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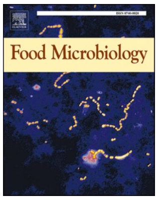

Conditional synergy: Impact of nutrient abundance on multispecies biofilm formation and sanitizer tolerance

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ABSTRACT

Bacteria exist in varying nutrient conditions and complex microbial consortia. *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Listeria monocytogenes* are commonly occurring biofilm-formers, share a similar nutritional niche, and have been isolated from common surfaces in multispecies environments. Biofilm properties, including biomass (O.D$_{590}$ nm), cell concentration (log CFU/cm^2), exopolysaccharide content (μg/cm^2), structure, and sanitizer tolerance (sodium hypochlorite), were observed under varying nutrient (full-strength TSB and 10 % TSB) conditions on polystyrene surfaces for single and multispecies biofilm. The synergistic interactions between the bacteria in multispecies biofilm were found to be nutrient-dependent, with significantly higher ($p < 0.05$) biofilm formation, exopolysaccharide content, and sanitizer tolerance in high nutrient conditions (TSB) compared with low nutrient conditions (10 % TSB). The cell concentrations in the biofilm (single and multispecies) were found to be comparable between TSB and 10 % TSB. All three bacteria involved showed increased tolerance against sanitizers in the multispecies arrangement compared to their single-species counterparts, with significantly higher survival for *L. monocytogenes* (5.3 log CFU/cm^2) in a multispecies biofilm compared to its single-species counterpart (2.3 log CFU/cm^2). A positive correlation was observed between exopolysaccharide concentration and sanitizer tolerance. This study highlights the importance of taking multiple bacteria and their growth environment into account when understanding sanitizer response, as it varies in multispecies biofilm setups and according to nutrient availability.

1. Introduction

The presence of multispecies bacteria in the natural biofilms has been observed in a multitude of industrial settings and food processing areas, often leading to contamination and microbial corrosion (Bonneville et al., 2021; Di Pippo et al., 2018; Dula et al., 2021). Biofilm formers such as *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Listeria monocytogenes* have been isolated in combination from food surfaces (Cherif-Antar et al., 2016), such as those in the dairy industry (Oxaran et al., 2018). *Staphylococcus* and *Pseudomonas* are good biofilm formers with common nutritional niches such as meat, milk and dairy products, fresh fruits and vegetables, with *Pseudomonas* causing spoilage and *Staphylococcus* capable of causing food poisoning (Xu et al., 2019). *L. monocytogenes* can adhere and multiply on industrial surfaces in the presence of *Pseudomonas* (Agustín and Brugnoni, 2018) and has been isolated in multispecies communities from drains (Dzieciol et al., 2016). *Pseudomonas* can protect and harbor *L. monocytogenes* against sanitizers on various surfaces (Dos Santos et al., 2023; Fagerlund et al., 2017; Kocot and Olszewska, 2020; Thomassen et al., 2023). The multispecies biofilms show increased resistance to cleaning agents under synergistic interactions compared to single-species biofilms, which has been associated with the exchange of public goods, quorum sensing between the bacteria involved, and added resistance provided by the biofilm matrix (Alonso et al., 2020; Sanchez-Vizuete et al., 2015; Wicaksono et al., 2022; Yuan et al., 2020). The bacteria that survive these treatments on industrial processing surfaces can be released into the passing bulk fluid/substrate, partly due to shear stress, and contaminate the food (Rückerl et al., 2014), which has been previously noted for pathogens such as *L. monocytogenes* (Rodríguez-Campos et al., 2019). The cross-contamination that leads from failure to remove biofilm on food industry surfaces has so far accounted for 25 % of the world's food safety outbreak cases (Yushina et al., 2024).

The sanitizers commonly used in the food industry for cleaning and sanitation are quaternary ammonium compounds, peracetic acid, and chlorine compounds such as sodium hypochlorite (Chaves et al., 2024; Simões et al., 2010). Chlorine compounds are also strong oxidizers and

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can interact 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 group. Additionally, chlorine oxidizes the organic matter in the biofilm before diffusing into the layers for a bactericidal effect (Sarjit and Dykes, 2017). The recommended concentration of sanitizer, such as sodium hypochlorite (active chlorine 13 %), ranges from 0.05 to 2 % (500–20,000 ppm), depending on the intended application and treatment time (Aarnisalo et al., 2007). Environmental variables such as temperature (Yushina et al., 2024), nutrients, and substrate variables such as topography, material, and coating/conditioning impact the interspecies interaction in the biofilm properties, such as key species, chemical composition, and sanitizer resistance (Ramstedt and Burmølle, 2022). In the food industry, surfaces are cleaned and sanitized frequently, which results in a nutrient-depleted surface for bacterial biofilms. Hence, it is important to understand the responses of biofilm-forming bacteria under different nutrient conditions (Pang et al., 2019). The biofilm and its components, including exopolysaccharide components, proteins, and eDNA, are affected by the nutrition conditions and are reported to be different for nutrient-limiting conditions (Wang et al., 2022).

The bacteria on the food contact surfaces rarely exist by themselves (Sadiq et al., 2017) hence the sanitizer response is defined by the complex interaction dynamics of the multiple bacteria present in the biofilm (Yuan et al., 2020). Although several researchers have studied the multispecies behavior of bacteria in the biofilm, few have focused on the relationship between nutrient conditions and interspecies interactions (Alonso and Kabuki, 2019; Halim et al., 2025; Pant et al., 2025). Nutrient concentration is a critical parameter for biofilm formation when the interspecies interactions of multiple species of bacteria are observed (Ibusquiza et al., 2012; Wang et al., 2022). This study aimed to understand the effect of nutrient variation on the single, dual, and triple-species biofilm formed by *Pseudomonas fluorescens*, *S. aureus*, and *L. monocytogenes* and their tolerance to sub-lethal concentrations of sodium hypochlorite (50 ppm/5 min).

2. Materials and methods

2.1. Bacterial strains and culture media used

Stock cultures (−80 °C in glycerol) of *P. fluorescens* (dairy), *S. aureus* ATCC 9144, and *L. monocytogenes* (environment) were inoculated into tryptone soy broth (TSB) (Difco™, Becton, Dickinson and Company, USA) and incubated at 30 °C for 18 h. All three bacteria are from a previously established culture collection of the Food Microbiology Lab, School of Natural Sciences and Food Technology, Massey University, Palmerston North, New Zealand. 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 solution (0.85 % w/v) via vortex mixing (30 s, highest setting) (Scilogex, Germany) and centrifugation (3000 *g* for 10 min). The concentrated cells were collected by removing the saline solution and dissolving the final pellet into fresh sterile saline solution to a 6 log CFU/mL cell concentration. The cell concentration of each bacterium was set to 6 log CFU/mL using a graph of optical density (O. D_{600nm}) vs. bacterial concentration (log CFU/mL) ($R^2 = 0.97$).

2.2. Biofilm formation

Polystyrene 96-well plates (Falcon, USA) (0.34 cm^2) were used as substrates. For single-species biofilm formation, 20 μL of bacterial solution (in saline) (7 log CFU/mL) was added to 180 μL of 10 % tryptone soy broth (TSB) in each well for biofilm formation. For multispecies biofilm formation, *P. fluorescens*, *S. aureus*, and *L. monocytogenes* in 1:1 (10 μL each) and 1:1:1 (10 μL each) was added into the 180 μL media (TSB and 10 % TSB) in single, dual, and triple species in each well. The final cell concentration was fixed at 6 log CFU/mL. The wells were incubated for 24 h at 30 °C under static conditions. For blank samples, respective sterile media were added to the wells. The full-strength TSB and 10 % TSB were selected to create nutrient-abundant and nutrient-deficient conditions, respectively (Chen et al., 2020; Folsom et al., 2006).

2.3. Biofilm biomass

The biofilm biomass was determined according to (Stepanović et al., 2004) with crystal violet dye (Acros Oranics, USA). Crystal violet stains all biofilm components, including cells and extracellular components. The 24 h incubated plates from section 2.2 were washed with saline solution (× 3) (200 μL) and air-dried at room temperature for 20 min. Crystal violet dye (200 μL) (0.5 % w/v in water) was added to each well of a 96-well plate and allowed to rest for 20 min before washing with saline solution until the washed saline runs clear. The stained plate was allowed to air-dry at room temperature (30 min) before adding 200 μL of 95 % ethanol. After 20 min of ethanol leeching, the absorbance value was noted at an optical density of 590 nm using a spectrophotometer (Varioskan Lux 3020–1333, Thermo Fisher Scientific Ltd., USA).

Depending on the crystal violet value for the single and multispecies biofilm, the interaction was classified into synergistic, neutral, and antagonistic. For synergy to be effective, the absorbance for the multispecies biofilm (OD_{590} MS) needed to be higher than the absorbance obtained from the highest biofilm producer in the single-species biofilm (OD_{590} HS). This can be written as (OD_{590} MS- S.D) > (O.D_{590} HS + S.D), where S.D is the standard deviation of respective values. Similarly for neutral interaction, (OD_{590} MS- S.D) = (O.D_{590} HS + S.D) and for competitive interactions, (OD_{590} MS- S.D) < (O.D_{590} HS + S.D) (Ren et al., 2015).

2.4. Sanitizer treatment

The response of single and multiple species bacteria in biofilm to sub-lethal concentrations of sodium hypochlorite sanitizer was analyzed according to (Lin et al., 2022). After 24 h incubation at 30 °C, the wells were washed with sterile saline (x2) to remove planktonic cells from the wells before treating with sodium hypochlorite (200 μL, 50 ppm, pH: 6.8–7) (Janola, Australia) for 5 min at room temperature immediately followed by 200 μL of 1 % (w/v) sodium thiosulphate neutralizer (AnalaR®, VWR International, England) for 2 min. The wells were then promptly saline solution washed and air dried. The wells were swabbed with cotton buds (Citoswab® Citotest Labware, China) in saline solution and then mixed by vortex (highest setting/1 min). The serial 10-fold dilutions for plate counting were carried out using saline solution (0.85 % w/v) and plated on selective agars. *Pseudomonas* Isolation agar (Difco™, USA) for *Pseudomonas*, Mannitol salt agar (Oxoid, UK) for *Staphylococcus,* and Modified Oxford agar (HiMedia, USA) for *Listeria*.

2.5. Exopolysaccharide concentration

The biofilm matrix from the polystyrene surface was extracted using sonication and then quantified using phenol-sulphuric acid hydrolysis (Zhou et al., 2024). The 96-well plates with single and multispecies biofilms (3 days) were washed (× 3) with sterile saline solutions to remove planktonic cells. Sterile distilled water (200 μL) was added into each well and sealed using microplate sealing tape (Nunc™, Thermo Scientific™, Denmark) before sonication for 20 min (40 kHz) (Ultrasonic cleaner, DAIHAN Scientific Co., Ltd, Korea). The extraction process was completed by centrifugation (14,400 *g*/5 min) of the sample solution collected from the wells. The supernatant pooled from a plate (96-wells) was collected for filtration (0.20 μm), and the pellet was discarded. To 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 (Supplementary 1), and the EPS was determined in ug/cm^2 of the polystyrene surface.

To confirm the removal of biofilm from the surface through sonication, the wells incubated with the sample and blank were stained with crystal violet (0.5 % w/v) after sonication and compared.

2.6. Confocal microscopy and surface plots

The co-localization of the bacteria and the structure of the biofilm formed under varying nutrient conditions was analyzed using two different stains: 4′,6-diamino-2-phenylindole (DAPI) (Invitrogen, Thermo Fisher Scientific, USA) for *P. fluorescens* and wheat germ agglutinin, Texas Red™- X conjugate (Invitrogen, Thermo-Fischer Scientific, Life Technologies corporation, USA) for *L. monocytogenes* (Zhou et al., 2024). Biofilm formed in 10 % TSB and full-strength TSB for 72 h at 30 °C on polystyrene surfaces was selected for staining and confocal microscopy (Nikon D-eclipse C1, Japan). The polystyrene coupons were washed with PBS (Oxoid, Oxoid Ltd, UK) and a staining solution containing 6 μL of DAPI (1 mg/mL) and 6 μL of wheat germ agglutinin-Texas red (1 mg/mL) in 6 mL of TSB. The staining was completed in the dark for 60 min at room temperature, after which the coupons were washed with PBS and allowed to air-dry before proceeding to the confocal imaging. The z-stack sequence was obtained using EZ-C1 (Gold version 3.80 build 860) and processing in ImageJ software (ImageJ 1.54g, National Institute of Health, USA) for surface plots. *P. fluorescens* and *L. monocytogenes* dual-species biofilm was chosen for imaging based on the lowest log reduction of each bacterium (higher sanitizer tolerance) observed compared to other combinations and their single counterparts (Table 2).

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 post-hoc analysis. The normality of the data was verified using the Shapiro-wilk ($p > 0.05$) and Kolmogorov-Smirnov test ($p > 0.05$). The correlation between the properties of the biofilm was analyzed using Pearson's Correlation Analysis (IBM SPSS version 29). All experiments had at least 2 biological replicates and 6 technical replicates, and results were presented as mean ± standard deviation.

3. Results

3.1. Biofilm formation

All three bacteria formed biofilm on the polystyrene surface under both nutrient conditions (TSB and 10 % TSB) as observed through the O. D$_{590}$ nm value after crystal violet staining. Significantly higher biofilm ($p < 0.001$) was observed in full-strength TSB compared to 10 % TSB for all three bacteria in single and multispecies (Fig. 1). In 10 % TSB, *P. fluorescens* (0.09) and *S. aureus* (0.06) formed significantly higher biofilm ($p < 0.001$) compared to *L. monocytogenes* (0.02) in single species. A similar pattern was observed for single-species biofilm formed in TSB, with *L. monocytogenes* being the poor biofilm former (0.11) compared to *P. fluorescens* (0.21) and *S. aureus* (0.37) (Fig. 1).

The relationship equation by (Ren et al., 2015) provided insight into the interaction between the bacteria in both nutrient-sufficient (TSB) and nutrient-deficient (10 % TSB) conditions. In TSB, the combination of *P. fluorescens*, *S. aureus*, and *L. monocytogenes* in dual and triple species biofilm resulted in synergistic interactions except for *P. fluorescens* and *L. monocytogenes* dual species biofilm, which resulted in neutral interaction (0.2) (Table 1).

Synergistic interaction: MS (O.D$_{590}$ - S. D) > SS (O. D$_{590}$ + S. D); Neutral interaction: MS (O.D$_{590}$ - S. D) = SS (O. D$_{590}$ + S. D);

Table 1
Interaction between *P. fluorescens* (P1), *S. aureus* (S2), and *L. monocytogenes* (LM1) in dual and triple species biofilm formed in TSB and 10 % TSB based on the relationship equation by (Ren et al., 2015).

Media	Sample	Multispecies biofilm MS (O. D$_{590}$ - S. D)	Highest biofilm former- single species SS (O. D$_{590}$ + S. D)	Interaction
10 % TSB	P1+S2	0.09	0.16	Antagonistic
	P1+LM1	0.07	0.11	Antagonistic
	S2+LM1	0.11	0.07	Synergistic
	P1+S2+LM1	0.06	0.11	Antagonistic
TSB	P1+S2	0.75	0.37	Synergistic
	P1+LM1	0.22	0.23	Neutral
	S2+LM1	0.78	0.37	Synergistic
	P1+S2+LM1	0.81	0.37	Synergistic

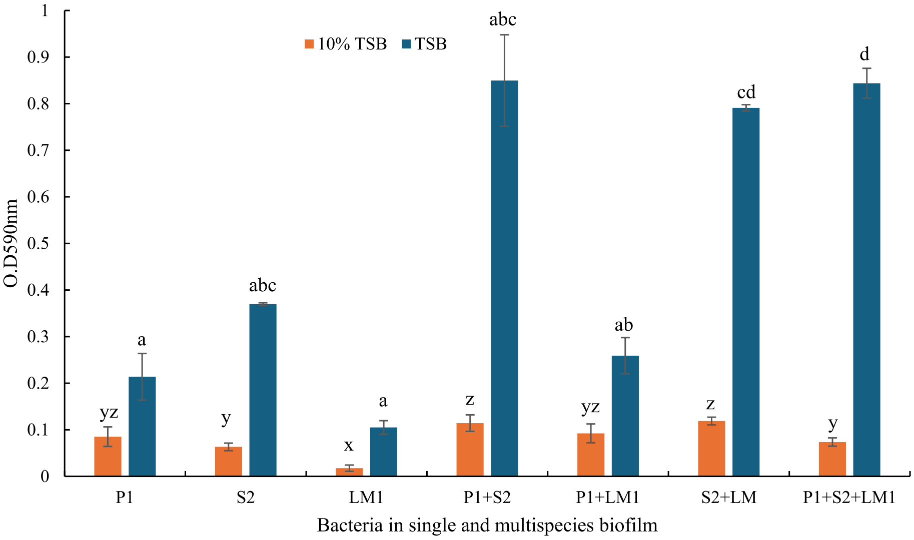

Fig. 1. Crystal violet values (O.D$_{590}$ nm) of single, dual, and triple species biofilm (24h) formed in 10 % TSB and full-strength TSB. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Antagonistic interac*tion MS (O.D590 - S. D)* $<$ *SS (O. D590 + S. D)*

Biofilm formed under 10 % TSB resulted in antagonistic interactions between all combinations of dual and triple species, except *S. aureus* and *L. monocytogenes* dual species, indicating the nutrient content-based synergy of the bacteria involved (Table 1).

3.2. *Biofilm formation: cell concentrations*

Under nutrient-limiting concentrations (10 % TSB), there was no significant difference ($p > 0.05$) in the cell concentration between single and multispecies biofilm for all three bacteria involved (*P. fluorescens*, *S. aureus*, and *L. monocytogenes*). *P. fluorescens*, *S. aureus*, and *L. monocytogenes* reached the cell concentration of 6.1–6.3 log CFU/cm^2, 7.4–7.7 log CFU/cm^2, and 6.1–7.0 log CFU/cm^2, respectively, indicating strong biofilm formation in both single and multispecies biofilms (Fig. 2). The co-inoculation of the second bacterium did not impact the cell concentrations of any bacteria involved in the dual and triple species biofilm formed in 10 % TSB (Fig. 2).

In full-strength TSB, the cell concentration of all three bacteria involved, *P. fluorescens*, *S. aureus*, and *L. monocytogenes,* were comparable in single and dual species, reaching 7.0–7.7 log CFU/cm^2, 6.5–7.6 log CFU/cm^2, and 6.1–6.6 log CFU/cm^2, respectively, for each bacterium. Significantly higher cell concentration ($p < 0.001$) was observed for *L. monocytogenes* in the three-species biofilm (7.9 log CFU/cm^2) compared to its single (6.6 log CFU/cm^2) and dual species (6.1–6.4 log CFU/cm^2) counterparts (Fig. 2). There was no significant difference ($p > 0.05$) between the cell concentrations of biofilm in TSB and 10 % TSB.

In the three-species biofilm, the predominance of the bacteria varied depending on the media used. In the three-species biofilm formed in 10 % TSB, the cell concentration of *S. aureus* was higher (7.7 log CFU/cm^2) compared to *L. monocytogenes* (6.18 log CFU/cm^2) and *P. fluorescens* (6.76 log CFU/cm^2), indicating the predominance of *S. aureus*. In contrast, observations showed that the three species biofilm formed in full-strength TSB had no significant differences ($p > 0.05$) in the cell concentration among *P. fluorescens* (7.7 log CFU/cm^2), *S. aureus* (8.3 log CFU/cm^2), and *L. monocytogenes* (7.9 log CFU/cm^2) (Fig. 2).

3.3. *Exopolysaccharide content*

The exopolysaccharide (EPS) content of the biofilm formed under nutrient-abundant conditions (TSB) was significantly higher ($p < 0.001$) than that under nutrient-limiting conditions (10 % TSB) (Fig. 3). Under both nutrient conditions, *L. monocytogenes* produced significantly lower exopolysaccharides (2.5–11.8 μg/cm^2) compared to *S. aureus* and *P. fluorescens*. In nutrient-limited conditions, the triple species combination resulted in significantly higher ($p < 0.001$) EPS content (16.9 μg/cm^2) compared to their single species counterparts (1.3–8.0 μg/cm^2) but was comparable to their dual species combinations (13.0–15.0 μg/cm^2). A similar observation was made for a multispecies biofilm formed in full-strength TSB, where the three species biofilm showed significantly higher ($p < 0.001$) EPS concentration (60.9 μg/cm^2) compared to their single species (11.8–33.5 μg/cm^2) and dual species counterpart (36.7–42.3 μg/cm^2). The EPS concentration (Fig. 3) reflected the findings of biofilm formation (crystal violet staining) (Fig. 1).

3.4. *Sanitizer tolerance*

The sanitizer treatment of biofilms (single and multispecies) formed in nutrient-limiting conditions (10 % TSB) reduced the cell concentration of all three bacteria, *P. fluorescens*, *S. aureus*, and *L. monocytogenes* below the detection limit (<2.1 log CFU/cm^2) (Fig. 4).

In full-strength TSB, the highest reduction post-sanitizer treatment was observed for *L. monocytogenes* in single species (4.3 log reduction), which improved in dual species, in the presence of *P. fluorescens* (1.1 log reduction) and *S. aureus* (2.2 log reduction). Similar observations were made for *P. fluorescens* with 2.8 log reduction in single species, 2.0 log reduction with *S. aureus* in dual species*,* and 1.4 log reduction with *L. monocytogenes* in dual species. The lowest log reduction was observed for *S. aureus* in single species (2.4 log reduction), which further improved in dual species (1.9 log reduction with *P. fluorescens,* and 2.2 log reduction with *L. monocytogenes*) (Table 2).

In the three-species biofilm formed in full-strength TSB, the survival of all three bacteria increased with the log reduction ranging 1.3 log CFU/cm^2, 1.8 log CFU/cm^2, and 3.3 log CFU/cm^2 for *P. fluorescens*, *S. aureus*, and *L. monocytogenes,* respectively. This indicated that all three bacteria in this study benefit from the synergy in dual and triple-species biofilm (Fig. 4).

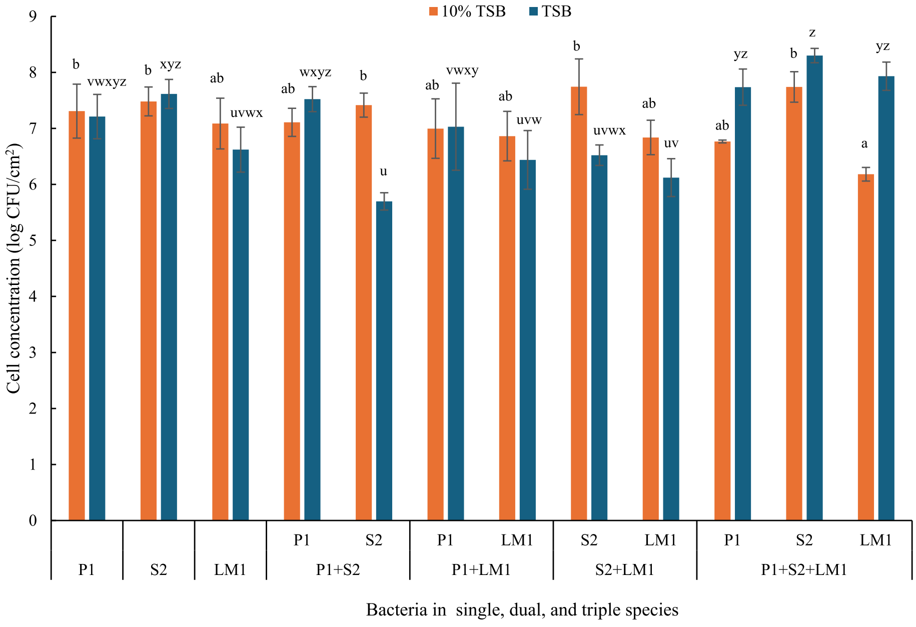

Fig. 2. Cell concentrations of *P. fluorescens* (P1), *S. aureus* (S2), and *L. monocytogenes* (LM1) in single, dual, and triple species biofilm formed in nutrient-limited conditions (10 % TSB) and nutrient-abundant conditions (TSB).

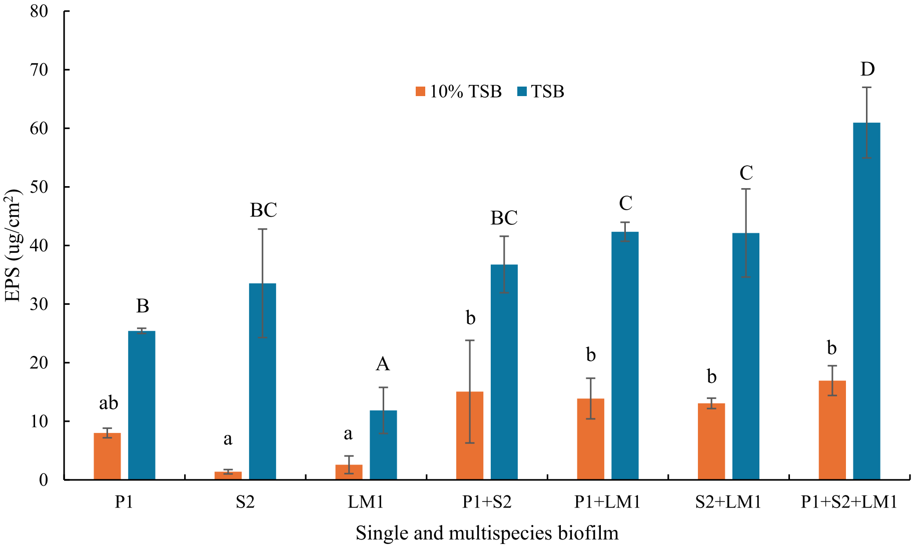

Fig. 3. Exopolysaccharide concentration (μg/cm^2) of single, dual, and triple species biofilm consisting of *P. fluorescens* (P1), *S. aureus* (S2), and *L. monocytogenes* (LM1).

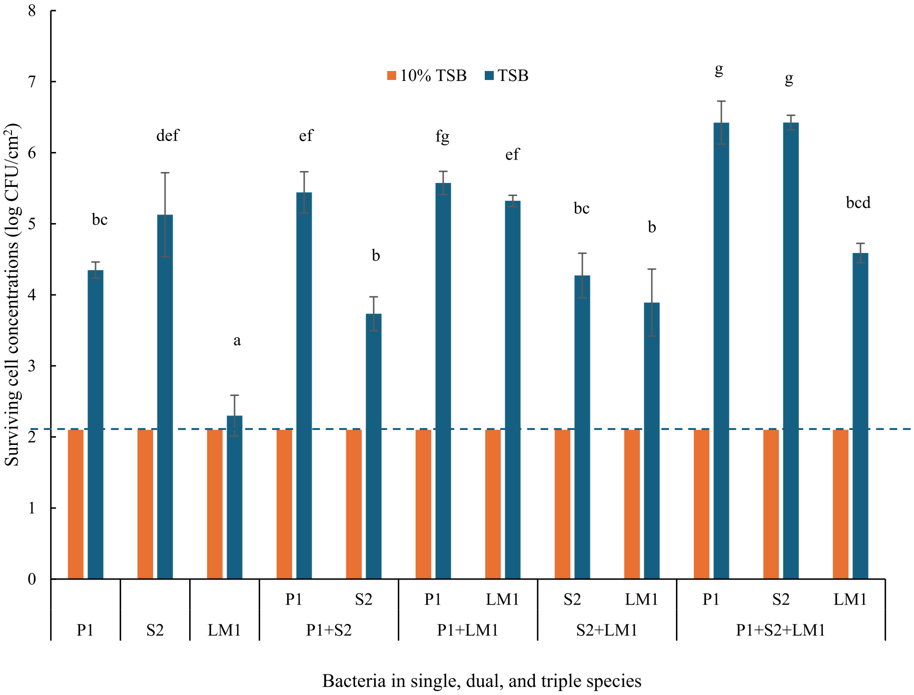

Fig. 4. Surviving cell concentrations (log CFU/cm^2) of *P. fluorescens* (P1), *S. aureus* (S2), and *L. monocytogenes* (LM1) in single, dual, and triple species biofilm formed in 10 % TSB and full-strength TSB after treatment with sodium hypochlorite (50 ppm/5 min). The dotted line represents the detection limit (2.1 log CFU/cm^2).

3.5. Correlation between biofilm formation and sanitizer resistance

A significant positive correlation (0.6) was observed between exopolysaccharide and sanitizer tolerance (Table 3), indicating that the higher exopolysaccharide concentration in combinations could be one of the reasons for increased tolerance in multispecies biofilm (Fig. 3). The cell concentration in the biofilm and the sanitizer tolerance did not have a significant correlation (0.1) (Table 3), clarifying the variation between sanitizer tolerance for biofilm formed in 10 % TSB and TSB despite having comparable cell concentrations (Fig. 4).

3.6. Structural variation as the result of nutrient conditions

The biofilm formed in 10 % TSB was observed to consist of mound-like protrusions compared to the compact layers formed in full-strength TSB. (Fig. 5A and B). The compact biofilm formed in full-strength TSB consisted of higher EPS concentration (Fig. 3) and higher tolerance to sanitizer (Table 2) compared to the biofilm with uneven surfaces formed in 10 % TSB.

4. Discussion

Significantly higher ($p < 0.05$) biofilm formation was observed for single and multispecies biofilms formed in TSB, compared to 10 % TSB

Table 2
Log reduction of *P. fluorescens* (P1), *S. aureus* (S2), and *L. monocytogenes* (LM1) in single, dual, and triple species biofilm formed in 10 % TSB and full-TSB.

Biofilm	Bacteria	Log reduction (log CFU/cm^2)	
		10 % TSB	TSB
P. fluorescens		5.21 ± 0.39^{y1}	2.86 ± 0.23^{cd2}
S. aureus		5.38 ± 0.21^{y1}	2.49 ± 0.41^{bcd2}
L. monocytogenes		4.99 ± 0.37^{xy1}	4.32 ± 0.09^{e2}
P. fluorescens + *S. aureus*	*P. fluorescens*	5.01 ± 0.20^{xy1}	2.08 ± 0.42^{abcd2}
	S. aureus	5.32 ± 0.17^{y1}	1.96 ± 0.06^{abc2}
P. fluorescens + *L. monocytogenes*	*P. fluorescens*	4.90 ± 0.43^{xy1}	1.46 ± 0.76^{ab2}
	L. monocytogenes	4.76 ± 0.36^{xy1}	1.12 ± 0.36^{a2}
S. aureus + *L. monocytogenes*	*S. aureus*	5.64 ± 0.40^{y1}	2.25 ± 0.40^{abcd2}
	L. monocytogenes	4.74 ± 0.25^{xy1}	2.23 ± 0.10^{abcd2}
P. fluorescens + *S. aureus* + *L. monocytogenes*	*P. fluorescens*	4.67 ± 0.02^{xy1}	1.31 ± 0.51^{ab2}
	S. aureus	5.64 ± 0.22^{y1}	1.88 ± 0.02^{abc2}
	L. monocytogenes	4.08 ± 0.10^{x1}	3.34 ± 0.09^{de2}

Note: letters (x,y) indicate the significant differences between the log reduction of bacteria for biofilm formed in 10 % TSB (between rows). Letters (a,b,c,d,e) indicate the significant differences between the log reduction of bacteria formed in TSB (between rows). Numbers (1,2) indicate the significant differences between the log reduction of bacteria for biofilm formed in 10 % TSB and TSB (between columns).

Table 3
Pearson's coefficient analysis between biofilm biomass (CV), biofilm cells, sanitizer tolerance, and the exopolysaccharide concentration.

	CV	Biofilm cells	Tolerance	EPS
CV	1	0.247	0.555**	0.807**
Biofilm cells	0.247	1	0.146	0.284
Tolerance	0.555**	0.146	1	0.618**
EPS	0.807**	0.284	0.618**	1

** Correlation is significant at the 0.01 level (2-tailed).

for all three bacteria involved: *P. fluorescens*, *S. aureus*, and *L. monocytogenes,* as observed through crystal violet staining (Fig. 1) and confirmed by exopolysaccharide concentrations (Fig. 3). Similar variations in the biofilm as a result of nutrient conditions have been mentioned before (S. Yuan et al., 2020). observed that the increased access to nutrients resulted in biofilms with higher thickness, biovolume, and roughness. Interestingly, the variation in nutrient content had no significant impact ($p > 0.05$) on the cell concentrations in single and multispecies biofilms (Fig. 2). *Pseudomonas* species have been previously reported as one of the major microbial contamination risks in the food industry because of their ability to grow and form biofilm even under low nutrient conditions and at low temperatures (frequently isolated from water - Centre for Disease Control and Prevention) (Xu et al., 2019). The supplementation of media with glucose, sucrose, and sodium chloride yielded higher biofilm (biomass) measured by crystal violet staining for *Staphylococcus* (Liu et al., 2020; Singh et al., 2017). A similar observation was made by (Karatan and Watnick, 2009) for *Staphylococcus aureus* and *Staphylococcus epidermis* where the presence of glucose and related sugars resulted in multilayer biofilm formation. Another study shows that the growth and attachment of *Pseudomonas* and *Staphylococcus* in pure and mixed (1:1) cultures in the presence of four different media (BHI, NB, LB, and RPMI 1640) resulted in higher growth and attachment in BHI broth (Wijesinghe et al., 2019). Enhanced planktonic growth and biofilm attachment have been positively associated with high nutrient concentrations. In a study of biofilm formation in different media, such as chicken juice and TSB, *L. monocytogenes* showed higher biofilm formation in TSB (Dong et al., 2022). In contrast, Wang et al. (2022) reported the increased production of exopolysaccharides for *L. monocytogenes* strains under nutrient-limiting conditions (dTSB-YE) compared with nutrient-rich conditions (TSB-YE), indicating the response is strain dependent. The order of predominance of bacteria in a three-species biofilm was found to be dependent on the media as well. In 10 % TSB media, *S. aureus* predominated the biofilm, followed by *P. fluorescens* and *L. monocytogenes* (Fig. 2). Previous reports have mentioned the predominance of *P. fragi* and *S. xylosus* in the steady-state multispecies setup containing *P. fragi*, *S. xylosus*, and *L. monocytogenes* in dilute tryptone soy broth (2 g/L) formed in a constant-depth film fermenter (Norwood and Gilmour, 2000). The order of dominance between *Acinetobacter* and *Enterobacter* in dual-species biofilm was also found to be dependent on the nutrient concentration in an 8-day study (Yuan et al., 2020). The nutrient abundance was associated with higher biofilm formation (Fig. 1) and synergistic interactions (Table 1) between the bacteria in multispecies biofilm.

In this study, synergistic interaction in multispecies biofilm was observed to be limited to high nutrient conditions (full-strength TSB). Under nutrient-deficient conditions, the relationship equations showed higher antagonistic combinations than nutrient-sufficient conditions (Table 1). While the biofilm formation of *S. aureus* and *L. monocytogenes* was found to be lowered in 10 % TSB, the dual species combination of these resulted in synergistic interactions under both nutrient conditions, indicating the species-dependent interactions despite the nutrient unavailability. These observations were based solely on biofilm biomass and are indicative of interactions between bacteria in multispecies biofilm (Røder et al., 2020). Depending on the nature of the bacteria, a similar shift in interaction is noted for a combination of oligotrophic bacteria (stress-preferring) and a fungus, which shifts their interaction from competition to cooperation when evaluated in a low-nutrient environment (Velez et al., 2018). While the ¼ TSB did not have a significant effect on the growth of the strains, lowered synergy was observed in the multispecies biofilm formed by *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans*, and *Paenibacillus amylolyticus* compared to the multispecies biofilm formed in full TSB (Olsen et al., 2019). Competitive interactions are reported to occur when there is competition for nutritional niches and space (Parijs and Steenackers, 2018). Hence, under nutrient-limiting conditions, the interaction between the bacteria has been reported to evolve into competition (Xu et al., 2019). Under stressful conditions, bacteria can shut down their regulatory mechanisms to focus on survival, giving rise to cells that are resistant to other stresses, such as antibiotics (Mok and Brynildsen, 2019). In biofilm, starvation causes changes in the composition of EPS, and also modifies the cell envelope for cell maintenance (Myszka and Czaczyk, 2009). The synergistic interaction occurs in many ways: physical interaction (co-aggregation) and metabolite interaction (secretion of enzymes, metabolic cross-feeding), but does not necessarily assure higher fitness of the biofilm (Burmølle et al., 2006). The interaction between the dual-species biofilm formed by *P. aeruginosa* and *K. pneumoniae* changed from neutral to competition when the nutrient feeding and waste removal rates were decreased (Tan et al., 2017). These observations collectively indicate that the interaction between the bacteria is conditional and dynamic.

In this study, the synergy between the bacteria in the multispecies biofilm provided the increased fitness of the mixed biofilm, showing increased survival of all three bacteria in the multispecies biofilm formed in full-strength TSB (Fig. 4). The sanitizer tolerance of the pathogen *L. monocytogenes* increased significantly ($p < 0.05$) in dual and triple species combinations (Fig. 4). In a similar disinfection study, eighteen *L. monocytogenes* cultures in single and dual species (with

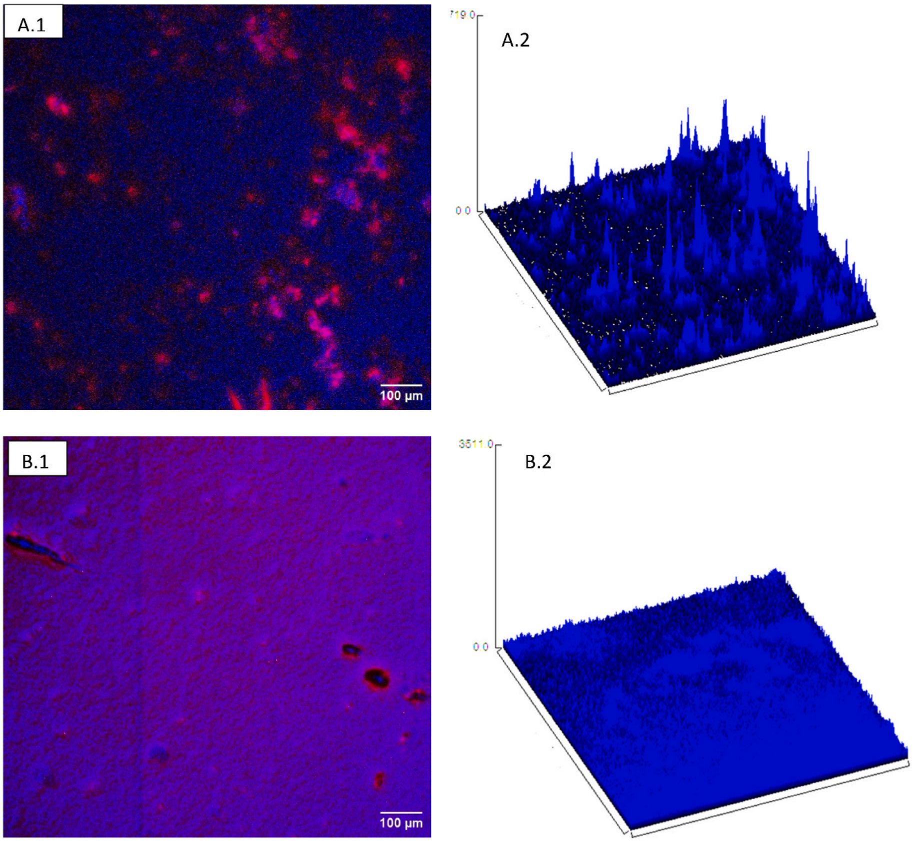

Fig. 5. Confocal microscopy images showing dual-species biofilm formed with *P. fluorescens* (blue) and *L. monocytogenes* (red) in A) 10 % TSB and B) full-strength TSB. A.1 and B.1 are 2-D images obtained from the microscope after dual staining. A.2 and B.2 are the surface plots showing the structural differences between the biofilm formed under varying nutrient conditions for 10 % TSB and full-TSB, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Pseudomonas) were treated with peracetic acid and antibiotics. After the disinfection, survival of ten cultures of *Listeria* was noted in dual species, whereas in single species, all eighteen cultures of *Listeria* were reduced to undetectable levels (Thomassen et al., 2023). The dual species of *Pseudomonas* and *S. enteritidis* showed increased tolerance against quaternary ammonium compounds in a dual species combination (Pang et al., 2020). The protective effect of the binary biofilm of *E. coli* and *P. aeruginosa* against benzalkonium chloride has been noted before (Machado et al., 2012). In addition to the binary biofilm, the persistence of *L. monocytogenes* was also found to be elevated in the presence of *S.* Typhimurium and *P. aeruginosa* against black pepper essential oil in a three-species biofilm (Dos Santos et al., 2023). The results show enhanced resistance of the *Listeria* in binary biofilm with *Pseudomonas*, which can be added to the fact that *Pseudomonas* produces higher EPS, which acts as a barrier for the sanitizer before it reaches the *Listeria* or *Staphylococcus* (Kocot and Olszewska, 2020). The formation of denser biofilm (higher EPS) in multiple species and its correlation with increased sanitizer resistance has been discussed before (Table 2). In resistance studies, the survival of susceptible bacteria was found to be largely dependent on the bacteria present alongside them, owing to the thickness and distribution of EPS around the accompanying bacteria (Dos Santos et al., 2023). *P. aeruginosa* cells are good producers of exopolysaccharides, which can delay/inhibit the penetration of sanitizer in the biofilm (Bridier et al., 2015; Thomassen et al., 2023). In a multispecies biofilm, a 'sheltering effect' can be observed, where a low EPS producer, such as *Listeria*, benefits from higher EPS-producing bacteria by association (Sanchez-Vizuete et al., 2015; Wagner et al., 2020).

In this study, despite the concentration of exopolysaccharide increasing significantly in three species biofilm (Fig. 3), the surviving bacteria in the biofilm after sanitizer treatment were comparable to dual species (Fig. 4), which might suggest that in addition to exopolysaccharides, the higher survival of *Listeria* in multispecies biofilm could be the result of quorum sensing (horizontal gene transfer) and/or spatial arrangement (Wagner et al., 2020) with one specific bacterium present in the combination (Guillonneau et al., 2018). observed that irrespective of the number of bacteria (dual and triple) in the biofilm, *P. mediterranea* was able to benefit from only one bacterium (*Shewanella* sp.) in the combination. Previous reports have indicated the importance of spatial arrangement on the composition and behavior of multispecies biofilm (Dong et al., 2023; Ibusquiza et al., 2012). In the dual-species biofilm consisting of *P. aeruginosa* and *S. aureus*, *S. aureus* was located at the bottom of the biofilm (facultative anaerobic), whereas *P. aeruginosa* was found on the top layers owing to its aerobic metabolism. The polysaccharide *Psl* produced by *P. aeruginosa* was found to form a protective barrier between *S. aureus* and *P. aeruginosa,* protecting the former bacteria from the lytic effect of the latter (Xu et al., 2019). In addition to sanitizer tolerance, the synergistic interaction in multispecies biofilm has been noted to provide the bacteria with higher resistance towards invasive bacterial species (Burmølle et al., 2006) and surfactant and antibiotics (Lee et al., 2014).

In this study, major structural differences were observed for biofilm formed under different nutrient conditions (Fig. 5). Heydorn et al. (2000) concluded that the *Pseudomonas* biofilm formed under nutrient-abundant conditions had a thick, compact, and uniform structural appearance compared to the biofilm formed under nutrient-deficient conditions, where a porous biofilm containing interstitial channels and voids can be observed. The observations made in Fig. 5 have also been previously recognized as rugose morphotype (uneven) vs smooth morphotype in *S.* Typhimurium, observed as the result of continuous exposure to sodium hypochlorite (Bansal et al., 2019). Nutrient stress can cause a nutrient gradient across the biofilm (rapid proliferation of bacteria on the surface compared to deeper layers), resulting in wrinkled, mound-like, or towered structures, and overall mechanical instability (Flemming et al., 2016). The relationship between the nutrient content and structure of the biofilm has direct implications for the sanitizer tolerance (Bas et al., 2017). Compact biofilm with higher EPS density has been noted to increase resistance against external factors (hydraulic flow, antimicrobials). Mathematical modelling of biofilm showed that the structure of the biofilm is dependent on the nutrient uptake and diffusion of the nutrient throughout the biofilm. Once the microcolonies are formed, the proliferation of the bacteria is dependent on the nutrient uptake by the bacteria on the outer layer of the structures (Lobo-Cabrera et al., 2024). A porous and heterogeneous structure can be observed when diffusion of the nutrients is limited in the biofilm, compared to a homogenous and compact biofilm when the diffusion limitations are absent (Van Loosdrecht et al., 2002). These structural observations are significant because the efficiency of antimicrobials (hydrogen peroxide, chlorine dioxide, and quaternary ammonium compounds) was found to be dependent on the proportion of biofilm coverage on the surface (Bas et al., 2017). Under different nutrient conditions, different genes may be upregulated, leading to differences in the EPS production and hence structural properties (Campanac et al., 2002). While the mechanisms are yet to be understood, the mound-like protrusions relative to mushrooms or towers have been observed for biofilm formed under stress, in contrast to non-stressed ones showing flat biofilm (Stoodley et al., 2002). This higher EPS production and compact biofilm result in higher proximity of the cells to each other and allow cell-cell interactions and the formation of a synergistic biofilm (Flemming and Wingender, 2010).

While this study explores the nutrient variation on polystyrene surfaces under static conditions, providing fundamental information on multispecies arrangements, the interactions on industrial surfaces (e.g, stainless steel) under dynamic conditions (continuous supply of media/shear stress) might vary. Further studies are required to understand the variations in the interactions for practical applications such as industry surfaces.

5. Conclusion

While the O.D$_{595}$ nm was an accurate indicator of the biofilm biomass, the relationship equation based on these values did not correlate with the synergistic interactions in all the cases studied. Between the three bacteria analyzed in this study, *L. monocytogenes* was the weakest biofilm former with the highest susceptibility to sanitizers in single-species and benefited most in the multispecies arrangement. While it's clear that higher exopolysaccharide concentration results in higher tolerance, the uneven log reduction of three bacteria (compared to dual species) indicates that the role of spatial arrangement might be significant in defining which bacteria will be sheltered more in multispecies biofilms. Additionally, the difference in the sanitizer tolerance between biofilms formed under different nutrient conditions raises the question for future study: Is there a threshold concentration of extracellular matrix that the sanitizer would be able to penetrate? Further studies relating to the spatial arrangement and thickness of the biofilms could answer this question. In addition to spatial arrangement, gene expression related to resistant genes and compositional changes (protein, eDNA) are also vital to understand in multispecies arrangements. In conclusion, by limiting the nutrient condition, more impactful removal methodologies can be developed without having to increase the concentration of the chemical sanitizers. Overall, understanding the interactions between bacteria in a multispecies arrangement and their response to sanitizers under different environmental variables can help to develop a targeted, efficient, sustainable cleaning regime.

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 known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.104952.

Data availability

Data will be made available on request.

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