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Evaluation of single and multispecies biofilm formed in the static and continuous systems

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ABSTRACT

Biofilms consisting of multiple species of bacteria compared to biofilms of single species are common in natural environments including food contact surfaces. The objective of this study was to understand the biofilm formation and the efficiency of sodium hypochlorite (50 ppm/5 mins) on the single and multiple species biofilm of *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Listeria monocytogenes* formed on stainless steel surfaces in static and continuous systems. The cell concentration of *Listeria* in the dual and triple species biofilm in the continuous system (7.3–8.4 log CFU/cm^2) was higher compared to the static system (4.7–4.9 log CFU/cm^2) while the concentration remained consistent in the single species biofilm (6.4–6.7 log CFU/cm^2) for both systems. Biofilm formed in the static system was significantly ($p < 0.001$) more susceptible to sodium hypochlorite than biofilm formed in the continuous system. This observation agrees with the exopolysaccharide concentration which was found to be higher in the continuous system (8.0–15.6 μg/cm^2) than in the static system (3.2–6.3 μg/cm^2) indicating a positive correlation between EPS production and sanitizer resistance. Epifluorescence microscopy images showed the formation of interstitial voids within the three-species biofilm and filaments in the single and dual species *Listeria* biofilms in the continuous system which were absent in the static system. Overall, results showed that the biofilm formation and sanitizer resistance vary due to multispecies interaction and the presence of flow and should be considered an important variable in multispecies sanitizer resistance studies.

1. Introduction

Listeria monocytogenes, commonly isolated from outbreaks in food products such as milk, soft cheese, and delicatessen meats (pork tongue in jelly) (Leriche and Carpentier, 2000), can adhere and multiply on industrial surfaces in the presence of *Pseudomonas* (Agustín and Brugnoni, 2018; Fagerlund et al., 2017; Kocot and Olszewska, 2020; Thomassen et al., 2023). *Staphylococcus* and *Pseudomonas* are known biofilm formers with common nutritional niches such as meat, dairy, fresh fruits, milk, and vegetables with *Pseudomonas* causing spoilage and *Staphylococcus* capable of causing food poisoning (Xu et al., 2019). Multiple species of planktonic bacteria can come together in the biofilm which defines the biofilm's characteristics and stability (Brooks and Flint, 2008; Papaioannou et al., 2018). In the food industry, stainless steel surfaces that are cleaned and sanitized are frequently contaminated with multispecies bacteria within a biofilm along with nutrient-depleted conditions (Pang et al., 2019). The interactions between the bacteria involved in multispecies biofilm depend on strains of the bacteria, temperature, pH, oxygen content, and shear forces. The changes in these variables lead to interactions leading to synergistic interactions because of cooperation and utilization of common goods; antagonism because of the competition for nutrients and space (Ramstedt and Burmølle, 2022). Depending on the interaction, exopolysaccharides/metabolites produced by one bacterium in a polymicrobial biofilm can protect other bacteria resulting in increased survival during cleaning and disinfection (Sanchez-Vizuete et al., 2015). The commonly used sanitizers used in the food industry for cleaning and sanitation are quaternary ammonium compounds, peracetic acid, chlorine compounds, and sodium hypochlorite (Chaves et al., 2024; Simões et al., 2010). The recommended in-use concentration of sodium hypochlorite with 13 % active chlorine ranges from 0.05 to 2 % (Aarnisalo et al., 2007), depending upon the treatment time. Sodium hypochlorite and chlorine compounds are strong oxidizers and show bactericidal effects by interacting with proteins, enzymes, lipids, DNA, and RNA (Barnes and Greive, 2013; Ran et al., 2019). The active compound in sodium hypochlorite is hypochlorous acid which interacts with the enzyme's sulfhydryl groups. Additionally, chlorine reacts with the organic matter in the biofilm before diffusing into the layers for a bactericidal effect (Chaves et al.,

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2024).

In the last decade, biofilm research in dynamic conditions has been extensively focused on single species (single strain/multi-strain) (Lineback et al., 2018; Mendez et al., 2020; Mettler et al., 2022; Nkemngong et al., 2020). Considering the dynamic nature of biofilm formation on the food industry surfaces, a continuous system with a multispecies bacteria model provides a more realistic representation of the biofilm (Diarra et al., 2023; Yuan et al., 2020). The viability of the Centre for Disease Control and Prevention (CDC) reactor to replicate the dairy industry settings in laboratory-controlled conditions has been mentioned before (Lindsay et al., 2022). This study aimed to understand the effect of flow on the biofilm formation and sanitizer resistance of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Listeria monocytogenes* in single, dual, and triple-species biofilm using the CDC bioreactor.

2. Methods

2.1. Culture preparation

Stock cultures (−80 °C) of *Pseudomonas fluorescens* S1 (dairy source), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 9144), and *Listeria monocytogenes* H1 (environment source) were inoculated into tryptone soy broth (TSB) (Difco™, Becton, Dickinson and Company, USA) and incubated at 30 °C for 18 h. The cultures were then inoculated into TSB and incubated again in 15 mL centrifuge tubes (Falcon®, Corning, the USA) for another 18 h to prepare the working culture. After the secondary incubation, the cultures were centrifuged (Sigma® 6–16, John Morris Scientific Ltd., New Zealand) at 3000*g* for 20 min at room temperature. The cell pellets after centrifugation were washed with sterile saline (0.85 %) via vortex (30 s, highest setting) (Scilogex, Germany) and centrifugation (3000 *g* for 10 min). The concentrated cells were collected by removing saline and dissolving the final pellet into fresh sterile saline to a cell concentration of 10^6 CFU/mL.

2.2. Biofilm formation in the static system

Stainless-steel coupons (316-2B; Advanced Sheetmetals Ltd., Palmerston North) (2.4cm^2) were used as substrates for biofilm formation in the static system. The coupons were passivated to generate a chromium oxide coat by dipping into 50 % nitric acid and heating to 70 °C for 30 min followed by washing, drying, and autoclaving (121 °C, 15 mins). Sterile coupons were inoculated with 6 log CFU/mL bacteria in 10 % tryptone soy broth (TSB) for single species and a 1:1 ratio of each bacterium at the same cell concentration for dual species and triple species inoculations. The sterile coupons were placed in 48-well plates (Costar®, Corning, USA) in the vertical position and incubated for 24 h at 30 °C. The media (10 % TSB) was refreshed every 24 h. The cell count was analyzed every 24 h and exopolysaccharide and microscopic observations were made at 72 h to obtain mature biofilms. The experiment was repeated for two biological replicates and each biological replicate had 3 technical replicates.

2.3. Biofilm formation in the continuous system

The CDC bioreactor® (Biosurface Technologies, USA) was used to form biofilm in a continuous system for 72 h. The CDC reactor system consisted of 4 components 1) the influent tank 2) the pump and flow rate control system 3) the bioreactor 4) the effluent tank. These components were connected via silicone 16 tubing and passed through the pump using neoprene 16 tubing. The bioreactor consisted of 8 coupon rods, each rod consisting of three circular stainless-steel coupon (2.26 cm^2). The media (10 % TSB) was prepared in the influent tank and connected to the bioreactor via a pump (Masterflex®, Cole-Parmer®, USA) which fixed the constant flow rate of 5 mL/min. The doubling time for *Pseudomonas*, *Staphylococcus,* and *Listeria* in 10 % TSB at 30 °C was found to be 1.22 h, 1.5 h, and 1.27 h following the growth curve (Data not shown). The bioreactor was run at 120 RPM and 30 °C using a hot plate setup (VWR International, U.S.A). Every 24 h, 2 coupon rods (each coupon-2.26 cm^2) were removed aseptically from the bioreactor, and cell counts in the biofilm were calculated. The system was continuously run for three days for single species and multiple species combinations. The exopolysaccharide (EPS) content and sanitizer resistance were determined at 72 h to analyze mature biofilm. The experiment was repeated at least twice (2 biological replicates), and three technical replicates were acquired from each biological replicate for analysis.

2.4. Sanitizer treatment

The incubated stainless-steel coupons obtained from static and continuous systems incubated for 72 h were washed with saline to remove planktonic cells before being treated with sodium hypochlorite (SH) (50 ppm) (Janola, Australia) (Lindsay et al., 2022; Pang et al., 2019). The sodium hypochlorite was prepared by diluting the commercial sodium hypochlorite solution with sterile distilled water and the pH was adjusted to 6.7–7.0 using 1 M HCL. After 5 min of treatment, the coupons were dipped in neutralizer solution (1 % sodium thiosulphate) (AnalaR®, VWR International, England) for at least 1 min before being washed with saline. The coupons were dipped in sterile saline instead of sanitizer for untreated samples followed by sodium thiosulphate. The cells were detached from the coupons using the glass bead beating method, vortex mixing with 10 g of sterilized glass beads (diameter − 5 mm, Sigma-Aldrich, Germany) with 5 mL saline, for 1 min. Selective agars were used to enumerate cells (*Pseudomonas* Isolation agar (Difco™, USA) for *Pseudomonas*, Mannitol salt agar (Oxoid, UK) for *Staphylococcus* and Modified Oxford agar (HiMedia, USA) for *Listeria*). The treatments were conducted in biological duplicates with each duplicate containing a technical triplicate.

2.5. Extracellular polymeric substances (EPS) quantification

The biofilm matrix from the coupon surface was extracted using glass beads and mixed by vortex and sonication then quantified using a phenol-sulphuric acid hydrolysis (Zhou et al., 2024). Stainless-steel coupons with single and multispecies biofilms were washed (×3) with sterile distilled water to remove planktonic cells. The washed coupons were then mixed by vortex with sterile glass beads (5 g) and sterile distilled water (2 mL) for 5 min at the maximum setting to remove the adhered cells and EPS. This was followed by the sonication of the tubes containing coupons, saline, and glass beads for 5 min (40 kHz). The extraction process was completed by centrifugation at 10,000 RPM for 5 min. The supernatant was collected for filtration (0.20 μm) whereas the pellet was discarded. 200 μL of filtrate, 100 μL of 6 % phenol, and 500 μL of 98 % sulphuric acid were added and left to react for 20 min at room temperature. The optical density reading was taken at 490 nm and compared with the standard curve for dextran (Fig. S.1) where the EPS was determined in ug/cm^2 of the stainless-steel coupons.

2.6. Epifluorescence microscopy

The coupons obtained from the static and the CDC system at 72 h were washed in saline before air drying the surface at room temperature followed by staining with acridine orange (10 g/L) (BDH, England) for 5 min (Jha et al., 2020). The coupons were again washed with saline and dried at room temperature before being visualized with an epifluorescence microscope (Nikon eclipse Ni, USA) at excitation filter 485/20 nm and emission filter 500–538 nm (TRITC/filter 3). Three coupons were taken for each biological replicate for microscopic analysis, and at least five images were captured from each coupon across the surface. The observations were made at a magnification of 100 μm. The epifluorescence images were acquired using NIS-elements D software.

2.7. Statistical analysis

The significant differences between the samples were determined with one-way ANOVA (IBM SPSS version 29) with 95 % confidence ($p < 0.05$) together with Tukey analysis for a post-hoc test. The normality of the data was verified using the Shapiro-walk ($p > 0.05$) and Kolmogorov test ($p > 0.05$). All experiments had at least two biological replicates and six technical replicates and results were presented as mean ± standard deviation.

3. Results

3.1. Biofilm formation in the static system

In the static system, the presence of *S. aureus* and L. *monocytogenes* did not affect the cell concentration of *P. fluorescens* in both dual and triple species (Fig. 1). The cell concentration of *Pseudomonas* was found to be consistent in single species (6.4 log CFU/cm^2), dual species (6.0–6.4 log CFU/cm^2), and triple species (6.5 log CFU/cm^2). Similar observations were made for *Staphylococcus* and *Listeria* in single and multi-species. The cell concentration of *Staphylococcus* reached 6.9 log CFU/cm^2 in single species, 5.8–6.3 log CFU/cm^2 in dual species, and 5.6 log CFU/cm^2 in triple species biofilm. In single species, the cell concentration of *S. aureus* (5.1–6.9 log CFU/cm^2) was found to be comparable with *P. fluorescens* (6.3–6.9 log CFU/cm^2) but reduced significantly ($p < 0.05$) in dual species (3.7–5.8 log CFU/cm^2) with *P. fluorescens*. There were no significant changes ($p > 0.05$) in the cell concentration of all three bacteria involved in their single and multiple species counterparts as the result of incubation time (Fig. 1).

3.2. Biofilm formation in continuous system

A gradual increase of cell concentration in the biofilm can be observed for *Staphylococcus* and *Listeria* in both single species and dual species setups (Fig. 2). In contrast, for *Pseudomonas,* the cell concentration reached 6.4 log CFU/cm^2 at 24 h and stayed comparable over the next 48 h (6.9–7.2 log CFU/cm^2) in both single and dual species.

The cell concentration of *Pseudomonas* in single species biofilm (6.4, 7.2, and 6.9 log CFU/cm^2) was found to be comparable ($p > 0.05$) with cell concentration of *Pseudomonas* in dual species (with *Staphylococcus* - 6.0, 6.7, and 6.6 log CFU/cm^2 and *Listeria* – 7.0, 7.1, and 6.6 log CFU/cm^2) and in triple species (6.8, 6.9, and 6.3 log CFU/cm^2) at 24 h, 48 h, and 72 h respectively (Fig. 2). In contrast, at 24 h, significantly ($p < 0.05$) higher cell concentration of *Staphylococcus* was observed in dual species biofilm with *Pseudomonas* (3.7 log CFU/cm^2), and *Listeria* (3.5 log CFU/cm^2) and triple species (5.5 log CFU/cm^2) (both *Pseudomonas* and *Listeria*) compared to single species biofilm (<1.5 log CFU/cm^2). A similar trend of improved biofilm formation of *S. aureus* could be observed for 48 h and 72 h in combination biofilm (dual and triple species) as well. Similarly, the presence of a second bacteria (*Pseudomonas*) improved the cell concentration of *Listeria*, increasing it to 7.2 log CFU/cm^2, 8.2 log CFU/cm^2, and 8.0 log CFU/cm^2 for 24 h, 48 h, and 72 h respectively. For the same incubation time, the cell concentration of *Listeria* in single species was limited to 4.4 log CFU/cm^2, 5.7 log CFU/cm^2, and 6.4 log CFU/cm^2 respectively. Additionally, the *Listeria* cell concentration in dual species biofilm with *Staphylococcus* was found to be comparable ($p > 0.05$) with single species (*Listeria*), with cell concentrations reaching 3.9 log CFU/cm^2, 5.2 log CFU/cm^2, and 7.3 log CFU/cm^2 at 24 h, 48 h, and 72 h respectively. As the result of the influence of *Pseudomonas* (as observed in dual species), the cell concentration of *Listeria* in triple species was found to be significantly higher than single species at 7.2 log CFU/cm^2, 8.9 log CFU/cm^2, and 8.4 log

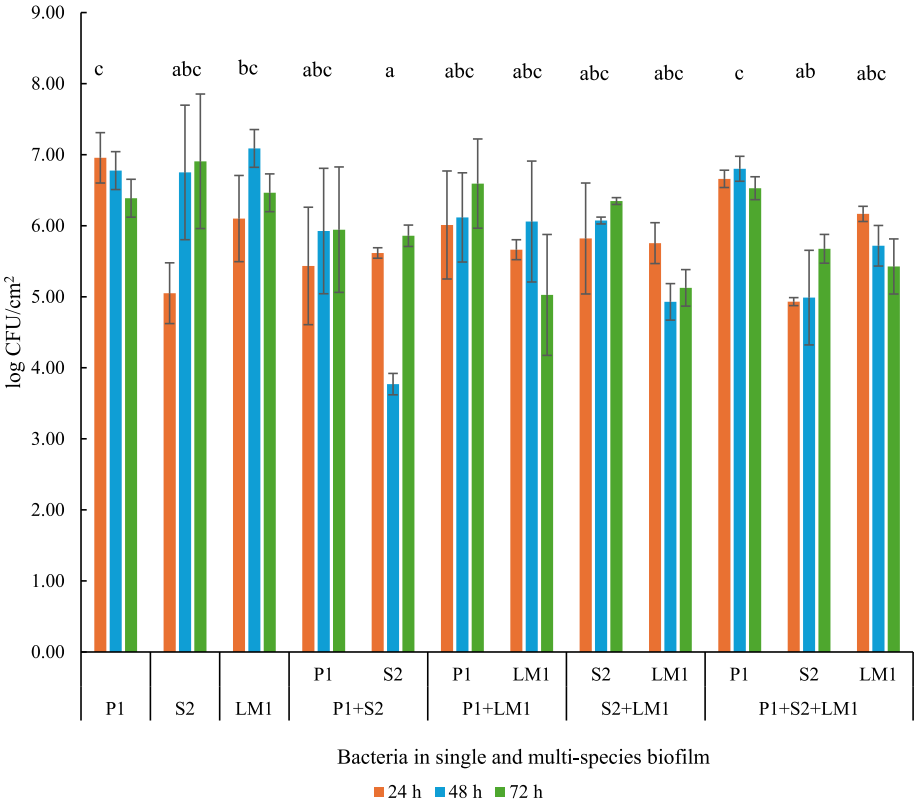

Fig. 1. Cell concentration (log CFU/cm^2) of *Pseudomonas fluorescens* (P1), *Staphylococcus aureus* (S2), and *Listeria monocytogenes* (LM1) in single, dual, and triple species biofilm formed in the static system in 10 % TSB at 30 °C over 72 h. Note: The lower-case superscripts (a-c) indicate the significant differences among the bacteria in single and multiple species biofilm.

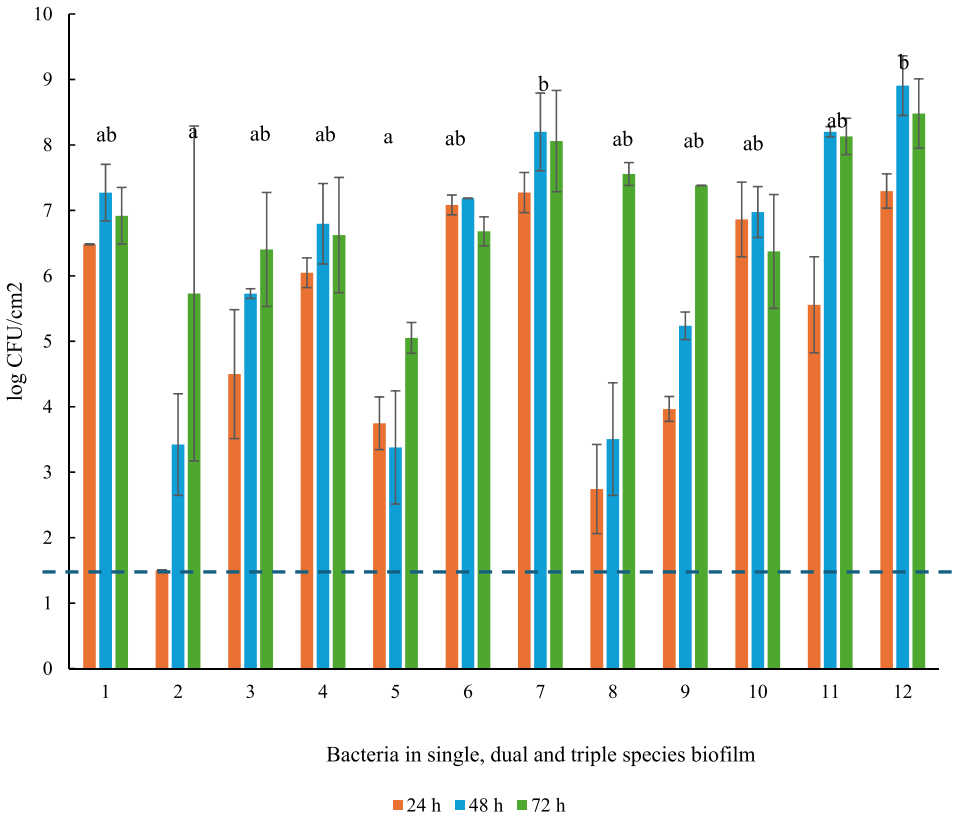

Fig. 2. Cell concentration (log CFU/cm^2) of *Pseudomonas fluorescens* (P1), *Staphylococcus aureus* (S2), and *Listeria monocytogenes* (LM1) in single, dual, and triple species biofilm formed in the CDC system in 10 % TSB at 30 °C over 72 h. Note: The lower-case superscripts (a-b) indicate the significant differences among the bacteria in single and multiple species biofilm. The dotted line indicates the level of detections (1.5 log CFU/cm^2).

CFU/cm^2 for 24 h, 48 h, and 72 h respectively. Overall, in the three-species biofilm (for all three time periods), the cell concentration of both *Staphylococcus* and *Listeria* improves significantly ($p < 0.05$) whereas *Pseudomonas* is consistent with single species.

Additionally, thread-like structures with one end attached to the coupon rod and another end floating in the impeller direction were observed for *Staphylococcus* biofilm in single and dual species (with *Listeria*) (Fig. 4). In dual species threads, in addition to *Staphylococcus* at cell concentration 7.4 log CFU/mL, *Listeria* was also detected at cell concentration 7.2 log CFU/mL.

3.3. Sanitizer tolerance

A significantly higher ($p < 0.001$) reduction of the bacteria was observed for biofilm formed in static systems compared to the continuous system when treated with sodium hypochlorite (Table 1). The log reduction was lowered significantly ($p < 0.05$) for *Pseudomonas* in dual species in both static (3.7–3.9 log CFU/cm^2) and continuous systems (0.1–2.3 log CFU/cm^2) compared to their single species counterpart (4.5–6.9 log CFU/cm^2). For *Staphylococcus*, the lowered log reduction in the presence of secondary bacteria (3.8–5.9 log CFU/cm^2) compared to single species (6.1 log CFU/cm^2) was limited to the static system. In the continuous system, the highest reduction of *Staphylococcus* (5.0 log CFU/cm^2) was observed with *Pseudomonas* and lowest in single species (0.1 log CFU/cm^2) and comparable log reduction with *Listeria* (3.1 log CFU/cm^2). Higher resistivity was observed for *Listeria* in both static and continuous systems in multispecies compared to single species. In the static system, there was a 5-log reduction in single species which significantly ($p < 0.05$) reduced to 3.3 log CFU/cm^2 (with *Pseudomonas*), and 4.7 log CFU/cm^2 (with *Staphylococcus*). In the continuous system, the resistance of single (1.3 log CFU/cm^2) and dual species (with *Staphylococcus*) (1.3 log CFU/cm^2) was found comparable but reduced with *Pseudomonas* (1.0 log CFU/cm^2).

Table 1
Log reduction of *Pseudomonas* (P1), *Staphylococcus* (S2), and *Listeria* (LM1) in single, dual, and triple species 72 h biofilm after treatment with sodium hypochlorite in the static and continuous systems.

Biofilm	Bacteria	Log reduction after sodium hypochlorite treatment	
		Static (log CFU/cm^2)	Continuous (log CFU/cm^2)
P1		4.5 ± 1.5^{eA}	6.9 ± 0.4^{jB}
S2		6.2 ± 0.7^{iA}	0.1 ± 0.1^{aB}
LM1		5.1 ± 0.2^{gA}	1.3 ± 0.1^{cB}
P1 + S2	P1	3.9 ± 1.5^{cdA}	1.7 ± 0.5^{dB}
	S2	4.0 ± 0.2^{dA}	5.0 ± 0.2^{iB}
P1 + LM1	P1	3.7 ± 1.5^{bA}	0.1 ± 0.1^{aB}
	LM1	3.3 ± 1.5^{aA}	1.0 ± 0.1^{bB}
S2 + LM1	S2	5.9 ± 0.4^{hA}	3.1 ± 0.1^{gB}
	LM1	4.7 ± 0.4^{fA}	1.3 ± 0.1^{cB}
P1 + S2 + LM1	P1	6.5 ± 0.2^{jA}	2.3 ± 0.1^{eB}
	S2	3.8 ± 0.2^{cA}	3.3 ± 0.1^{hB}
	LM1	4.7 ± 0.1^{fA}	2.7 ± 0.1^{fB}

Note: The same superscript lowercase letters (a-j) indicate the significant differences between the bacteria within the same column. The same superscript uppercase letters (A-B) indicate the significant differences between the columns (static and continuous).

In triple species biofilm, higher reduction was observed for the predominating bacteria in both systems. In the static system, *Pseudomonas* was found to be the predominating bacteria (Fig. 1) and showed the highest reduction in triple species (6.5 log CFU/cm^2) upon sanitizer treatment compared to single species (4.5 log CFU/cm^2) and dual species (3.7–3.9 log CFU/cm^2) biofilm. Similarly, *Listeria* was the predominating bacteria in the continuous system (Fig. 2) and showed the highest reduction in triple species (2.6 log CFU/cm^2) compared to single

(1.3) and dual species counterparts (1.0–1.3 log CFU/cm^2).

3.4. Exopolysaccharide production

Significantly higher ($p < 0.001$) exopolysaccharide was produced by the bacteria in the biofilm formed in the CDC reactor compared to the static system irrespective of the number of species of bacteria present in the biofilm (Fig. 3). In the static system, *Listeria* was found to be the lowest producer (3.2 μg/cm^2) of EPS together with the dual species combination of *Pseudomonas* and *Listeria* (3.6 μg/cm^2). Comparatively, *Listeria* in the CDC system produced higher EPS (7.6 μg/cm^2). In a continuous system, the dual species combination of *Pseudomonas* + *Staphylococcus* (6.3 μg/cm^2) and *Staphylococcus* + *Listeria* (6.4 μg/cm^2) produced comparable EPS with single species biofilm of *Pseudomonas* (6.0 μg/cm^2) and *Staphylococcus* (5.9 μg/cm^2). In both flow systems, there was no significant difference ($p > 0.05$) between the EPS produced by the three-species combination compared to two-species combinations.

3.5. Epifluorescence microscopy

Acridine orange staining of the single species biofilm of *Listeria* biofilm structure showed filament-like structures at 48 h (Fig. 4. A) where the cell concentration reached 5.7 log CFU/cm^2. These filamentous structures were absent in the static system (Fig. 4. B) with 7.1 log CFU/cm^2 cell concentration. A similar filamentous structure was observed in dual species with *Staphylococcus* (Fig. 4. C), where the *Listeria* cell concentration reached 5.2 log CFU/cm^2. In contrast, *Listeria* in dual species with *Pseudomonas* did not appear to have filaments but instead, a layer of *Pseudomonas* cells could be observed. With *Pseudomonas*, the cell concentration of *Listeria* was significantly higher ($p < 0.05$) at 8.2 log CFU/cm^2. Additionally, the formation of wide channel-like structures was observed in the three species of biofilm formed in a continuous system (Fig. 5. A). In contrast, the static system showed a different distribution of bacteria with a dense arrangement of bacteria throughout the surface of the coupon (Fig. 5. B).

4. Discussion

Significantly higher ($p < 0.05$) cell concentration (Figs. 1 and 2), exopolysaccharides (Fig. 3), and tolerance to a hypochlorite sanitizer (Table 1) were noted for biofilms formed in continuous systems as compared to static systems. The importance of hydrodynamics and flow in the biofilm formation and biofilm community shift in drinking water systems has been stated before (Liu et al., 2020; Wang et al., 2014). The flow plays a vital role in the access of nutrients, metabolites, shear stress, and oxygen ultimately shaping the three-dimensional structure of single species biofilm. In addition, the bacteria in the biofilm formed in flow conditions produce higher EPS and are more resistant to detachment from the surface (Paul et al., 2012; Wang et al., 2014). Depending on the bacterial species, the formation of exopolysaccharide is regulated by cyclic-di-guanosine-5′-monophosphate (*c*-di-GMP), quorum sensing molecules, and small RNAs. When these genetic variables are impacted by the stress factors, variations in the production of matrix compounds such as exopolysaccharides can be noted (Flemming et al., 2023). In triple species combinations, the highest reduction upon sanitizer treatment was observed for bacteria that exist in predominance in both systems which could be the result of a higher chance of exposure to the sanitizer in the biofilm. In this study, the three-species biofilm formed in the continuous system showed the formation of interstitial voids, also known as fluid channels (Fig. 5). This is normally established by the bacteria primarily for the transportation of metabolites, nutrients, and waste (Chitlapilly Dass and Wang, 2022). A study on the biofilm architecture of *P. aeruginosa* revealed a similar formation of open, hydrated structures with large voids in the biofilm structure where fresh media was continuously supplied (Davey et al., 2003). This continued access to the nutrients and metabolites could be the reason for the favorable growth of *Listeria* in the continuous system in this study. In contrast, at the same incubation time for static culture, dense patches of bacteria could be observed where *Pseudomonas* was the predominating species with *Staphylococcus* and *Listeria* in the three-species biofilm. Some research has hypothesized that the domination of *Pseudomonas* in multispecies set-ups is the result of higher EPS production (Chaggar et al., 2024). In contrast, this study found that even though biofilms in the CDC system had significantly higher EPS content, the predominant

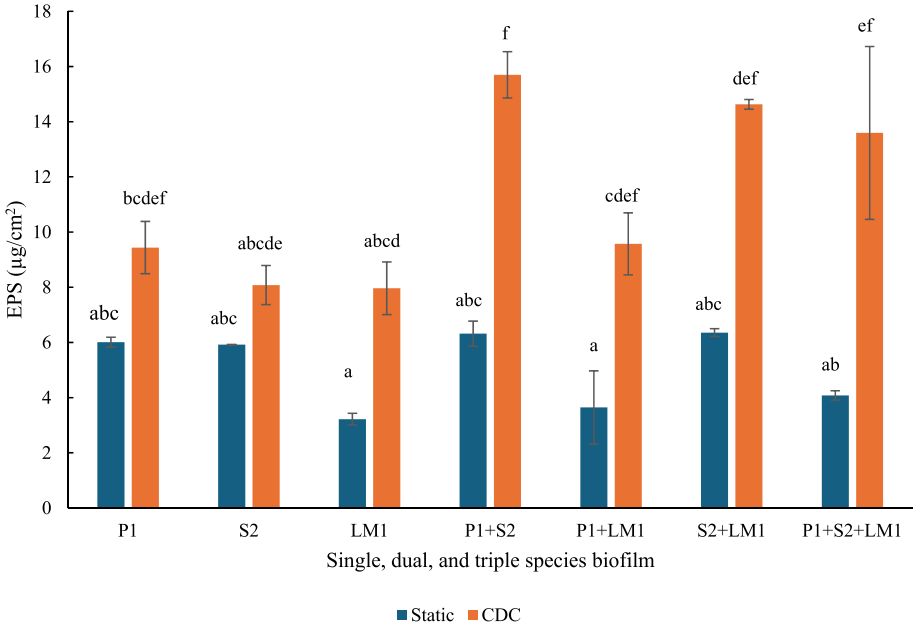

Fig. 3. Exopolysaccharide content (μg dextran/cm^2) determined for single, dual, and triple species combination of *Pseudomonas fluorescens* (P1), *Staphylococcus aureus* (S2), and *Listeria monocytogenes* (LM1) in static and CDC system. Note: The lowercase superscript (a-f) indicates the significant differences ($p < 0.05$) between the exopolysaccharide content produced by single and multiple species bacteria in static and CDC system.

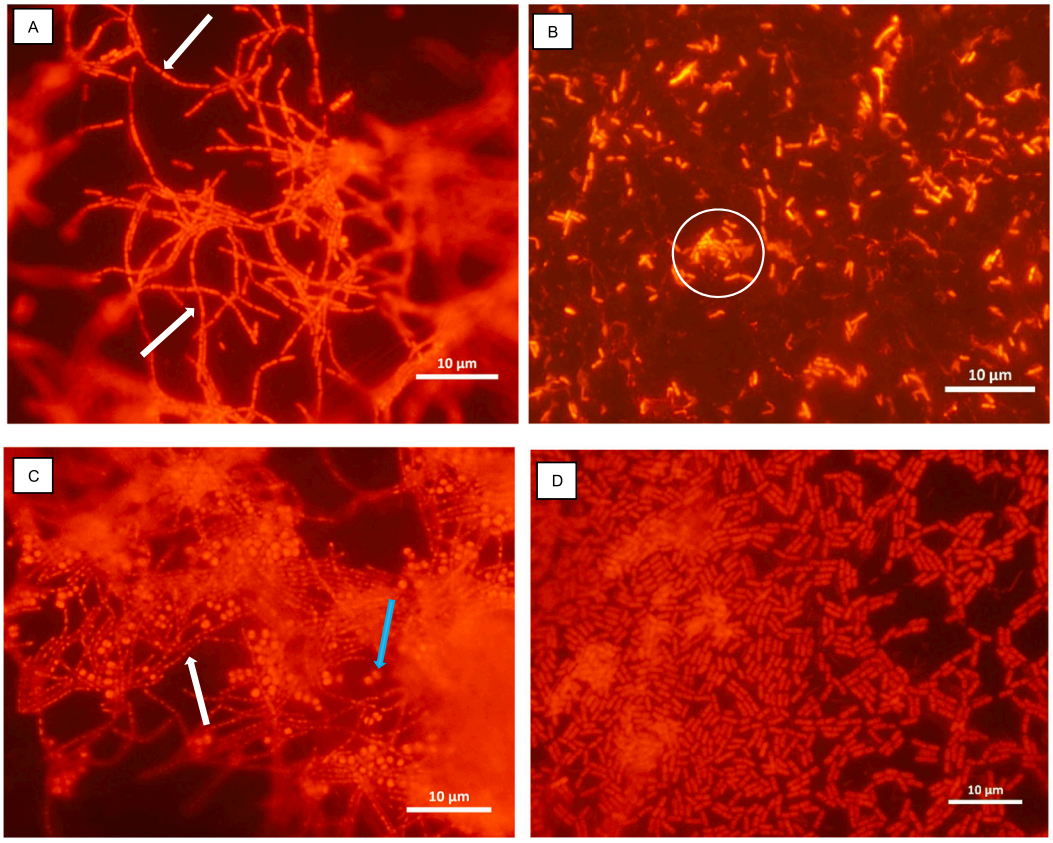

Fig. 4. *L. monocytogenes* biofilm formed at 48 h in 10 % TSB and 30 °C in A) single species in CDC bioreactor system B) single species in static system C) *Listeria monocytogenes* in dual species biofilm with *Staphylococcus aureus* in CDC bioreactor, D) *Listeria monocytogenes* in dual species with *Pseudomonas fluorescens* in CDC bioreactor, observed with acridine orange staining. Scale bar: 10 µm. Note: A) The white arrows indicate the *Listeria monocytogenes* filaments in the single species biofilm formed in the CDC bioreactor B) The white circle highlights the *Listeria monocytogenes* cells in single cell clusters on the stainless steel surface formed in static system C) White arrow indicates the filamentous *Listeria monocytogenes* and the blue arrow indicates the spherical *Staphylococcus aureus* cells entangles in the *Listeria monocytogenes* filaments in dual-species biofilm formed in CDC system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species was L. *monocytogenes* indicating the importance of flow for the order of predominance in a multispecies setup. The biofilm formation of *S. aureus* and L. *monocytogenes* in single species was found to be lowered in the first 24 h in the presence of flow, indicating that the shear has a higher impact on the weaker biofilm formers as compared to strong biofilm formers (*P. fluorescens*) which remained unaffected. Significantly ($p < 0.05$) higher cell concentration of L. *monocytogenes* was observed in dual and triple species in the presence of flow (Fig. 2). This could be the result of synergy between the bacteria in the presence of stress factors such as shear. The growth and the interaction of L. *monocytogenes* in multiple species biofilms are known to be different according to the type of stress present (Puga et al., 2016). The results showed a significant ($p < 0.05$) increase in the tolerance against hypochlorite sanitizer when multiple species of bacteria were present in the biofilm in both static and continuous systems (Table 1). Various studies have indicated that the elevated protection of bacteria in single and dual species biofilm is the result of the sheltering effect produced by a higher amount of EPS produced in the combination (Chaggar et al., 2024; Nkemngong et al., 2020). In this study, the EPS produced by single species biofilm was found to be comparable with dual and triple-species biofilms under a specific flow condition (Fig. 3) indicating other factors such as quorum sensing, and spatial organization could be the reasons behind elevated resistance in multiple-species biofilms formed under shear stress. Previous studies have noted that the biofilm properties (thickness, composition), spatial arrangement, and resistivity to the physical removal of the bacteria from the biofilm are significantly impacted by the presence of turbulent flow (Berne et al., 2018; Chawla et al., 2020).

In this study, biofilm streamers were observed for *Staphylococcus* biofilm in single and dual species (with *Listeria*) (Supplementary S2). In addition, *Listeria* could attach itself to the streamers formed by *S. aureus,* indicating tolerance between the bacteria. These interactions were found to be highly strain dependent. The inhibitory effect of L. *monocytogenes* by *Staphylococcus* via the inhibitory compounds has been noted before (Bockelmann et al., 2017; Lynch et al., 202). Biofilm formed in static conditions with no shear forces often forms biofilm through layering and ends up forming mushroom and tower structures (Secchi et al., 2022). With the addition of shear forces going unidirectional, the formation of filamentous structures referred to as biofilm streamers formed in a microfluidic channel has previously been observed for *P. aeruginosa* (Drescher et al., 2013; Secchi et al., 2022; Stoodley et al., 2002) and *S. aureus* (Kim et al., 2014). The foundation of *P. aeruginosa* biofilm streamers was found to be EPS and eDNA (Secchi et al., 2022), and they elongate by attaching the passing cells onto the EPS in the initial stages (Drescher et al., 2013). Once the initial attachment is complete, the elongation of the streamer depends on the type of biofilm formed. In solid biofilm, further elongation of the streamer is mostly through cell growth rather than attachment from the passing fluid. But, in the case of porous biofilm, the cells flowing through attach themselves to the streamers (Drescher et al., 2013). In addition to the gene expression for enhanced attachment and EPS production, flow decides the structure of the *S. aureus* biofilm. When flow is present, the motility of the bacteria does not play a vital role in the streamer type of biofilm formation (Kim et al., 2014). Like any other type of biofilm, dislodgement of the streamer type of biofilm results in the attachment of the

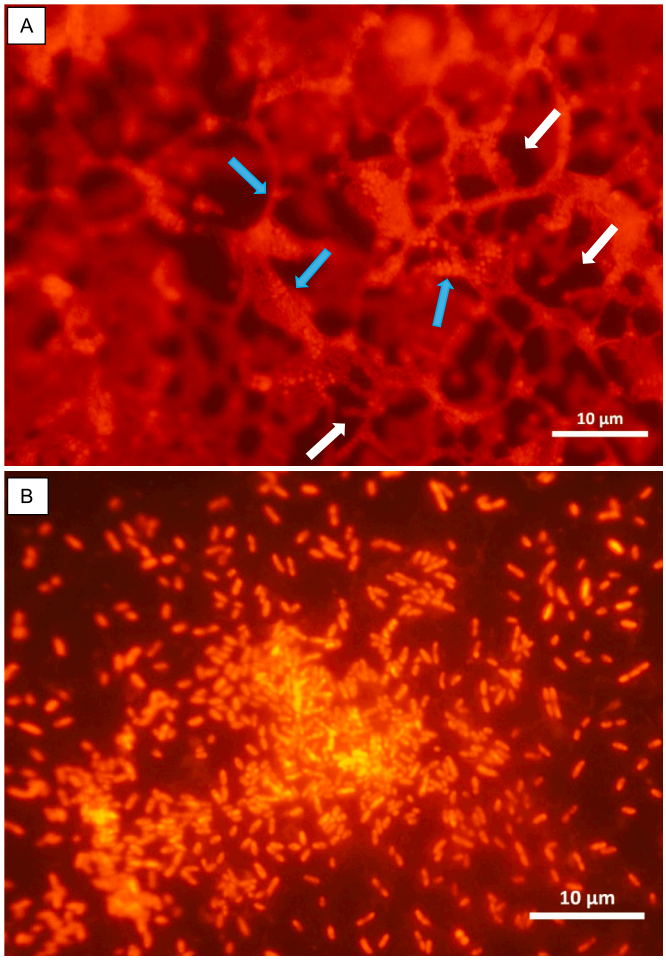

Fig. 5. Three-species (*Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Listeria monocytogenes*) biofilm formed at 72 h in 10 % TSB and 30 °C in A) CDC bioreactor system B) Static system, observed with acridine orange staining. Scale bar: 10 µm. Note: A) Blue arrows indicate the 3-D cell arrangement on the stainless-steel surface and white arrows indicate the interstitial voids in the 3-D structure formed in CDC bioreactor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detached biofilm to another site, continuing the process. Additionally, the location of the streamers plays an important role in deciding the flow in a microfluidic channel. The presence of *P. aeruginosa* streamers in the center of the channel results in a significantly decreased flow rate compared to the streamers attached to the wall of the channel (Drescher et al., 2013), which ultimately results in clogging (Kim et al., 2014) and infection challenges (Secchi et al., 2022) for biofilms formed in medical contexts.

Another interesting observation was made for the *Listeria* biofilm formed in a continuous system where filamentous structures of *Listeria* could be observed in single species (Fig. 4. A) and dual species (with *Staphylococcus*) (Fig. 4. C) but were absent in the biofilm formed in the static system (Fig. 4. B). In this study, the *Listeria* filaments were only observed when the cell concentration was <6 log CFU/cm^2 indicating the structural variation being the result of poor biofilm formation. These filamentous structures have previously been observed for L. *monocytogenes* in a planktonic state under sublethal sodium chloride concentrations (Hazeleger et al., 2006; Yamaki et al., 2021), antimicrobial stress (Rocha et al., 2019), alkaline stress (Giotis et al., 2007) and acidic stress in combination with sodium chloride (Bereksi et al., 2002). Furthermore, the filamentous *Listeria* was found to have higher stress tolerance compared to non-filamentous *Listeria* and multiplied rapidly once the cells ended the filamentous phase (Yamaki et al., 2021). So far, these filaments have not yet been reported in biofilm form. The formation of stress-resistant filamentous *Listeria* in the single and dual species biofilm in the continuous system has important implications in the food industry.

5. Conclusion

The cell concentration, sanitizer tolerance, and spatial organization of the biofilm are significantly affected by the presence of flow indicating the importance of shear stress and access to nutrients in the overall biofilm structure and characteristics. The ability of the L. *monocytogenes* to enhance its biofilm formation and attach to the *S. aureus* biofilm streamers in the presence of flow is concerning, considering the pathogenicity of L. *monocytogenes*. Additionally, the ability of L. *monocytogenes* to form filamentous cells under flow conditions in single and dual species biofilms with elevated chemical stress tolerance could be a serious concern in food safety. This study concludes that to understand the sanitizer tolerance of bacteria in multiple species biofilms in a dynamic system, in addition to the chemical components (EPS), the spatial arrangement of resistant bacteria in the biofilm architecture and variations in gene expression need to be studied.

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CRediT authorship contribution statement

Krisha Pant: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Jon Palmer:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Steve Flint:** Writing – review & editing, Supervision, Resources, Project administration, Methodology.

Declaration of competing interest

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

Data availability

Data will be made available on request.

References

Aarnisalo, K., Lundén, J., Korkeala, H., Wirtanen, G., 2007. Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. LWT-Food Sci. Technol. 40 (6), 1041–1048. https://doi.org/10.1016/j.lwt.2006.07.009.

Agustín, M.d.R., Brugnoni, L., 2018. Multispecies biofilms between *Listeria monocytogenes* and *Listeria innocua* with resident microbiota isolated from apple juice processing equipment. J. Food Saf. 38 (5), e12499. https://doi.org/10.1111/jfs.12499.

Barnes, T.M., Greive, K.A., 2013. Use of bleach baths for the treatment of infected atopic eczema. Australas. J. Dermatol. 54 (4), 251–258. https://doi.org/10.1111/ajd.12015.

Bereksi, N., Gavini, F., Benezech, T., Faille, C., 2002. Growth, morphology and surface properties of *Listeria monocytogenes* Scott A and LO28 under saline and acid environments. J. Appl. Microbiol. 92 (3), 556–565. https://doi.org/10.1046/j.1365-2672.2002.01564.x.

Berne, C., Ellison, C.K., Ducret, A., Brun, Y.V., 2018. Bacterial adhesion at the single-cell level. Nat. Rev. Microbiol. 16 (10), 616–627. https://doi.org/10.1038/s41579-018-0057-5.

Bockelmann, W., Koslowsky, M., Goerges, S., Scherer, S., Franz, C.M., Heller, K.J., 2017. Growth inhibition of *Listeria monocytogenes* by bacteriocin-producing *Staphylococcus equorum* SE3 in cheese models. Food Control 71, 50–56. https://doi.org/10.1016/j.foodcont.2016.06.019.

Brooks, J.D., Flint, S.H., 2008. Biofilms in the food industry: problems and potential solutions. Int. J. Food Sci. Technol. 43 (12), 2163–2176. https://doi.org/10.1111/j.1365-2621.2008.01839.x.

Chaggar, G.K., Bryant, D.B., Chen, R., Fajardo, D., Jules-Culver, Z.A., Drolia, R., Oliver, H.F., 2024. Development of *Salmonella enterica* serovar *typhimurium*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa* multi-species in vitro dry surface biofilm

models: insights into resilience and persistence in low-moisture environments. Food Control 166, 110703. https://doi.org/10.1016/j.foodcont.2024.110703.
Chaves, R.D., Kumazawa, S.H., Khaneghah, A.M., Alvarenga, V.O., Hungaro, H.M., Sant'Ana, A. S., 2024. Comparing the susceptibility to sanitizers, biofilm-forming ability, and biofilm resistance to quaternary ammonium and chlorine dioxide of 43 *Salmonella enterica* and *Listeria monocytogenes* strains. Food Microbiol. 117, 104380. https://doi.org/10.1016/j.fm.2023.104380.
Chawla, R., Gupta, R., Lele, T.P., Lele, P.P., 2020. A skeptic's guide to bacterial mechanosensing. J. Mol. Biol. 432 (2), 523–533. https://doi.org/10.1016/j.jmb.2019.09.004.
Chitlapilly Dass, S., Wang, R., 2022. Biofilm through the looking glass: A microbial food safety perspective. Pathogens 11 (3), 346. https://doi.org/10.3390/pathogens11030346.
Davey, M.E., Caiazza, N.C., O'Toole, G.A., 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 185 (3), 1027–1036. https://doi.org/10.1128/jb.185.3.1027-1036.2003.
Diarra, C., Goetz, C., Gagnon, M., Roy, D., Jean, J., 2023. Biofilm formation by heat-resistant dairy bacteria: multispecies biofilm model under static and dynamic conditions. Appl. Environ. Microbiol. 89 (10), e00713–e00723. https://doi.org/10.1128/aem.00713-23.
Drescher, K., Shen, Y., Bassler, B.L., Stone, H.A., 2013. Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems. Proc. Natl. Acad. Sci. 110 (11), 4345–4350. https://doi.org/10.1073/pnas.1300321110.
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). https://doi.org/10.1128/AEM.01046-17 e01046–01017.
Flemming, H.-C., van Hullebusch, E.D., Neu, T.R., Nielsen, P.H., Seviour, T., Stoodley, P., Wingender, J., Wuertz, S., 2023. The biofilm matrix: multitasking in a shared space. Nat. Rev. Microbiol. 21 (2), 70–86. https://doi.org/10.1038/s41579-022-00791-0.
Giotis, E.S., Blair, I.S., McDowell, D.A., 2007. Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. Int. J. Food Microbiol. 120 (3), 250–258. https://doi.org/10.1016/j.ijfoodmicro.2007.08.036.
Hazeleger, W.C., Dalvoorde, M., Beumer, R.R., 2006. Fluorescence microscopy of NaCl-stressed, elongated *Salmonella* and *Listeria* cells reveals the presence of septa in filaments. Int. J. Food Microbiol. 112 (3), 288–290. https://doi.org/10.1016/j.ijfoodmicro.2006.04.026.
Jha, P.K., Dallagi, H., Richard, E., Benezech, T., Faille, C., 2020. Formation and resistance to cleaning of biofilms at air-liquid-wall interface. Influence of bacterial strain and material. Food Control 118, 107384. https://doi.org/10.1016/j.foodcont.2020.107384.
Kim, M.K., Drescher, K., Pak, O.S., Bassler, B.L., Stone, H.A., 2014. Filaments in curved streamlines: rapid formation of *Staphylococcus aureus* biofilm streamers. New J. Phys. 16 (6), 065024. https://doi.org/10.1088/1367-2630/16/6/065024.
Kocot, A.M., Olszewska, M.A., 2020. Interaction of *Pseudomonas aeruginosa* and *Staphylococcus aureus* with *Listeria innocua* in dual species biofilms and inactivation following disinfectant treatments. Lwt 118, 108736. https://doi.org/10.1016/j.lwt.2019.108736.
Leriche, V., Carpentier, B., 2000. Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms. J. Appl. Microbiol. 88 (4), 594–605. https://doi.org/10.1046/j.1365-2672.2000.01000.x.
Lindsay, D., Killington, A., Fouhy, K., Loh, M., Malakar, P., 2022. The CDC biofilm bioreactor is a suitable method to grow biofilms, and test their sanitiser susceptibilities, in the dairy context. Int. Dairy J. 126, 105264. https://doi.org/10.1016/j.idairyj.2021.105264.
Lineback, C.B., Nkemngong, C.A., Wu, S.T., Li, X., Teska, P.J., Oliver, H.F., 2018. Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms than quaternary ammonium compounds. Antimicrob. Resist. Infect. Control 7, 1–7. https://doi.org/10.1186/s13756-018-0447-5.
Liu, Y., Shan, R., Chen, G., Liu, L., 2020. Linking flow velocity-regulated EPS production with early-stage biofilm formation in drinking water distribution systems. Water Supply 20 (4), 1253–1263. https://doi.org/10.2166/ws.2020.039.
Lynch, D., Hill, C., Field, D., Begley, M., 2021. Inhibition of *Listeria monocytogenes* by the Staphylococcus capitis-derived bacteriocin capidermicin. Food Microbiol. 94, 103661. doi: 10.1016/j.fm.2020.103661.
Mendez, E., Walker, D.K., Vipham, J., Trinetta, V., 2020. The use of a CDC biofilm reactor to grow multi-strain *Listeria monocytogenes* biofilm. Food Microbiol. 92, 103592. https://doi.org/10.1016/j.fm.2020.103592.
Mettler, M.K., Parker, C.W., Venkateswaran, K., Peyton, B.M., 2022. Antimicrobial coating efficacy for prevention of *Pseudomonas aeruginosa* biofilm growth on ISS water system materials. Front. Microbiol. 13, 874236. https://doi.org/10.3389/fmicb.2022.874236.
Nkemngong, C.A., Voorn, M.G., Li, X., Teska, P.J., Oliver, H.F., 2020. A rapid model for developing dry surface biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* for in vitro disinfectant efficacy testing. Antimicrob. Resist. Infect. Control 9, 1–9. https://doi.org/10.1186/s13756-020-00792-9.
Pang, X., Wong, C., Chung, H.-J., Yuk, H.-G., 2019. Biofilm formation of *Listeria monocytogenes* and its resistance to quaternary ammonium compounds in a simulated salmon processing environment. Food Control 98, 200–208. https://doi.org/10.1016/j.foodcont.2018.11.029.
Papaioannou, E., Giaouris, E.D., Berillis, P., Boziaris, I.S., 2018. Dynamics of biofilm formation by *Listeria monocytogenes* on stainless steel under mono-species and mixed-culture simulated fish processing conditions and chemical disinfection challenges. Int. J. Food Microbiol. 267, 9–19. https://doi.org/10.1016/j.ijfoodmicro.2017.12.020.
Paul, E., Ochoa, J.C., Pechaud, Y., Liu, Y., Liné, A., 2012. Effect of shear stress and growth conditions on detachment and physical properties of biofilms. Water Res. 46 (17), 5499–5508. https://doi.org/10.1016/j.watres.2012.07.029.
Puga, C.H., Orgaz, B., SanJose, C., 2016. Listeria monocytogenes impact on mature or old Pseudomonas fluorescens biofilms during growth at 4 and 20 C. Front. Microbiol. 7, 134. https://doi.org/10.3389/fmicb.2016.00134.
Ramstedt, M., Burmølle, M., 2022. Can multi-species biofilms defeat antimicrobial surfaces on medical devices? Curr. Opin. Biomed. Eng. 22, 100370. https://doi.org/10.1016/j.cobme.2022.100370.
Ran, Y., Qingmin, C., Maorun, F., 2019. Chlorine dioxide generation method and its action mechanism for removing harmful substances and maintaining quality attributes of agricultural products. Food Bioprocess Technol. 12, 1110–1122. https://doi.org/10.1007/s11947-019-02279-x.
Rocha, R., Sousa, J.M., Cerqueira, L., Vieira, M.J., Almeida, C., Azevedo, N.F., 2019. Development and application of peptide nucleic acid fluorescence in situ hybridization for the specific detection of *Listeria monocytogenes*. Food Microbiol. 80, 1–8. https://doi.org/10.1016/j.fm.2018.12.009.
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, 705. https://doi.org/10.3389/fmicb.2015.00705.
Secchi, E., Savorana, G., Vitale, A., Eberl, L., Stocker, R., Rusconi, R., 2022. The structural role of bacterial eDNA in the formation of biofilm streamers. Proc. Natl. Acad. Sci. 119 (12), e2113723119. https://doi.org/10.1073/pnas.2113723119.
Simões, M., Simões, L.C., Vieira, M.J., 2010. A review of current and emergent biofilm control strategies. LWT–Food Sci. Technol. 43 (4), 573–583. https://doi.org/10.1016/j.lwt.2009.12.008.
Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W., 2002. Biofilms as complex differentiated communities. Ann. Rev. Microbiol. 56 (1), 187–209. https://doi.org/10.1146/annurev.micro.56.012302.160705.
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.
Wang, C., Miao, L., Hou, J., Wang, P., Qian, J., Dai, S., 2014. The effect of flow velocity on the distribution and composition of extracellular polymeric substances in biofilms and the detachment mechanism of biofilms. Water Sci. Technol. 69 (4), 825–832. https://doi.org/10.2166/wst.2013.785.
Xu, Z., Xie, J., Soteyome, T., Peters, B.M., Shirtliff, M.E., Liu, J., Harro, J.M., 2019. Polymicrobial interaction and biofilms between *Staphylococcus aureus* and *Pseudomonas aeruginosa*: an underestimated concern in food safety. Curr. Opin. Food Sci. 26, 57–64. https://doi.org/10.1016/j.cofs.2019.03.006.
Yamaki, S., Kawai, Y., Yamazaki, K., 2021. Long filamentous state of *Listeria monocytogenes* induced by sublethal sodium chloride stress poses risk of rapid increase in colony-forming units. Food Control 124, 107860. https://doi.org/10.1016/j.foodcont.2020.107860.
Yuan, L., Wang, N., Sadiq, F.A., He, G., 2020. Interspecies interactions in dual-species biofilms formed by psychrotrophic bacteria and the tolerance of sessile communities to disinfectants. J. Food Prot. 83 (6), 951–958. https://doi.org/10.4315/0362-028X.JFP-19-396.
Zhou, G., Dong, P., Luo, X., Zhu, L., Mao, Y., Liu, Y., Zhang, Y., 2024. Combined effects of cold and acid on dual-species biofilms of *Pseudomonas fluorescens* and *Listeria monocytogenes* under simulated chilled beef processing conditions. Food Microbiol. 117, 104394. https://doi.org/10.1016/j.fm.2023.104394.