02303	*Pseudomonas mandelii*	88.2	SAMN31456480	JASCOM000000000
Xanthomonadaceae	PS02289	*Stenotrophomonas* spp.	28.6	SAMN31456466	JASCOZ000000000
	PS02297	*Stenotrophomonas nitritireducens*	85.3	SAMN31456474	JASCOS000000000
	PS02298	*Stenotrophomonas* spp.	34.0	SAMN31456475	JASCOR000000000
	PS02299	*Stenotrophomonas maltophilia*	81.0	SAMN31456476	JASCOQ000000000
	PS02300	*Stenotrophomonas* spp.	35.1	SAMN31456477	JASCOP000000000
	PS02301	*Stenotrophomonas* spp.	29.6	SAMN31456478	JASCOO000000000
	PS02304	*Xanthomonas* spp.	54.5	SAMN31456481	JASCOL000000000
	PS02340	*Luteimonas* spp.	30.0	SAMN31456491	JASCOC000000000

[a] Digital DNA-DNA hybridization d_4 score.

Table 2
Selection of *L. monocytogenes* strains used in this study.

L. monocytogenes strain	Facility	Multilocus sequence type clonal complex	Strain alias[a]	NCBI Biosample accession number
PS01272	F2	CC 554	CFSAN058345	SAMN22866631
PS01277	F2	CC 37	CFSAN062813	SAMN22866711
PS01278	F3	CC 5	CFSAN062927	SAMN22883931
PS01281	F1	ST-1509	CFSAN058407	SAMN22866792
PS01291	F1	CC 4	CFSAN058399	SAMN22866749
PS01293	F3	ST-1510	CFSAN062942	SAMN22883842
PS01295	F2	ST-1003	CFSAN058360	SAMN22887143

[a] Alias of *L. monocytogenes* strains isolated by Chen et al., (2022) [54].

(PS02289, PS02299, and PS02301) genomes. A multidrug resistance efflux pump gene *mexAB*, which was previously found to confer resistance to BAC in *Pseudomonas aeruginosa* [33], was detected in 5 *Pseudomonadaceae* isolates (PS01297, PS01303, PS01856, PS02288, and PS02302) and in *Xanthomonadaceae* strain PS02299. Interestingly, the *cnt* gene, which has been shown to aid in the degradation of quaternary ammonium compounds in *Pseudomonas* spp. [34]. was detected in three isolates (PS01859 *Microbacteriaceae*, PS01297 *Pseudomonadaceae*, and PS02303 *Pseudomonadaceae*).

2.3. The concentration of L. monocytogenes was higher in multi-family assemblage biofilms compared to L. monocytogenes mono-species biofilms

To assess the biofilm formation by selected environmental microbiota and *L. monocytogenes*, bacterial single- and multi-family assemblages were allowed to attach to pegs of a Minimal Biofilm Eradication Concentration (MBEC) device for three days in R2A broth at 15 °C. Biofilms were grown by co-culturing isolates from a same taxonomic family (i.e., single-family assemblage) or as multi-family assemblages in a full factorial design (Table 3). A known biofilm former, *P. aeruginosa* PAO1 (ATCC15692 = PS02184) was used as a positive control in the experiments. Biofilm formation was first assessed with a crystal violet assay which stains dead and live bacterial cells and some components of the biofilm matrix. The crystal violet assay serves as a proxy for biofilm formation and has been extensively used as a high throughput method to quantify total biofilm biomass of early-stage biofilms [35]. In addition to the crystal violet assay, we quantified the total bacteria in the attached biomass and the *L. monocytogenes* concentration using spread plating and the most probable number (MPN) method, respectively. *L. monocytogenes* strains formed the least biofilm when grown in single-species assemblages, as determined by the crystal violet method (Fig. 2a). Among single-family assemblages, *Pseudomonadaceae* assemblage formed significantly more biofilm ($p < 2 \times 10^{-16}$), compared to the *P. aeruginosa* positive control and *L. monocytogenes*, *Microbacteriaceae*, and *Flavobacteriaceae* single-family assemblages, as determined by the crystal violet assay (Fig. 2a). Furthermore, the total bacteria concentration in single-species *L. monocytogenes* assemblage biofilm was significantly lower than the biomass of *Pseudomonadaceae*, *Xanthomonadaceae*, and *Microbacteriaceae* single-family assemblage biofilms ($p = 0.03$) (Fig. 2b). Similarly, multi-family assemblages containing *L. monocytogenes*, *Pseudomonadaceae*, and/or *Xanthomonadaceae* formed significantly more biofilm (Fig. 2a) and had a significantly higher bacterial biomass compared to the *L. monocytogenes* single-family assemblage (Fig. 2b).

The concentration of *L. monocytogenes* in single-species biofilm was 5.87 ± 0.01 $\log_{10}$ MPN/peg, which was lower than the concentration of planktonic *L. monocytogenes* used as the inoculum at the beginning of the experiment (~6.3×10^6 CFU/peg). The concentration of *L. monocytogenes* in all multi-family assemblages was significantly higher compared to the single-species *L. monocytogenes* assemblage ($p = 1.27 \times 10^{-10}$). Given that all experiments started with the same total bacteria inoculum concentration, the concentration of *L. monocytogenes* was lower in assemblages that contained a higher diversity of bacteria (e.g., the 5-family assemblage had 5× lower initial concentration of *L. monocytogenes* compared to the single-family assemblage). To account for this difference, we normalized the concentration of *L. monocytogenes* in biofilms to the concentration of *L. monocytogenes* added at the beginning of the experiment (Fig. 2c). After normalization, we observed that *L. monocytogenes* attached and/or grew better in high diversity assemblages compared to low diversity assemblages (Fig. 2c).

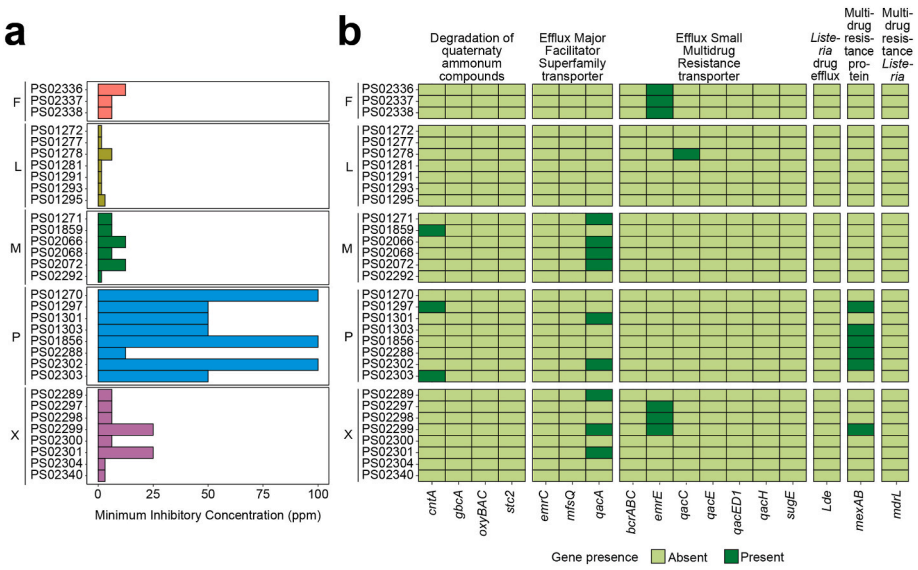

Fig. 1. Phenotypic and genotypic resistance to benzalkonium chloride of *L. monocytogenes* and environmental isolates from tree fruit packing facilities. (a) Bar plots show the minimal inhibitory concentration (MIC) to benzalkonium chloride (BAC), determined using the broth microdilution assay at 30°C. Each panel and distinct colors represent different taxonomic family of bacterial isolates. (b) Heatmap shows the presence (dark green) and absence (light green) of known genes associated with resistance to BAC. Isolates are grouped by taxonomic family: F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*. Genes are grouped by function. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Single- and multi-family assemblages tested for biofilm formation and tolerance to benzalkonium chloride.

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

[a] Labels for microbial assemblage treatments. F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*.

2.4. *Pseudomonadaceae and Xanthomonadaceae dominated multi-family assemblage biofilms*

Viability amplicon sequencing was used to characterize the strain-level composition of viable cells in biofilms formed by environmental microbiota and *L. monocytogenes* assemblages. For this purpose, the biomass that attached to the pegs of MBEC devices was resuspended, treated with propidium monoazide (PMA), followed by DNA extraction, amplification, and sequencing of the 16S rRNA V4 gene region. Mock microbial community controls were included in experiments to assess potential biases at the DNA extraction and amplification steps. Overall, there were some differences in the experimentally determined relative abundances of the three positive controls included in the DNA extraction step compared to the relative abundances declared by the manufacturer (Fig. S2). For example, the concentration of *Limosilactobacillus fermentum* was 11 % higher than the expected concentration declared by the manufacturer. Bias at the DNA extraction steps could have been introduced at the mechanical and chemical lysis steps [36,37]. In contrast, the positive control included at the PCR amplification step showed the expected composition based on the manufacturer's declaration.

To characterize the biofilms at the strain level, the Amplicon Sequence Variants (ASVs) were aligned to the database of 16S rRNA gene Sanger sequences of the isolates used in the experiments. Thirty one out of 32 isolates matched with 100 % identity to only one ASV in the database, while for strain PS02303 the closest match was to ASV11 with 97.6 % identity (Table S1). PS02338, which we had identified as *Flavobacteriaceae* using WGS (Table 1), matched to an ASV assigned to the *Sphingobacteriaceae* family (Table S1). This could have been due to increased representation of the sequence in the taxonomy database at the time of analysis of amplicon sequences, compared to when the isolate was identified. Further, this isolate was identified with low confidence based on its WGS, possibly due to low representation of the diversity of *Flavobacteriaceae* and *Sphingobacteriaceae* in the databases. It is also possible that this isolate represents a new uncharacterized species, thus further characterization is needed to determine its taxonomic identity.

To characterize the composition of living cells within the biofilms, we plotted the relative abundance of ASVs that matched with the strains

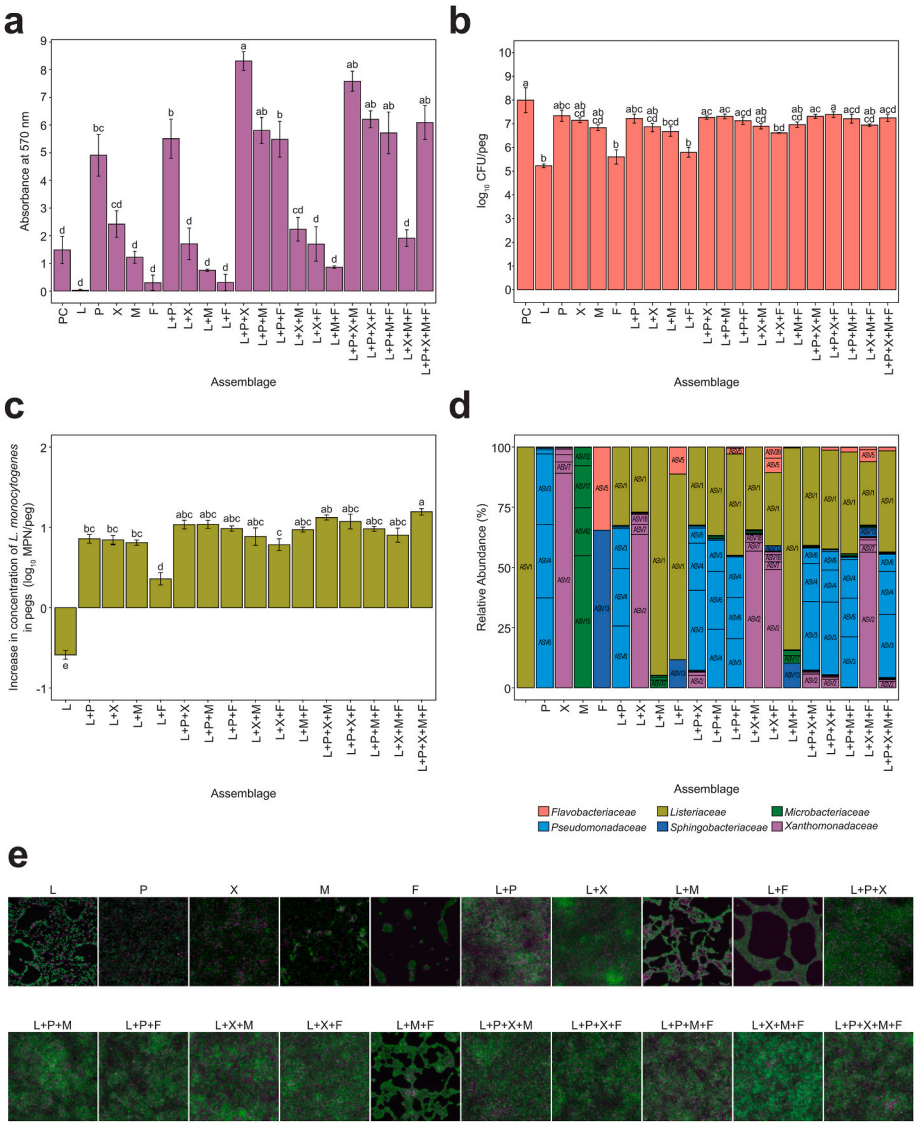

Fig. 2. Biofilm formation by single- and multi-family assemblages of environmental isolates and *L. monocytogenes*. (a) Biofilm formation after incubation of single- and multi-family assemblages in Minimal Biofilm Eradication Concentration (MBEC) devices in Reasoner's 2 A (R2A) broth for 3 days at 15 °C with daily replacement of media, as determined by the crystal violet assay. Biofilms with an absorbance above 1 were diluted and the final absorbance was calculated using the dilution factor. (b) Total concentration of bacteria in biofilms, as determined after detachment and spread plating onto R2A. (c) Change of *L. monocytogenes* concentration in single- and multi-family assemblage biofilms compared to the initial concentration of *L. monocytogenes*. (d) Relative abundance of bacteria in biofilms determined by viability amplicon sequencing. Bars are color coded by taxonomic genus of each Amplicon Sequence Variant (ASV) and, when possible, the ASV number was added to the bar. (e) Representative CLSM projections of single- and multi-family assemblage biofilms grown in R2A broth at 15 °C for 3 days in black microtiter plates with an optical grade base are shown. Biofilms were stained with LIVE/DEAD fluorescent stain; live cells shown in green (SYTO 9) and dead cells shown in magenta (propidium iodide). For each image, nine z-stacks were taken with 1 μm between each image and composed 3D projections of the biofilm structure were produced using ImageJ software. Each image represents an 84 × 84 μm square. In panels a, b, and c, error bars represent the mean ± standard error of the mean. Different lowercase letters show significant differences ($\alpha = 0.05$) between microbial assemblages, as determined using one-way ANOVA followed by Tukey's Honest Difference *post hoc* test for panels a, c, and d; or the Kruskal-Wallis tests followed by Dunn's test for panel b. Labels for microbial assemblage treatments were 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.)

included in assemblages (Fig. 2D). All *L. monocytogenes* isolates matched with 100 % accuracy with ASV1 (Table S1), which is likely because the V4 region of the 16S rRNA gene is not always sufficiently discriminatory to differentiate isolates within a species [38]. In single-family assemblage biofilms containing *Pseudomonadaceae* or *Microbacteriaceae* strains, three ASVs dominated the formed biofilms (*Pseudomonadaceae*: 29.3 % ASV3-PS01297, 30.5 % ASV4-PS01303, and 37.4 % ASV6-PS01270; *Microbacteriaceae*: 54.9 % ASV15-PS01271, 19.9 % ASV42-PS02292, and 17.5 % ASV17-PS02072) (Fig. 2d). In assemblages comprised of *Flavobacteriaceae* strains, only two strains (65.5 % ASV13-PS02338 and 34.5 % ASV5-PS02336) were detected in the formed biofilms. Single-family assemblages of *Xanthomonadaceae* were dominated (89.1 % relative abundance) by only one ASV, ASV2-PS02298. In multi-family assemblages, all assemblage biofilms contained a high relative abundance of *L. monocytogenes*. Specifically, in biofilms grown using assemblages that contained *Pseudomonadaceae* and/or *Xanthomonadaceae* strains, the relative abundance of *L. monocytogenes* was between 26 % and 42 % (Fig. 2d). In contrast, biofilms grown using assemblages containing only *Microbacteriaceae* and/or *Flavobacteriaceae* strains (and no *Pseudomonadaceae* or *Xanthomonadaceae*), the relative abundance of *L. monocytogenes* was between 77 % and 95 % (Fig. 2d). Furthermore, any biofilm containing

Pseudomonadaceae was predominated by this taxonomic family. For example, in biofilms grown from assemblages composed of *L. monocytogenes*, *Pseudomonadaceae*, and *Xanthomonadaceae*, *Pseudomonadaceae* were the predominant family (60.3 %), followed by *L. monocytogenes* (32.4 %), and *Xanthomonadaceae* (7.2 %).

2.5. Assemblages containing Pseudomonadaceae and Xanthomonadaceae formed continuous biofilms

Biofilms formed by single- and multi-family assemblages were also visualized using a confocal laser scanning microscope (CLSM) to determine the topology of a biofilm biomass in three dimensions. Biofilms formed by single-family assemblages formed microcolonies and did not continuously cover the surface (Fig. 2e). Notably, the assemblage containing only *L. monocytogenes* attached as single cells, and assemblages containing only *Microbacteriaceae* or *Flavobacteriaceae* attached as microcolonies. With an increased number of families in the assemblages, the continuity of biofilm coverage also increased (Fig. 2e). For example, biofilms formed by families with lower biofilm forming ability when cultured alone, as determined by the crystal violet assay (Fig. 2a) (i.e., *Flavobacteriaceae*), showed more continuous biomass coverage when cultured in two-family (e.g., L + F) or in three-family assemblages (e.g., L + F + M). In contrast, biofilms formed by families with good biofilm-forming ability (i.e., *Pseudomonadaceae* or *Xanthomonadaceae*) completely covered the surface, regardless of the diversity of the assemblages. CLSM images were analyzed to determine topological parameters of the biofilms, including biomass (defined as the volume with fluorescent signal over the surface area), maximum thickness, and roughness (i.e., porosity of the surface of the biofilm). Biofilms formed by three-family assemblages had a significantly greater amount of biomass compared to the biofilm formed by *L. monocytogenes* alone, except for the biofilm formed by *L. monocytogenes*, *Microbacteriaceae*, and *Flavobacteriaceae* (L + M + F) ($p = 5.75 \times 10^{-9}$) (Table S2). Furthermore, there was no significant difference in the biomass of single-family assemblages, and the two-family assemblage composed of *L. monocytogenes* and *Flavobacteriaceae* (L + F) and *L. monocytogenes* and *Microbacteriaceae* (L + M), further confirming the results obtained through the crystal violet assay and bacterial quantification detailed above (Fig. 2a and b). Interestingly, all the biofilms had a maximum thickness of 2 μm, suggesting that the conditions used to grow the biofilm structures did not allow for multilayer structures. The roughness of the biofilms, a measure of the surface topology, was significantly lower in assemblages with a larger number of families combined compared to two- and single-family assemblages ($p = 9.38 \times 10^{-10}$) (Table S2).

2.6. Genes involved in the production of extracellular matrix components were detected in environmental isolates' genomes

Production of extracellular matrix compounds is essential for bacteria to colonize surfaces [39]. In this study, the extracellular matrix produced by bacteria in biofilms was quantified indirectly via the crystal violet assay. Thus, the chemical composition of the matrix remains unknown. Nonetheless, the use of whole genome sequencing allowed for the detection of genes associated with the production of extracellular matrix compounds in genomes of studied isolates, which allowed for the inference of the genomic potential for expression of specific groups of extracellular matrix components. Genes related to alginate production (*algD* or *alg*U) were detected in the genomes of all *Flavobacteriaceae* and *Pseudomonadaceae* isolates, in 2 *Microbacteriaceae* (PS01859 and PS02292), and in 7 *Xanthomonadaceae* isolates (PS02289, PS02297, PS02299, PS02300, PS02301, PS02304, PS2340) (Fig. S3). Other genes related to polysaccharide production were detected in 5 *Microbacteriaceae* (*rnlA*; PS01859, PS02266, PS02268, PS02072, and PS02292), 1 *Xanthomonadaceae* (*pelF* and *pelD*; PS02304), and 2 *Pseudomonadaceae* (*peb*; PS01270 and PS01303). Genes related to release of extracellular DNA were detected in 7 *Pseudomonadaceae* (*cidA*; PS01270, PS01297, PS01303, PS01856, PS02288, PS02302, and PS02303) (Fig. S3).

2.7. Multi-family assemblage biofilms increased the tolerance of L. monocytogenes to benzalkonium chloride

The tolerance of *L. monocytogenes* to BAC was assessed by exposing single- and multi-family assemblage biofilms and planktonic cultures to BAC for 2 h. Biofilms and planktonic cultures were exposed to 12.5 ppm of BAC, which was twice the MIC of the most resistant *L. monocytogenes* strain (PS01278) included in the assemblages (Fig. 1a). The total bacterial (Fig. 3a) and *L. monocytogenes* (Fig. 3b) concentrations were measured by spread plating and MPN method, respectively, to determine the die-off kinetics.

L. monocytogenes was significantly reduced in biofilms formed only by *L. monocytogenes* strains or *L. monocytogenes* with *Flavobacteriaceae* (Fig. 3b) and a 30-min exposure to BAC was required for a significant reduction. In contrast, *L. monocytogenes* was not significantly reduced in any other assemblage biofilms even after a 2-h exposure to BAC (Fig. 3b). Compared to a biofilm, a longer, 2-h exposure to BAC was required to achieve a significant reduction of *L. monocytogenes* in planktonic monoculture or planktonic culture comprised of *L. monocytogenes* and *Flavobacteriaceae* (Fig. 3b). In contrast, the concentration of *L. monocytogenes* was significantly reduced in planktonic assemblages within 30 min to 2 h of exposure to BAC depending on the composition of the assemblage (Fig. 2b).

To quantify the tolerance of *L. monocytogenes* to BAC, we fitted the data to the Weibull model, which has been previously used to determine death kinetics of *L. monocytogenes* due to exposure to antimicrobials [40,41]. The goodness-of fit of the Weibull model was assessed by calculating the Root Mean Square Error (RMSE) and Akaike's Information Criterion (AIC) (Table 4). We then used the fitted curve to calculate the minimal duration of killing (MDK99) needed to reduce the *L. monocytogenes* concentration by 2-logs (Table 4). In planktonic cultures, the time necessary to reduce the population of *L. monocytogenes* by 2 logs in all tested assemblages was between 20 and 59 min. In biofilms, 0.35 and 3.9 s exposures to BAC were required in *L. monocytogenes* (L) and *L. monocytogenes* with *Flavobacteriaceae* (L + F), respectively, to reduce the *L. monocytogenes* by 2-logs. In contrast, the time required to reduce the population of *L. monocytogenes* by 2 logs was greater than 2 h when *L. monocytogenes* was grown in any other tested multi-family assemblage biofilm.

3. Discussion

3.1. Environmental microbiota that co-occurred with L. monocytogenes in tree fruit packing facilities differed in their resistance to benzalkonium chloride

Environmental microbiota previously identified as co-occurring with *L. monocytogenes* in environmental surfaces of tree fruit packing facilities [24] was isolated and characterized for phenotypic and genotypic resistance to benzalkonium chloride. Due to their broad-spectrum antimicrobial properties, quaternary ammonium compounds such as BAC are commonly used as sanitizers of environmental surfaces in food processing facilities [42] at concentrations ranging from 150 to 1000 ppm [7]. The MIC of BAC for the *L. monocytogenes* strains obtained from food processing environments has been extensively characterized [31,43–49]. While sanitizer resistance breakpoints have not been established for *L. monocytogenes*, studies typically consider *L. monocytogenes* strains to be resistant to BAC when their MIC is at least twice the average MIC of the population of strains under study [50]. In this study, strain PS01278 had twice the MIC compared to the average MIC of the *L. monocytogenes* strains under investigation. Compared to previous studies, PS01278's MIC falls in between low resistance (i.e., MIC below 4 ppm) and high resistance (i.e., MIC above 10 ppm) [31,43,44,46–51].

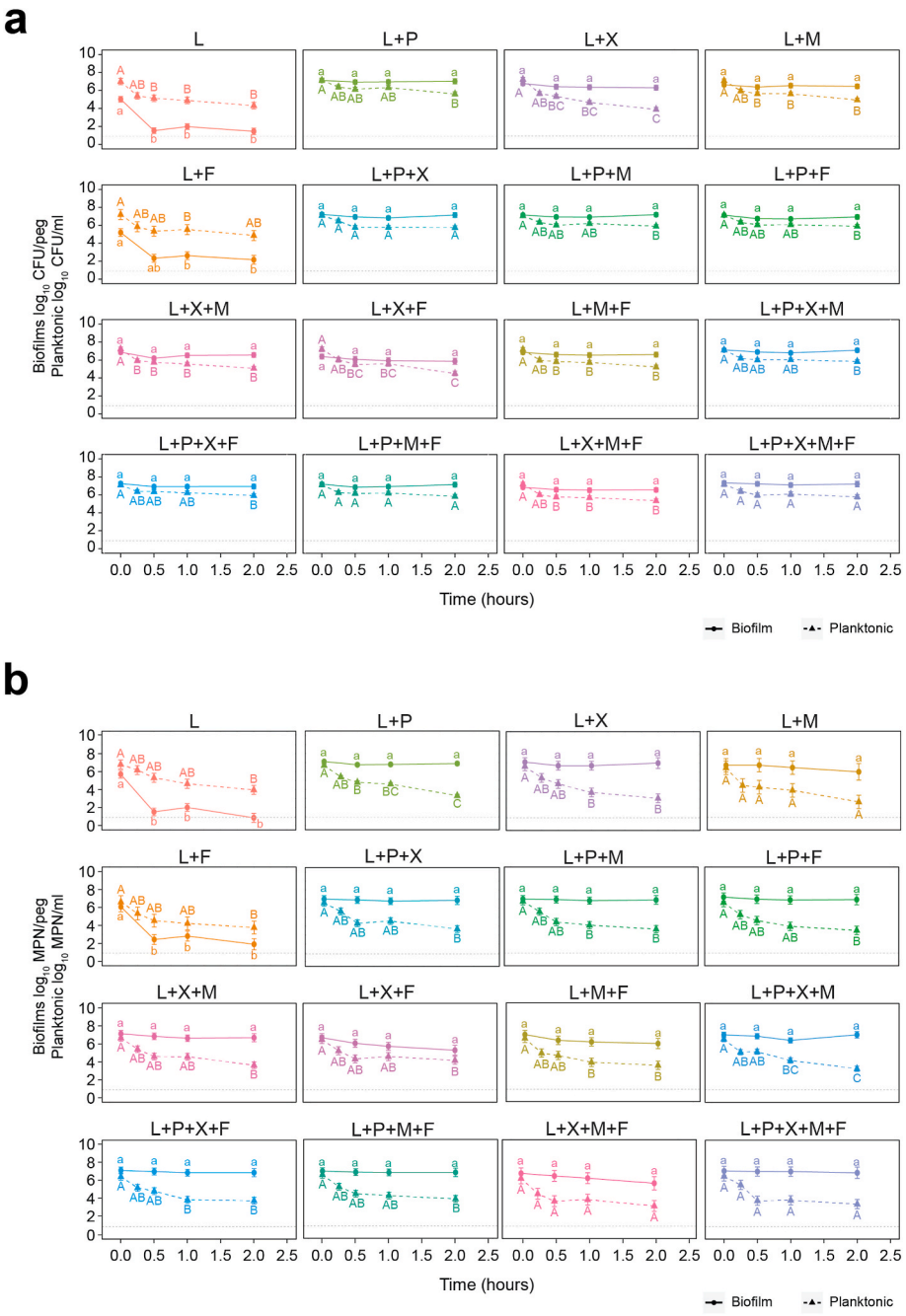

Fig. 3. Changes in microbial composition of multi-family assemblages during exposure to benzalkonium chloride. Reduction in total aerobic mesophilic bacteria (a) and *L. monocytogenes* (b) concentration in biofilms (circles with full lines) and planktonic cultures (triangles with dashed lines) exposed to 12.5 ppm of benzalkonium chloride (BAC). Data points represent the mean ± standard error of three independent biological replicates. Each microbial assemblage is color coded and presented in a separate panel. In each panel, different uppercase and lowercase letters indicate statistical differences ($\alpha = 0.05$) in the bacterial concentration of planktonic and biofilm assemblages, respectively, as determined using one-way ANOVA followed by *post-hoc* Tukey Honest Significant Different test. Dashed grey line represents the limit of quantification of the aerobic plate count method (0.6 $\log_{10}$ CFU/peg or ml) and of the most probable number method (0.9 $\log_{10}$ MPN/peg or ml). 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.)

Genetic mechanisms involved in decreased susceptibility to BAC include changes in cell membrane composition, increased expression of efflux pumps, and biodegradation [42]. In *L. monocytogenes*, the presence of BAC-specific efflux pumps encoded by *qacH* and *emrC* genes, and the *bcrABC* gene cassette are commonly associated with decreased susceptibility of strains to BAC [52]. Further, non-specific efflux pumps encoded by *mdrL* or *lde* can increase the resistance of *L. monocytogenes* to BAC [53]. In this study, only *L. monocytogenes* strain PS01278 possessed *qacH* gene (as previously reported by Chen et al. (2022) [54]) and showed twice the MIC compared to the other tested *L. monocytogenes* strains. No other BAC resistance genes were detected in the *L. monocytogenes* genomes.

Pseudomonas spp. can withstand concentrations of BAC above 50 ppm [42]. Consistent with our study, *Pseudomonas* spp. isolated from food sources reported in another study, including *P. fluorescens*, *P. lundensis*, and *P. fragi* had an MIC of BAC of 50–200 ppm [55]. Further, the *Pseudomonadaceae* isolates studied here exhibited a higher MIC of BAC compared to environmental isolates from strains from other

Table 4
Parameters of the Weibull model for the inactivation of *L. monocytogenes* in multi-family assemblage biofilms and planktonic cultures after exposure to 12.5 ppm benzalkonium chloride (BAC).

Assemblage[a]	Biofilm					Planktonic				
	p[b]	δ[c] (hours)	AIC[d]	RMSE[e]	MDK99[f]	p[b]	δ[c] (hours)	AIC[d]	RMSE[e]	MDK99[f]
L	0.08	1.6×10^{-8}	1.53	0.83	0.35 s	0.56	0.28	1.75	0.83	59 min
L + P	9.1×10^{-2}	0.68	−11.84	0.51	UD[g]	0.46	0.16	−27.12	0.34	42 min
L + X	1.1×10^{-8}	0.20	−8.07	0.51	UD[g]	0.49	0.11	7.96	1.01	27 min
L + M	1.58	2.58	−28.19	0.29	>2 h	0.36	0.05	13.54	1.20	23 min
L + F	0.09	3.8×10^{-7}	18.37	1.36	3.9 s	0.32	0.07	−0.92	0.77	38 min
L + P + X	3.9×10^{-9}	0.42	−13.00	0.49	UD[g]	0.38	0.12	6.97	0.98	44 min
L + P + M	1.6×10^{-8}	0.64	−12.07	0.50	UD[g]	0.36	0.07	2.55	0.82	30 min
L + P + F	0.15	4.4×10^{3}	−55.77	0.12	>2 h	0.36	0.08	9.44	1.06	32 min
L + X + M	0.26	34.13	−28.49	0.29	>2 h	0.37	0.11	−2.33	0.73	41 min
L + X + F	0.51	1.18	−7.95	0.51	>2 h	0.21	0.03	−4.49	0.68	56 min
L + M + F	0.28	2.31	−3.40	0.61	>2 h	0.30	0.05	−0.56	0.77	29 min
L + P + X + M	0.12	6.1×10^{3}	−5.78	0.55	>2 h	0.48	0.17	−9.90	0.58	44 min
L + P + X + F	0.54	17.08	−42.14	0.18	>2 h	0.36	0.11	−1.01	0.76	45 min
L + P + M + F	0.29	7.7×10^{2}	−38.83	0.21	>2 h	0.28	0.05	−2.59	0.73	38 min
L + X + M + F	0.84	2.15	−9.81	0.54	>2 h	0.23	0.02	13.55	1.20	20 min
L + P + X + M + F	1.15	8.30	−81.45	0.05	>2 h	0.35	0.06	8.86	1.04	28 min

[a] Labels for microbial assemblage treatments: F, *Flavobacteriaceae*; L, *L. monocytogenes*; M, *Microbacteriaceae*; P, *Pseudomonadaceae*; X, *Xanthomonadaceae*.
[b] p, dimensionless shape parameter describing the upward or downward concavity of the curve.
[c] δ, time scale parameter representing the time needed for the first logarithmic reduction in microbial population.
[d] AIC, Akaike's Information Criterium.
[e] RMSE, Root Mean Square Error.
[f] MDK99, Minimal Duration for Killing 2-logs of the microbial population. >2 is shown when MKD99 calculation resulted in a value above the experimental time scale.
[g] UD, undetermined - MDK99 calculation produced an infinity.

taxonomic family. Genetic determinants of BAC resistance in *Pseudomonas*, previously studied in *P. aeruginosa*, involve the expression of efflux pumps encoded by *cepA*, *mexAB*, *qacA*, and *sugE* genes [33,56,57]. The high MIC of the *Pseudomonadaceae* included in this study could potentially be attributed to the presence of *qacA* and *mexAB* genes in the strain's genome. In addition to genetic mechanisms of BAC resistance, previous research has shown that some *Pseudomonas* spp. can metabolize quaternary ammonium compounds into non-toxic compounds [16,34,58]. In this study, a gene related to the degradation of quaternary ammonium compounds (i.e., *cntA*) was detected in two *Pseudomonadaceae* and in one *Microbacteriaceae* strain.

Environmental microbiota belonging to the families *Xanthomonadaceae*, *Flavobacteriaceae*, and *Microbacteriaceae* had MIC of BAC below 12.5 ppm, except for *Xanthomonadaceae* PS02299 (*Stenotrophomonas maltophilia*) and PS02301 (*Stenotrophomonas* spp.) which had an MIC of 25 ppm. *Stenotrophomonas maltophilia*, an opportunistic human pathogen, can carry an efflux transporter involved in decreased susceptibility to BAC (i.e., *msfQ*) [59], however, this gene was not detected in any *Xanthomonadaceae* isolates included in this study. Other genes related to quaternary ammonium resistance were detected in some *Xanthomonadaceae* isolates, including *qacA*, *emrE*, and *mexAB* genes. To date, the resistance to BAC has not been extensively studied within strains of the *Xanthomonadaceae, Flavobacteriaceae,* and *Microbacteriaceae* families. This study presents the phenotypic resistance and associated genetic determinants to a sanitizer commonly used in food processing environments. However, our limited collection of environmental isolates does not allow for generalization of findings regarding the resistance of specific microbial families to BAC.

3.2. *L. monocytogenes* can attach and/or grow better in biofilms formed by multi-family assemblages, compared to *L. monocytogenes* alone

Biofilm formation can protect bacteria on environmental surfaces of food processing facilities from the action of the antimicrobials used during sanitizing operations. Previous studies on multi-species biofilms that included *L. monocytogenes* have been limited by the use of single strains of environmental microbiota [60–63], or the use of other foodborne pathogens that may not be relevant in a food production environment [62,64–66]. Our study aimed to investigate the biofilm formation ability of microbial assemblages composed of environmental microbiota that co-occur with *L. monocytogenes* to determine whether *L. monocytogenes* can benefit from the production of biofilms by other bacteria. Our results showed that the presence of other microbiota from the taxonomic families *Pseudomonadaceae, Xanthomonadaceae, Microbacteriaceae,* and *Flavobacteriaceae*, increases the attachment and/or growth of *L. monocytogenes* in biofilms. In contrast to our results, Heir et al. (2018) [67] showed increased growth of *L. monocytogenes* in mono-species biofilms compared to biofilms grown in co-culture with psychrotrophic Gram negative bacteria (i.e., *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Serratia liquefaciens*, *Stenotrophomonas maltophilia*, and *Acinetobacter* sp.), on stainless steel coupons for 9 days at 12 °C. Similarly, another study reported that biofilms formed by co-culturing *L. monocytogenes* and *P. fluorescens* on stainless steels coupons at 15 °C resulted in a lower *L. monocytogenes* concentrations compared to concentration in monoculture biofilms [68]. In concordance with our results, a two-week biofilm study that included *L. monocytogenes* and *P. fluorescens* showed that *L. monocytogenes* concentration in biofilms was 1-log higher than that of *P. fluorescens* [63]. Differences reported by other studies compared to our study could be attributed to the use of different surface materials for bacterial attachment (i.e., stainless steel vs. polystyrene), the media used for biofilm growth [69], as well as by differences in strains and incubation temperatures and durations. Both studies described above used highly nutritive media (i.e., brain heart infusion broth or tryptic soy broth), whereas our study used a minimal medium (i.e., R2A) to better represent the nutrient conditions in tree fruit packing facilities. Thus, research examining the effects of media and incubation conditions is needed to further unravel the survival of *L. monocytogenes* in multi-species biofilms. To date, there is a limited understanding of interactions between *L. monocytogenes* and resident microbiota which might enhance the survival and persistence of *L. monocytogenes* within multi-species biofilms. In future studies, transcriptomics and metabolomics could be used to study the gene expression and metabolites produced by strains in multi-family assemblages that could enhance *L. monocytogenes* survival in biofilms.

3.3. Pseudomonadaceae and Xanthomonadaceae dominate biofilms when present in microbial assemblages

To characterize the microbial composition of formed biofilms we used viability amplicon sequencing. While, to the best of our knowledge, this technique has not been previously used to characterize lab-grown biofilms before, we selected it to distinguish strain level differences in the composition of viable cells within formed biofilms. Overall, we observed that biofilms formed by multi-family assemblages containing *Pseudomonadaceae* and/or *Xanthomonadaceae* had a lower relative abundance of *L. monocytogenes* compared to the biofilms that included *Microbacteriaceae* and/or *Flavobacteriaceae*. Similar to our results, previous work has shown that biofilms composed of strains of *L. monocytogenes* and *Pseudomonas aeruginosa* were dominated by *Pseudomonas*, which represented 99 % of the biofilm composition [9]. Similarly, Thomassen et al. (2023) [70] grew multi-species biofilms composed of one strain of *L. monocytogenes* and five strains of *Pseudomonas* spp. for three days and determined that *L. monocytogenes* concentration represented 0.9 % of the total biofilm biomass [70]. Nonetheless, it should be noted that in these two studies [9,70], culture-based techniques were used to quantify bacteria and likely did not account for potential persister cells within the biofilms. To the best of our knowledge, no previous study has included strains of *Xanthomonadaceae, Microbacteriaceae*, or *Flavobacteriaceae* when studying multi-species biofilms and persistence of *L. monocytogenes*. It is possible that members of the *Pseudomonadaceae* and *Xanthomonadaceae* families can form biofilms faster than other bacterial species at the temperatures and nutrient conditions used in our experiments. However, further work is needed to understand the kinetics of biofilm formation within the used model system.

In this study, we grew biofilms by co-culturing all environmental strains with *L. monocytogenes* at similar concentrations. Nonetheless, it is likely that within food processing environments, *L. monocytogenes* enters facilities at a lower concentration than used in our experiments and integrates in biofilms pre-formed by other organisms, such as *Pseudomonadaceae* and *Xanthomonadaceae*.

3.4. Multi-family assemblage biofilms increased the tolerance of L. monocytogenes to benzalkonium chloride

The tolerance of *L. monocytogenes* to the sanitizer BAC was increased in biofilms formed by multi-family bacterial assemblages. In comparison, *L. monocytogenes* was inactivated by the same concentration of BAC when in planktonic assemblages. Furthermore, biofilms that had lower bacterial biomass, as determined by the crystal violet assay and total bacteria counts, such as *L. monocytogenes* alone and *L. monocytogenes* with *Flavobacteriaceae* were more susceptible to the action of the antimicrobial. Previous studies on multi-species biofilms containing *L. monocytogenes* have focused on the resistance of biofilms to antimicrobials, but not on the tolerance (i.e., reduced kinetics of bacterial killing) [9,63]. For example, a previous work on multi-species biofilms formed by *L. monocytogenes* and *P. aeruginosa* showed a higher resistance of the biofilm to sanitizing agents compared to mono-culture biofilms [9]. Similarly, *L. monocytogenes* single-species biofilms were shown to have lower resistance to BAC compared to biofilms of *L. monocytogenes* grown with *P. fluorescens* [63]. However, these studies reported the resistance of whole biofilms and did not show evidence of resistance of *L. monocytogenes* within the multi-species biofilms.

Previous work on *L. monocytogenes* and *Pseudomonas* spp. mixed species biofilms has shown that *L. monocytogenes* tends to locate in the inner layers of *P. fluorescens* pre-formed biofilms [9,71] and induce the production of the extracellular matrix by *P. fluorescens* [10]. While we did not test the location of *L. monocytogenes* within the multi-family assemblages tested in this study, it is possible that localization is one mechanism used by *L. monocytogenes* for survival under antimicrobial pressure. Another mechanism thought to enhance microorganisms' tolerance to chemical antimicrobials within biofilm is through extracellular matrix production. The viscous nature of the extracellular biofilm matrix can reduce the diffusion rate of chemicals into the deeper layers of a biofilm, resulting in a range of antimicrobial concentrations in microenvironments within a biofilm. While in this study we did not directly quantify or characterize the extracellular matrix of the multi-family assemblages, we detected the presence of genes related to matrix production in the genomes of most environmental strains. Remarkably, most of the environmental isolates carried genes that encode alginate production. Alginate production genes are well conserved within *Pseudomonas* and alginate has been previously identified as a key structural component of *Pseudomonas* spp. biofilms [39]. Alginate is highly viscous, can retain water and nutrients within the biofilm, and has been shown to increase the tolerance of *Pseudomonas* spp. to antimicrobials in cystic fibrosis infections [39]. However, the presence of extracellular matrix production related genes does not imply that they are being produced under the experimental conditions used to grow the biofilms. This warrants further research to determine the production of alginate and other matrix components of multi-family assemblages' biofilms and to determine if the production of extracellular matrix components aids in increasing the tolerance of *L. monocytogenes* to chemical sanitizers.

3.5. Conclusions

Control of *L. monocytogenes* in tree fruit packing facilities is challenging due to the potential repeated introduction of the pathogen with raw ingredients and the presence of environmental microbiota that may facilitate its persistence. We showed that *L. monocytogenes* can attach and/or grow better in biofilms formed by multiple species of environmental microbiota, including *Pseudomonadaceae*, *Xanthomonadaceae*, *Flavobacteriaceae*, and *Microbacteriaceae*. Furthermore, formation of multi-family assemblage biofilms resulted in an increased tolerance of *L. monocytogenes* to a commonly used sanitizer, benzalkonium chloride. Given that *Pseudomonadaceae, Xanthomonadaceae, Microbacteriaceae*, and *Flavobacteriaceae* have been shown to be consistently present in some tree fruit packing environments, they may play a role in the persistence of *L. monocytogenes* in such environments. Thus, cleaning and sanitizing should focus on not only the efficacy in reduction of *L. monocytogenes,* but also the reduction of strong biofilm producers such as *Pseudomonadaceae* or *Xanthomonadaceae*.

4. Methods

4.1. Selection of L. monocytogenes isolates

L. monocytogenes isolates were previously collected by Simonetti et el., (2021) [72] from three tree fruit packing facilities in the Northeastern U.S., and were whole genome sequenced by Chen et al. (2022) [54]. A subset of strains was used in this study to represent genomically diverse strains isolated from three facilities (Table 2).

4.2. Collection of environmental microbiomes from tree fruit packing facilities

Environmental samples were collected from three tree fruit packing facilities during processing hours, from surfaces underneath the conveyor belt that transports fruit through the washing, drying, and waxing operations [24,73]. Samples were collected using pre-hydrated sponges with neutralizing buffer (3 M, Saint Paul, MN), from 40 × 40 cm areas on the floor under conveyor belts at the washing, drying, and waxing processing stations [24,73]. Each sponge was placed into a sterile bag and stored in a cooler during transportation to the lab and processed within 24 h of collection. Each sample was homogenized with 90 ml of Brain Heart Infusion (BHI) broth (BD, Franklin Lakes, NJ) in a stomacher, at 230 rpm for 7 min. The homogenates of three samples

collected from a facility were transferred to a sterile bottle, supplemented with 20 % v/v sterile glycerol, and thoroughly mixed for 5 min using a magnetic stir plate. The composite sample of each facility was aliquoted into 15 ml conical tubes and preserved at −80°C until microbiota isolation. Each environmental microbiome sample tube was thawed at room temperature before isolation of bacteria and used one time.

4.3. Isolation of environmental microbiota

Four strategies were used to isolate environmental bacteria present in the composite samples.

(1) A high throughput isolation was conducted using Prospector (Galt Inc., San Carlos, CA). Environmental microbiome composite samples from each facility were diluted in 0.5 × BHI broth containing 100 μM resazurin (Sigma, St. Lois, MO) and loaded onto a Prospector array. The arrays were sealed and incubated for 48 h at 30°C. After incubation, the arrays were placed in the Prospector and wells with microbial growth were transferred to 96-well plates containing 0.5 × BHI broth supplemented with 100 μM resazurin, followed by incubation at 30°C for 48 h. Cultures were transferred to BHI, incubated for 48 h at 30°C, then supplemented with 20 % v/v sterile glycerol, and maintained frozen at −80 °C until further use.
(2) To obtain isolates that did not grow in medium with high concentration of nutrients (i.e., BHI), as well as slow-growing isolates (i.e., those that require more than 48 h to form colonies), a 1000-fold dilution of an environmental microbiome composite sample was spread plated onto an R2A agar (BD, Franklin Lakes, NJ) plate and incubated at 30°C. Colony development was monitored throughout the five days of incubation, and colonies that grew after 48 h were collected, purified, and cryopreserved at −80°C.
(3) To obtain isolates from the family *Flavobacteriaceae,* which are known to produce yellow-pigmented colonies, a 1000-fold dilution of a composite environmental microbiome sample was plated onto R2A agar and incubated at 30°C and 15°C for 5 days. All yellow colonies were collected, purified, and cryopreserved at −80°C.
(4) To obtain isolates from family *Xanthomonadaceae*, a composite sample was plated onto *Stenotrophomonas* selective agar (HiMedia Laboratories Pvt. Limited, Nashik, MH) and *Xanthomonas* agar [74–76] and incubated at 30°C for 48 h. Colonies with different morphologies were collected, purified, and cryopreserved at −80°C.

4.4. Preliminary identification of isolates

Bacterial isolates (n = 510) were randomly selected for preliminary identification using Sanger sequencing. Briefly, each selected isolate was streaked from a cryostock onto an R2A agar plate and grown at 30°C until colony formation. One colony was transferred to R2A broth (Neogen, Lansing, MI) and incubated at 30°C overnight or until turbidity was observed. After the incubation, 1 ml of culture was centrifuged at 20,000 g for 20 min, the supernatant was removed, the pellet was resuspended in 100 μl of DNase free water, and heat-lysed at 95°C for 30 min. A lysate was centrifuged at 6,000 g for 20 min, and the supernatant containing the genomic DNA was used to PCR amplify the 16S rRNA gene with universal primers 27F [5-AGAGTTTGATCMTGGCTCAG-3] and 1492R [5-GGTTACCTTGTTACGACTT-3], as previously described by Potomastowski et al., (2019) [77]. The PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (1U/μl, Applied BioSystems, Thermo Fisher Scientific) and submitted to the Penn State Genomic Core Facility (University Park, PA) for Sanger sequencing with 27F primer. Sanger sequences were trimmed to remove uncalled bases (N) at the beginning and end of each sequence. Mega11 software [78] was used to align sequences using MUSCLE and trim them to the same length. The trimmed and aligned sequences were then analyzed using the nucleotide BLAST database [79] to obtain a preliminary taxonomic genus identity for each isolate. To reduce the number of identical isolated, all sequences of isolates that belonged to the families *Pseudomonadaceae, Microbacteriaceae, Xanthomonadaceae*, and *Flavobacteriaceae* were aligned with the 16S rRNA gene sequences from target family type strains extracted from the Ribosomal Database Project (RDP) database [28] and used to build a maximum likelihood phylogenetic tree in Mega11, with 500 bootstraps. A representative isolate of each genotype within a bacterial family was selected from the phylogenetic tree. To select the final strains included in the experiments, V4 region of 16S rRNA Sanger sequences of each isolate were extracted using mothur v.1.43.0 [80] using primers 505F [5′-GTGCCAGCMGCCGCGGTAA-3’] and 806R [5′-GGACTACHVGGGTWTCTAAT-3’] [81]. Sequences were aligned using MUSCLE and pairwise distances were calculated using Mega11. Isolates with at least 2 single nucleotide polymorphisms (SNP) difference compared to other strains were selected for further experiments to allow for differentiation among individual strains in the following experiments.

4.5. Whole genome sequencing

Whole-genome sequencing (WGS) of the final collection of environmental isolates was performed to confirm their taxonomic identity. Isolates were grown on Brain Heart Infusion (BHI) at 30°C until turbidity was observed. After incubation, cultures were centrifuged at 16,000 g for 20 min to pellet the cells. DNA was extracted from pellets using the EZNA Bacterial DNA extraction kit (Omega Bio-Tek, Norcross, GA) by following the manufacturer’s protocol. The DNA concentration and quality of DNA samples were measured with Nanodrop (Thermo Fisher Scientific, Waltman, NJ) and Qubit broad range dsDNA kit (Thermo Fisher Scientific, Waltman, NJ). Extracted DNA was sent to Wright Labs (Huntington, PA) for library preparation and sequencing. Briefly, metagenomic libraries were prepared using the Nextera XT DNA Library Preparation kit (Illumina, San Diego, CA). The libraries were quality checked using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and dsDNA High Sensitivity kit (Thermo Fisher Scientific, Waltman, NJ) and then pooled in an equimolar ratio. The pool was purified using a 2% agarose gel and the Qiagen QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified pooled library was sequenced using a NextSeq 550 (Illumina, San Diego, CA) to produce 150 bp paired end reads. For the *L. monocytogenes* strains which had previously been whole genome sequenced [54], the sequences were retrieved from the NCBI SRA (Table 1). The quality of reads was analyzed using FastQC v.0.11.9 [82] and reads were trimmed using Trimmomatic v0.39 [83] to remove the adapters and low-quality bases. Trimmed reads were assembled using SPAdes v3.15.3 [84]. The quality of assembled genomes was analyzed using Quast v5.0.2 [85] and the sequencing coverage was calculated by alignment of sequencing reads to the assembled genomes using SAMtools v.1.16 [86]. To determine the taxonomic identity of each isolate, assembled genomes were submitted to the Type (Strain) Genome Server [29] in August 2022. The confidence of taxonomic identification was evaluated with the digital DNA-DNA hybridization method by using d4 (dDDH4) metric, which is recommended for draft genomes [87]. Isolates that matched a reference strain with dDDH4 value greater than 70 were identified at the species level, while isolates with dDDH4 value lower than 70 were identified at the genus level.

To investigate the genomic potential of isolates for antimicrobial resistance and biofilm formation, the assembled genomes were annotated with Prokka v.1.14.6 [88], using default parameters, and genome annotations of isolates belonging to the same taxonomic family were compared using Roary v.3.13.0 [89], using default parameters, in the GalaxyTrakr v.21.09 platform. Genome annotations were reviewed to detect the presence of genes previously known to confer resistance to benzalkonium chloride. Target genes were selected based on a literature

review of genes involved in resistance to benzalkonium chloride in *L. monocytogenes* (*bcrABC, emrC, emrE, lde, mdrL, qacA, qacC, qacH, sugE*) [30–32,43,50,51,90–92] and *Pseudomonas* spp. (*mexAB, qacE, sugE, cntA, gbcA, oxyBAC, stc2*) [32,33,48,49]. To the best of our knowledge, genes involved in resistance to benzalkonium chloride have not been reported for *Microbacteriaceae*, *Flavobacteriaceae*, and *Xanthomonadaceae*. We also searched annotated genomes for the presence of genes linked with biofilm formation. Target genes were selected based on a literature review of genes involved in the production of extracellular matrix components needed for biofilm formation. We searched for relevant genes previously reported in *Pseudomonas aeruginosa* (*alg 44, algD, algU, fimX, lecA, lecB, pelD, pelF, pslD*) [93–95], *Pseudomonas putida* (*bcs, pea, peb*) [96], and *Stenotrophomonas maltophilia* (*rmlA and spgM*) [97]. To the best of our knowledge, genes involved in the production of extracellular matrix components have not been reported for *Microbacteriaceae* or *Flavobacteriaceae*.

4.6. Antimicrobial susceptibility assay

The minimal inhibitory concentration (MIC) of the antimicrobial benzalkonium chloride (BAC) was determined for each selected isolate using the broth microdilution assay [98]. A stock solution of BAC (Spectrum, New Brunswick, NJ) was prepared in Mueller Hinton (MH) broth (BD, Franklin Lakes, NJ) in a concentration of 400 ppm, followed by sterile filtration and storage at −20 °C until use. Each isolate was streaked from a cryo-stock onto MH agar for isolation (BD, Franklin Lakes, NJ) and grown at 30°C until colony formation. An isolated colony was suspended in MH broth and incubated under agitation at 30°C until the turbidity exceeded an optical density at 600 nm (OD_{600}) of 0.2, measured with an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). Following the incubation, the culture was diluted to an OD_{600} equal to 0.2 in MH broth and further diluted to achieve the final concentration of ~5×10^5 CFU/ml in the assay plate. BAC concentrations ranging from 100 to 0.29 ppm were tested in a microtiter plate. Four wells of each microtiter plate were used as positive controls to verify growth of each strain. Positive control wells contained the bacterial culture and MH broth without BAC. Four additional wells were used as negative controls. Negative control wells contained only MH broth without BAC or bacterial culture. Microtiter plates were incubated for 20–24 h at 30°C. The temperature of incubation was modified from the standard method due to the inability of some environmental isolates to grow at 35°C. The MIC for each antimicrobial was defined as the lowest concentration of an antimicrobial that resulted in no microbial growth [98] determined by visual inspection and measurement of the OD_{600} using a microplate reader (BioTek Synergy Neo2 Hybrid Multi-Mode Microplate Reader, Thermo Fisher Scientific, Waltman, NJ). Each test was conducted in at least two independent biological replicates, each with two technical replicates.

4.7. Biofilm formation by environmental microbiota and *L. monocytogenes*

Selected isolates from the families *Pseudomonadaceae, Xanthomonadaceae*, *Flavobacteriaceae*, and *Microbacteriaceae*, along with *L. monocytogenes* strains were combined into single-and multi-family assemblages to assess their ability to form biofilms. Single family assemblages contained all isolates from the same taxonomic family (i.e., *Listeriaceae, Microbacteriaceae, Pseudomonadaceae, Xanthomonadaceae*, or *Flavobacteriaceae*), and multi-family assemblages included all combinations of selected bacterial families with *L. monocytogenes*, in a full factorial design, resulting in 20 treatments (Table 3). Each strain was streaked from a cryostock onto an R2A agar plate and grown at 20 or 30°C until visible colony formation. R2A was selected as the growth medium since it is a low-nutrient medium that can mimic the nutrient-restricted conditions of environmental surfaces in tree fruit packing facilities. Isolated colonies from each strain were resuspended in 10 ml of R2A broth and grown at 20 or 30°C until $OD_{600} > 0.2$, measured with an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). Cultures were then diluted to a final concentration of ~1×10^7 CFU/ml in R2A broth (~10 ml) and combined in equal volumes into single-family assemblages (Table 3) to achieve a final volume of 20 ml. Multi-family assemblages (Table 3) were prepared by combining equal volumes of single-family assemblages cocktails to obtain a final volume of 5 ml per assemblage. The final bacterial concentration in single- and multi-family assemblages (~1×10^7 CFU/ml) was confirmed by spiral-plating (EasySpiral, Interscience, Saint Nom la Brétèche, France) onto R2A agar plates followed by incubation at 30°C for 72 h. For each assemblage, 150 μl were added to 8 wells of a Minimal Biofilm Eradication Concentration (MBEC) Assay plate (Innovotech, Edmonton, Canada). MBEC plates, formerly known as Calgary device, are 96-well plates with polystyrene pegs attached to the lids, which allow for biofilm formation on pegs, reducing the inter-well variation previously reported for biofilms grown in regular 96-well plates that are washed by pipetting [35]. A positive control culture of *Pseudomonas aeruginosa* PAO1 (ATCC15692 = PS02184), was included in each experiment, and was selected as it is the model organism used to study biofilm formation [99,100]. Negative control wells contained 150 μl of sterile R2A broth and were included to control for contamination. MBEC plates were incubated at 15°C for 3 days, and medium was replenished every day by replacing the base of the MBEC device with a 96-well plate containing 150 μl of sterile R2A broth in each well. The incubation temperature for biofilm selection was selected because it was the mean temperature measured in tree fruit packing facilities in our previous studies [24,73].

4.8. Quantification of biofilm formation using a crystal violet assay

Biofilm formation on pegs was quantified using the crystal violet assay as previously described by Piercey et al. (2016) [101], with a few modifications. Briefly, MBEC pegs were rinsed for 10 s with sterile 0.85 % NaCl solution, transferred to a sterile 96-well plate base and heat fixed statically at 65°C for 40 min in a Multi-Therm microplate shaker (Benchmark Scientific, Sayreville, NJ). Two hundred microliters of crystal violet (1% w/v) (Ward's Science, Rochester, NY) were 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 biofilms was quantified by measuring the absorbance of the crystal violet-ethanol solution at 570 nm on a Synergy microplate reader (BioTek Synergy Neo2 Hybrid Multi-Mode Microplate Reader, Thermo Fisher Scientific, Waltman, NJ). The experiment was conducted in three independent biological replicates.

4.9. Quantification of total bacteria and *L. monocytogenes* in biofilms

To quantify and characterize the bacteria and *L. monocytogenes* present in the formed biofilms, MBEC peg lids were rinsed for 10 s with sterile 0.85 % NaCl solution, transferred to a 96-well plate containing Dey Engley (DE) Neutralizing broth (BD, Franklin Lakes, NJ), and sonicated for 30 min (VWR, Radnor, PA). Eight technical replicates were combined into one sample per treatment and ten-fold dilutions of each treatment were prepared in sterile 0.85 % NaCl solution. The concentration of bacteria in biofilms was quantified by spread plating of dilutions of onto R2A agar, followed by incubation at 30°C for 3 days.

To determine the concentration of *L. monocytogenes* in single-family and multi-family assemblages, a miniaturized MPN assay was performed [102]. The MPN method was selected to avoid growth of other microbiota on selective media using direct quantification and to allow for recovery of dormant and sub-lethally injured cells which may not be detected by direct plating methods. Briefly, 100 μl of serial dilutions were transferred to three tubes containing 900 μl of Buffered *Listeria* Enrichment broth (BLEB) (Criterion, Santa Maria, CA), mixed thoroughly, and incubated at 30°C for 4 h. After incubation, 4 μl of *Listeria*

Selective Supplement (90 mg acriflavine, 450 mg cycloheximide and 360 mg sodium nalidixic acid in 40 ml of sterile reverse osmosis water) (Sigma Aldrich, St. Lois, MO) were added to each tube, mixed, and incubated for an additional 44 h at 30°C. After incubation, enrichment tubes were spot inoculated onto Agar *Listeria* according to Ottavani & Agosti (ALOA) (Biomerieux, Marcy-l'Étoile, France) and incubated at 37°C for 24 h. The number of positive tubes for *L. monocytogenes* (i.e., blue colonies with a halo on an ALOA agar) were counted and the MPN was calculated using the MPN calculator spreadsheet available on the FDA BAM website [103].

4.10. Characterization of microbiota composition in biofilms

To further characterize the bacterial composition of biofilms, viability amplicon sequencing [104,105] was used. A propidium monoazide (PMA) treatment was performed to bind the DNA of dead cells and prevent its amplification. Briefly, the detached biofilm biomass (600 μl) was treated with 1.5 μl of 20 mM propidium monoazide (PMA) (Biotium, Fremont, CA) (final concentration 50 μM) [104]. Tubes containing detached biomass and PMA were placed on a Multi-Therm microplate shaker (Benchmark Scientific, Sayreville, NJ), covered with aluminum foil and incubated at 30°C for 10 min with agitation (700 rpm) to allow for the PMA to permeate injured cells. After incubation, photoactivation of PMA was carried out in PMA-Lite (Biotium, Fremont, CA) with a blue LED light source ($\lambda = 465–475$ nm) for 15 min at room temperature. After light exposure, tubes were centrifuged for 5 min at 16,000 g and the pelleted cells were stored at −80°C until DNA extraction. The DNA present in PMA treated samples was extracted using the DNeasy PowerBiofilm DNA extraction kit (Qiagen, Hilden, Germany), by following manufacturer's protocol. Three negative controls (i.e., no sample added to a lysis tube) were included at the DNA extraction step to detect potential contaminants in the kits. DNA concentration was quantified with the High Sensitivity double strand DNA kit (Thermo Fisher Scientific, Waltham, MA) and sent for amplification of the 16S V4 rRNA region, library preparation, and sequencing to CD Genomics (Shirley, NY). Briefly, the V4 region of the bacterial 16S rRNA gene was amplified and indexed by PCR using the primers 515F and 806R. PCR products was purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA) and sequencing libraries were generated using NEB Next Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) following manufacturer's recommendations. The constructed libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Paired-end sequencing was conducted using an Illumina NovaSeq 6000 platform (Illumina, Inc., San Diego, CA) with 2 × 250 bp configuration at CD Genomics, Co., Ltd. (Shirley, NY). Sequence reads were analyzed using the R package DADA2 v.3.2 [106] following the standard protocol for 16S rRNA V4 sequence reads in R [107]. Briefly, low-quality sequence reads were discarded, and low-quality bases were trimmed from remaining reads. Errors in reads were predicted and ASVs were inferred. Paired-end sequence variants were merged into contigs and sequences shorter than 251 bp or longer than 255 bp were discarded. Chimeras were detected and removed from the dataset. The remaining ASVs were assigned taxonomy using the reference database Silva v.138 [108]. To evaluate biases in DNA extraction, amplification, library preparation, and sequencing, the ASVs from the mock community controls were aligned against the reference genomes provided by the manufacturer. Pairwise differences were calculated using MEGA11 [78] to determine which ASVs matched with the reference genomes. Similarly, all ASV sequences were aligned against a database containing the 16S rRNA V4 gene region Sanger sequences of the strains used in the experiment to detect the ASV that matched the reference database with 100 % accuracy, using vsearch v.2.23.0 [109] with the exact and global search options [110]. The ASVs corresponding to the strains used in the biofilm experiments were extracted with phyloseq R package v.1.44.0 [111], and relative abundances were calculated using a compositional analysis approach [112,113]. Briefly, the data was normalized using the center-log ratio (CLR) transformation and relative abundances were calculated using the Aitchison method using the compositions v.2.0–6 [114] R package. The composition of biofilms was visualized with barplots using ggplot2 R package v.3.4.2 [115].

4.11. Characterization of biofilm structure using confocal laser scanning microscopy

The morphology of single- and multi-family assemblage biofilms were visualized and quantified using confocal laser scanning microscopy (CLSM) using the procedure described by Guilbaud et al. (2015) [116] with modifications. Briefly, biofilms of microbial assemblages were grown on black microtiter plates with a microscopy grade base (Thermo Fisher Scientific, Rochester, NY) in the same conditions as previously described. After 3 days of incubation, the medium was removed, biofilms were washed once with sterile 0.85% NaCl solution and stained with the LIVE/DEAD viability kit (Invitrogen, Thermofisher Scientific, Waltman, NJ). The LIVE/DEAD kit stains viable cells with the green fluorophore Syto9 (3 mM) and dead cells with the red fluorophore propidium iodide (PI) (20 mM). Biofilms were visualized with an inverted CLSM Zeiss LSM880 microscope (Carl Zeiss, Oberkochen, Germany) with a diode-pumped solid-state (DPSS) laser with excitation at 561 nm and an argon laser with excitation at 488 nm. The fluorescent signal was collected at 589–718 nm for PI and at 500–589 nm for Syto9. Nine images were taken at 1 μm distance in the z-plane. ImageJ v.1.48 [117] was used to create 3D projections of the biofilm by combining the stacks of image along with an orthogonal view of the stack. COMSTAT v.2.1 [118] was used to calculate topographical parameters of each treatment biofilm, including biomass (i.e., number of voxels, which are pixels in 3 dimensions with a grey level above a threshold divided by the area), maximum thickness (i.e., highest point in the biofilm relative to the substratum minus the empty space), and roughness coefficient (i.e., variability in height of the biofilm). The experiment was conducted in two independent biological replicates with three technical replicates.

4.12. Kinetics of L. monocytogenes die-off due to benzalkonium chloride in single and multi-family assemblage biofilms and planktonic cultures

To determine whether the presence of environmental microbiota affects the die-off kinetics of *L. monocytogenes* exposed to BAC, planktonic cultures and biofilms formed by multi-family assemblages (Table 3) were exposed to sterile 12.5 ppm BAC (prepared in 0.85 % sterile saline) for 2 h at 15 °C. The applied concentration of BAC was two times the MIC of the most resistant *L. monocytogenes* strain used in the assemblages (PS01278; Fig. 1). While the concentration of BAC used in this experiment was lower than the typical concentrations of quaternary ammonium compound applied in food processing facilities (i.e., 150 to 1,000 ppm [7]), it allowed us to test our hypothesis without completely eliminating the biofilm. Planktonic cultures of bacterial assemblages were prepared by mixing bacterial strains at ~10^7 CFU/ml in R2A broth and exposing them to 12.5 ppm BAC for 0, 0.25, 0.5, 1, and 2 h. At each timepoint, the cultures were neutralized with DE neutralizing buffer, serially diluted in 0.85 % sterile saline, spread plated onto R2A agar plates and incubated at 30 °C for 3 days to determine total bacterial concentration. Viable *L. monocytogenes* remaining in the planktonic cultures after exposure to the sanitizer was quantified using the MPN method as previously described. Microbial assemblages were grown as biofilms as previously described and were exposed to BAC for 0, 0.5, 1, and 2 h. At each timepoint, the biofilms were neutralized in DE neutralizing buffer and detached by sonication as previously described. Total bacteria concentration, and viable *L. monocytogenes* remaining in biofilms after exposure to sanitizer were quantified by aerobic plate counts and the MPN method, respectively, as previously described. Survival of *L. monocytogenes* in multi-family assemblages was fitted to

the Weibull model equation [119,120]:

$$\log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^p$$

where N is the number of surviving *L. monocytogenes* at each timepoint, N_0 is the initial concentration of *L. monocytogenes*, t is the exposure time in hours, δ is the sale parameter in hours, and p is the dimensionless shape parameter. When $p > 1$, the survivor curve shows a downward concavity, when $p < 1$ the curve shows a downward concavity, and when $p = 1$, the model is linear [41]. Curves were fitted to the Weibull equation using the USDA Integrated Pathogen Modelling Program (v. 2013) [121]. The goodness-of-fit of the models was evaluated through the Root Mean Square Error (RMSE) and the Akaike's Information Criterium (AIC). To quantify the tolerance of *L. monocytogenes* to BAC in multi-family assemblage biofilms and planktonic cultures, the time needed to achieve a 2-log reduction in *L. monocytogenes* concentration, also known as the minimum duration for killing (MDK99) [11], was calculated from the Weibull fitted curves.

4.13. Statistical analyses of microbiological data

One-way Analysis of Variance (ANOVA) ($\alpha = 0.05$) was used to assess the effect of each microbial assemblage on biofilm formation data using the R package stats v4.3.1 [107], followed by Tukey's Honest Significant Differences test using the R package agricolae v.1.3–6 [122]. The ANOVA assumptions of normality and homogeneity of variance were confirmed with the Shapiro and Levene tests, respectively, using the R package rstatix v0.7.2 [123]. When the assumptions for ANOVA were not met, a non-parametric Kruskal-Wallis test was applied using the R package rstatix, followed by the Dunn's multiple comparison *post-hoc* test using the R package FSA v0.9.5 [124]. Similarly, for each microbial assemblage a one-way ANOVA was used to determine significant changes in bacteria and *L. monocytogenes* concentration due to exposure of sanitizer through time in biofilm and planktonic form, independently. All results are presented as mean ± standard error of the mean. Statistical and bioinformatics analysis were carried out in R v.4.3.1 [107].

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All sequence data presented in this study can be retrieved from NCBI BioProject PRNJA894476. Whole genome sequence reads and assembled genomes were submitted to the Sequence Read Archive and GeneBank, respectively (accession numbers are listed in Table 3). Amplicon sequences obtained for the characterization of biofilms were deposited in NCBI SRA under BioProject PRNJA1004302 (accession numbers are listed in Table S3).

Code availability

Scripts used for data analysis and bioinformatics are available on GitHub: https://github.com/LauRolon/BiofilmsAndTolerance.

Funding

This research was supported by the U.S. Department of Agriculture (USDA), National Institute of Food and Agriculture (NIFA) under grant number 2022-67017-36556, Hatch Appropriations under project number PEN04853, and Multistate Project S1007 (4666). The funding agency was not involved in the design of the study and collection, analysis, and interpretation of data presented in this study. The findings and conclusions in this publication have not been formally disseminated by the U.S. Department of Agriculture and should not be construed to represent any agency determination or policy.

CRediT authorship contribution statement

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

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.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank the three tree fruit packing facilities that provided environmental samples for this project, and Dr. Carolee Bull for her technical assistance in the isolation of *Xanthomonadaceae* strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2024.100177.

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