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Sequential attachment and *Listeria* predominance under turbulent flow

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ABSTRACT

The exploration of multispecies biofilms has provided significant insight into how bacteria interact in natural environments. However, crucial information is still lacking when the assumption that all the bacteria approach the substrate simultaneously during multispecies biofilm formation is challenged. The objective of this study was to analyse the multispecies biofilm formed by industrially relevant bacteria, including *Pseudomonas fluorescens* and *Listeria monocytogenes*, focusing on sequential colonisation under turbulent flow and static conditions. Under flow conditions, the attachment of *Listeria* on preformed *Pseudomonas* biofilm was significantly higher ($p < 0.001$) and reached its highest cell concentration (8.9 log CFU cm^{-2}) earlier (by 24 h) compared to control (sterile stainless-steel surfaces) and co-inoculated (with *P. fluorescens*) conditions. The conditioning of stainless-steel surfaces with freeze-dried and rehydrated exopolysaccharides (0–27.5 μg ml^{-1}) extracted from *P. fluorescens* did not affect *L. monocytogenes* attachment, indicating the importance of biofilm architecture. The cell concentration of *P. fluorescens* was not affected by either the flow rate or the colonisation order in a multispecies biofilm. Overall, the sequence of colonisation affects biofilm formation in multispecies biofilms under turbulent flow and is an important variable in studies of the community lifestyle of multispecies biofilms.

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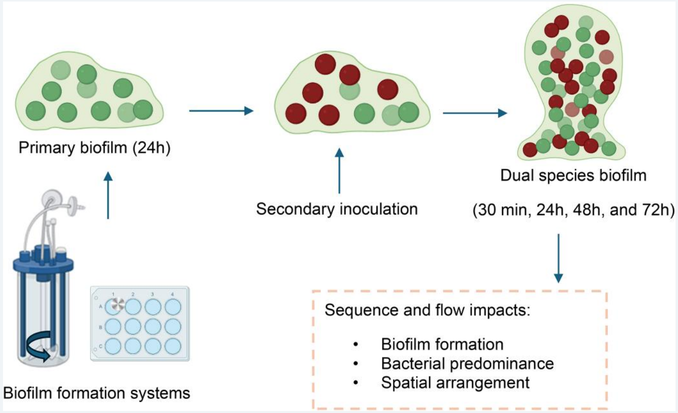
Preformed biofilm; colonisation sequence; multispecies detachment; species predominance; spatial arrangement

GRAPHICAL ABSTRACT



Introduction

The persistence of pathogens such as *Listeria monocytogenes* on food processing surfaces has been attributed to the multispecies composition, particularly in the presence of high levels of exopolysaccharide-producing spoilage bacteria such as *Pseudomonads* (Wang et al. 2020). The affinity of *Listeria* to colonise preformed, mature biofilms of *Pseudomonas* and to dictate interspecies interactions has been reported by several researchers (Puga et al. 2018; Rodríguez-López et al. 2022; Zhou et al. 2024b). When microbes attach to the surface as a primary coloniser, they impact and regulate the attachment and biofilm formation of secondary colonisers by the accumulation of exopolysaccharides (EPS), soluble microbial products (SMP) (Hwang et al. 2013), and cell extracts (total cell extract, cytoplasm with cellular debris, periplasmic

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extract) (Moreira et al. 2017). Bacterial attachment is the first stage of biofilm formation, with early colonisers influencing overall interactions and, ultimately, the fate of the biofilm (Puga CH, SanJose C, et al. 2016).

The exopolysaccharides produced by *Pseudomonas aeruginosa*, also known as 'EPS footprints' due to their resistance to cleaning and disinfection, can affect sequential bacterial attachment (Liu et al. 2007). Any positive or negative impact on subsequent attachment is dependent on the physicochemical composition of the extracellular matrix produced by the primary attaching bacteria. For example, interactions between surface polymers on the bacteria and the EPS polymers on the matrix have been reported to result in greater bacterial attachment (Hwang et al. 2012). The preconditioning matrix, which contained predominantly protein, can reduce bacterial attachment by repelling proteins on the *P. aeruginosa* cell surface and the matrix (Hwang et al. 2012).

Another factor that affects bacterial attachment to the surface is hydrodynamic conditions. Research on the impact of turbulence in annular bioreactors shows that higher turbulence levels lead to greater attachment and the subsequent formation of biofilms (Tsagkari and Sloan 2018). The hydrodynamic conditions surrounding the attachment surface influence the nutrient transport rate into the microcolonies and, subsequently, the mechanical stability of the biofilm (Simões et al. 2005). The flow also affects the structure of the biofilm, with turbulent flow resulting in a filamentous biofilm (streamers) (Stoodley et al. 1999) compared to static or non-turbulent flows. In terms of biofilm composition, biofilms formed under flow were found to be thinner, with higher, more compact exopolysaccharide layers compared to those formed in the absence of turbulent flow (Pereira et al. 2002). The flow had a significant impact on the architecture of the biofilm when the nutrient was limiting during the biofilm formation ($\leq 100\,\mathrm{mg\ l^{-1}}$ glucose) (Pereira et al. 2002). The architecture of *P. fluorescens* can vary significantly under flow conditions in different flow systems: a flow cell reactor (Pereira et al. 2002) *vs* a microfluidic system (Di Somma et al. 2020), which could impact the bacteria subsequently attaching. The colonisation of *L. monocytogenes* on preformed *P. fluorescens* in a bioreactor with continuous shaking (80 rpm) showed enhanced growth of *L. monocytogenes* and overproduction of exopolysaccharide by *Pseudomonas* (Puga et al. 2018). The biofilm matrix produced by the primary bacteria is likely to serve as an attachment site for free-floating bacteria (Toté et al. 2009). In addition to providing a preferred attachment surface, the leftover matrix can also serve as a nutrient source for incoming bacteria (Simões et al. 2005).

The importance of studying *L. monocytogenes* and *P. fluorescens* in a multispecies biofilm setup has been highlighted by researchers (Puga CH, SanJose C, et al. 2016; Puga et al. 2018). As we step into multispecies studies, we must naturally consider a sequence of colonisation, since realistically, not all bacteria may attach at the same time, and some may serve as the foundation of the multispecies biofilm complex, creating a conditioning film for subsequent bacteria to attach. The literature on sequential multispecies biofilms focuses on the colonisation of a glass surface by *Pseudomonas* under flow, followed by *Listeria* (Puga CH, SanJose C, et al. 2016). While the impact of surface conditioning by primarily attached bacteria is clear, it does not delve into whether it is the cells, the matrix, or the architecture of the biofilm that traps *Listeria* cells within the *Pseudomonas*-preformed biofilm. Additionally, the impact of flow on the migration of *Listeria* into deeper layers of *Pseudomonas* biofilm requires the observation of similar sequential biofilm under static conditions and on a more industrially relevant surface, such as stainless steel. All these aspects are addressed in the present study. The extracellular matrix (ECM) has been shown to impact sequential colonisation by modifying surface properties, such as hydrophobicity, surface charge, and roughness (Hwang et al. 2012). A research gap exists regarding how matrix modifications can modulate subsequent attachment of other bacteria and the dynamics of multispecies biofilm formation. This study explores the different stages of biofilm formation (attachment, maturation and detachment) and spatial arrangement of multispecies biofilm formed by *P. fluorescens* and *L. monocytogenes*, with a focus on sequential colonisation under turbulent and static conditions. The impact of the exopolysaccharide (EPS) conditioning layer was analysed at a range of exopolysaccharide concentrations (extracted from *P. fluorescens*), used as anchoring sites for *L. monocytogenes* attachment under turbulent flow, and in the absence of the conditioning layer (static).

Materials and methods

Bacteria and media

Stock cultures of *P. fluorescens* (dairy) and *L. monocytogenes* H1 (environment-soil) stored in −80 °C glycerol stock were inoculated into tryptone soy broth

(TSB) (Difco™, Becton, Dickinson and Company, New Jersey, USA) and incubated at 30 °C for 18 h. Both cultures were centrifuged (Sigma® 6–16, John Morris Scientific Ltd, Auckland, New Zealand) at 12,000 g for 10 min, after which the pellet was collected. The cell concentration was fixed at 6 log CFU ml^{-1} for each bacterium, as determined by the correlation of OD at 600 nm vs log CFU ml^{-1} ($R^2 = 0.97$). Both isolates are from the strain collection in the Food Microbiology Laboratory, Massey University, Palmerston North, with access codes P1 and H1KP for *P. fluorescens* and *L. monocytogenes*, respectively.

Biofilm formation system

The following two systems were used to form biofilm with three conditions: (a) *Pseudomonas* preformed biofilm – *P. fluorescens* was allowed to form biofilm for 48 h before the introduction of *L. monocytogenes* into the system; (b) *Listeria* preformed biofilm – *L. monocytogenes* was allowed to form biofilm for 48 h before the introduction of *P. fluorescens* into the system; and (c) co-inoculated biofilm where both *P. fluorescens* and *L. monocytogenes* were inoculated at the same time. The time (48 h) was selected to allow for mature biofilm formation before the introduction of the secondary coloniser (Daneshvar Alavi and Truelstrup Hansen 2013; Puga CH, Orgaz B, et al. 2016).

Static system

Co-inoculation. Sterile stainless-steel coupons (2.4 cm^2) (316-2B; Advanced Sheetmetals Ltd, Palmerston North, New Zealand) were prepared for experiments by creating a chromium oxide coating using 50% nitric acid and heating at 70 °C for 30 min, followed by washing, drying, and autoclaving (121 °C, 15 min). The coupons were placed vertically on the wells of a 48-well plate (Costar®, Corning, New York, USA). Each well contained *P. fluorescens* and *L. monocytogenes* in cell concentrations (1:1) (6 log CFU ml^{-1}) in 10% TSB. The coupons containing wells were incubated for seven days at 30 °C with media refreshed every 24 h. After 30 min of attachment and every 24 h after that, triplicate coupons were used to enumerate cell concentrations in the biofilm using the glass bead beating assay (5 g of glass beads (diameter – 5 mm, Sigma-Aldrich, Darmstadt, Germany) in 5 ml saline solution mixed by vortex for 5 min) and plating on selective agar. *Pseudomonas* isolation agar (Difco™) was used for *P. fluorescens*, and Modified Oxford *Listeria* agar (HiMedia, Pennsylvania, USA) was used for *L. monocytogenes*.

Sequential. The sterile stainless-steel coupons (2.4 cm^2) were prepared under the same conditions as mentioned above in the co-inoculation section. The coupons were placed vertically in a 48-well plate with each well containing primary bacteria (*P. fluorescens* or *L. monocytogenes*) (6 log CFU ml^{-1}) in 10% TSB. The wells were incubated for 48 h at 30 °C to promote mature biofilm formation by the foundation bacteria, with media refreshed every 24 h. After 48 h, the coupons were washed with sterile saline solution and placed vertically into a new well containing secondary coloniser (*P. fluorescens* or *L. monocytogenes*) (6 log CFU ml^{-1}) in 10% TSB at 30 °C. The coupons were analysed for attachment (30 min) and growth (1–5 days) on preformed biofilm. The media (10% TSB) was refreshed every 24 h. Cell concentration was analysed using the glass bead beating method and selective agar plating, as described above, in the co-inoculation system.

For single-species biofilm formation, coupons were inoculated into wells containing a single species of bacteria (*P. fluorescens* or *L. monocytogenes*) (6 log CFU ml^{-1}) and incubated for seven days with media refresh every day. Similar to the dual-species assay, coupons were collected at 30 min to analyse attachment on a clean, sterile surface and every 24 h to analyse the dynamics of biofilm formation. The cell concentration was analysed using the glass bead beating method and plated into tryptone soy agar (TSA) (Difco™) as mentioned above in the co-inoculated system.

Continuous system

The Centre for Disease Control (CDC) bioreactor was used to form biofilm under a continuous flow of media (10% TSB) and turbulent flow (250 rpm). The CDC system consists of four components: (a) inlet tank (sterile 10% TSB media), (b) pump system (5 ml min^{-1}), (c) bioreactor (330 ml) containing eight rods (three stainless-steel coupons on each) (2.26 cm^2) and a stirring system, and (d) outlet tank for waste.

Co-inoculation. Both *P. fluorescens* and *L. monocytogenes* were co-inoculated at a cell concentration (1:1) (6 log CFU ml^{-1}) into the bioreactor (10% TSB) connected with an inlet tank (10% TSB) *via* a peristaltic pump (5 ml min^{-1}) (Masterflex®, Cole-Parmer®, USA), and an outlet tank (waste). The incubation temperature (30 °C) and the stirring rate (250 rpm) were set using a magnetic stirrer hot plate (VWR International, Radnor, Pennsylvania, USA). After 30 min of run time, a rod was removed from the bioreactor (replaced with a blank rod) to analyse attachment during co-inoculation. The cell concentration on the surface was enumerated post-washing with sterile saline solution using the glass-bead beating method as described in 'Static system'. Additionally, cell counts were enumerated every 24 h for the next seven days.

Sequential. Using the above CDC setup, a mature biofilm (48 h) was formed for primary bacteria, i.e. single species (*P. fluorescens* or *L. monocytogenes*), in bioreactor A at 30 °C. After 48 h of biofilm formation, the rods were removed from bioreactor A, washed with sterile saline to remove planktonic cells, and introduced into sterile bioreactor B. The sterile bioreactor B was inoculated with the secondary coloniser (*P. fluorescens* or *L. monocytogenes*) (6 log CFU ml^{-1}), and the system was run for the next five days at 30 °C and 250 rpm with a constant supply of fresh media (10% TSB) using a peristaltic pump. For the attachment assay, the system was run for 30 min before the rods were taken out to analyse attachment of secondary coloniser on preformed biofilm, and every 24 h for the next five days to analyse the dynamics of biofilm formation using glass bead beating and selective agar plating for the attachment assay as mentioned in 'Static system'.

For single-species biofilm formation under flow, single species of *P. fluorescens* and *L. monocytogenes* were inoculated into each bioreactor, which was set up as described above. The system was run for seven - days with a continuous media supply (5 ml min^{-1}) and high turbulence (250 rpm) at 30 °C. Every 24 h, rods were removed from the bioreactor and replaced with a blank rod. The cell concentration in the biofilm was estimated using the glass-bead beating method and plated onto tryptone soy agar (TSA) as described above.

L. monocytogenes *attachment to preformed* **Pseudomonas** *biofilm*

To further understand the improved attachment of *L. monocytogenes* on preformed *P. fluorescens* biofilm, *L. monocytogenes* was attached to *Pseudomonas* biofilm of different ages (24, 48, and 72 h). The single-species biofilm of *P. fluorescens* was formed in static and continuous systems, as described in 'Static system' and 'Continuous system', respectively. Triplicate coupons (rods) of preformed *Pseudomonas* biofilm were removed from the system at 24, 48 and 72 h, then washed with saline and introduced into another system containing an *L. monocytogenes* inoculum (in 10% TSB). The attachment was allowed for 30 min at 30 °C under static conditions for preformed biofilm formed under the static system, and for 30 min under turbulent flow for preformed biofilm formed under turbulent conditions. The stainless-steel coupons were then saline-washed before proceeding with glass-bead beating and cell enumeration using selective agar, as described in 'Static system'.

Extraction, freeze-drying, and quantification of exopolysaccharides

Microtitre plates (96-well plates) (Falcon, Glendale, Arizona, USA) were inoculated with *P. fluorescens* (6 log CFU ml^{-1}) in TSB. After 72 h incubation at 30 °C, the media were removed, and the wells were washed with sterile water. Sterile distilled water (100 µl) was added to each well, and the plate was sonicated (40 kHz) for 10 min (Zhou et al. 2024a). The collected extract was centrifuged at 12,000 g for 10 min, and the supernatant was collected for filtration (0.20 µm) to remove cells. Extracts were freeze-dried (Lab-Kits®, Hong Kong, China) before redissolving (sterile distilled water) to a range of concentrations for the *L. monocytogenes* attachment assay.

The exopolysaccharide (EPS) was quantified using the phenol sulphuric acid method (Zhou et al. 2024a). To 200 µl of rehydrated EPS solution, 100 µl of 6% phenol were added, immediately followed by 500 µl of 95% sulphuric acid. The reaction was allowed at room temperature for 20 min before the OD was analysed at 490 nm using a spectrophotometer. The exopolysaccharide content was determined using a dextran standard curve (Supplementary Figure A).

Preparation of conditioned surfaces and attachment assays

For conditioned surface preparation, the sterile stainless-steel coupons were coated with different concentrations (0–27.5 µg ml^{-1}) of EPS solution and allowed to air-dry. After the surfaces dried, they were introduced into the static and turbulent systems. Each system contained 10% TSB and a *L. monocytogenes* cell concentration of 6 log CFU ml^{-1}. The attachment assays were run for 30 min at 30 °C. After the attachment assay, the coupons were washed with saline solution to remove planktonic cells, and the adhered cells were enumerated using the glass-bead beating

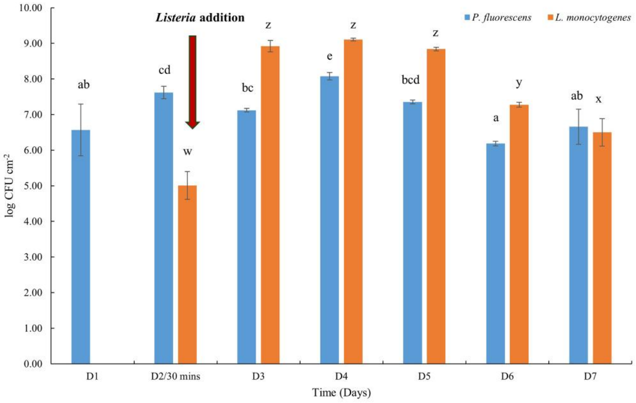


Figure 1. The cell concentrations (log CFU cm^{-2}) of *P. fluorescens* (P1) and *L. monocytogenes* (LM1) in sequentially formed dual species biofilm under turbulent flow during a seven-day incubation period. The red arrow represents the inoculation of *L. monocytogenes* in *P. fluorescens* 48 h mature biofilm. Note: the superscripts (a–e) and (w–z) indicate the significant differences between cell concentrations during the seven-day incubation period for *P. fluorescens* and *L. monocytogenes*, respectively.

method and plated on tryptone soy agar according to 'Static system'.

Spatial arrangements via confocal microscopy

The samples collected from 'Biofilm formation system' (static-co, static-P1 pre, static-LM1 pre, continuous-co, continuous-P1 pre, and static-LM1 pre) on their fifth day of incubation were washed with phosphate buffer saline (PBS) and air-dried before staining with wheat germ agglutinin (WGA)-Texas red conjugate (1 mg ml^{-1}) and counterstaining with DAPI (1 mg ml^{-1}) (Rodríguez-Melcón et al. 2021). WGA-Texas red (6 µl) and 6 µl of DAPI were mixed with 6 ml of TSB and used to stain the coupons at room temperature in the dark for 50 min. After staining, the coupons were washed with PBS and allowed to air-dry before imaging through confocal microscopy (Nikon D-eclipse C1, Tokyo, Japan) (10X) and EZ-C1 software (Gold version 3.80 build 860, Nikon Instruments, Tokyo, Japan) for image acquisition.

Statistical analysis

The significant differences between the samples were analysed using one-way ANOVA (IBM SPSS Statistics 29, New York, USA) and post hoc analysis with Tukey's test. The results were represented as mean ± SD Image analysis of the confocal microscope images was performed using ImageJ (ImageJ 1.54 g, National Institutes of Health, Maryland, USA). Biological replicates and technical triplicates were repeated for each analysis.

Results

Sequential biofilm formation under flow

Attachment

The attachment of *L. monocytogenes* on a 48-h preformed *Pseudomonas* biofilm was significantly higher ($p < 0.001$) (5.0 ± 0.4 log CFU cm^{-2}) (Figure 1) compared to its attachment on the sterile stainless-steel surface (4.0 ± 0.2 log CFU cm^{-2}), and in co-inoculation (2.57 ± 0.3 log CFU cm^{-2}) (with *P. fluorescens*) (Figure 3). In contrast, the attachment of *P. fluorescens* on preformed *Listeria* biofilm (2.32 ± 0.13 log CFU cm^{-2}) (Figure 2) was comparable with attachment in co-inoculation (2.47 ± 0.24 log CFU cm^{-2}) with *L. monocytogenes* (Figure 3). The attachment of *P. fluorescens* on sterile stainless-steel surfaces in single species was higher compared to other conditions at 4.0 ± 0.3 log CFU cm^{-2}.

Maturation

L. monocytogenes reached its plateau (8.9 ± 0.2 log CFU cm^{-2}) earlier (Day 1 of co-growth) when inoculated onto the *Pseudomonas* preformed biofilm surface (Figure 1) compared to co-inoculated in mixed species (8.8 ± 0.4 log CFU cm^{-2} on Day 3) (Figure 3), and when *L. monocytogenes* was the primary attached bacteria (9.0 log CFU cm^{-2} on Day 3 of co-growth) (Figure 2). *P. fluorescens* did not show preferential growth in any conditions of biofilm formation. They reached their plateau of 7.5 ± 0.1 log CFU cm^{-2} on Day 3 in co-inoculation (Figure 3), 8.0 ± 0.1 log CFU cm^{-2} on Day 2 when *Pseudomonas* was preformed (Figure 1), and 7.8 ± 0.2 log CFU cm^{-2} on

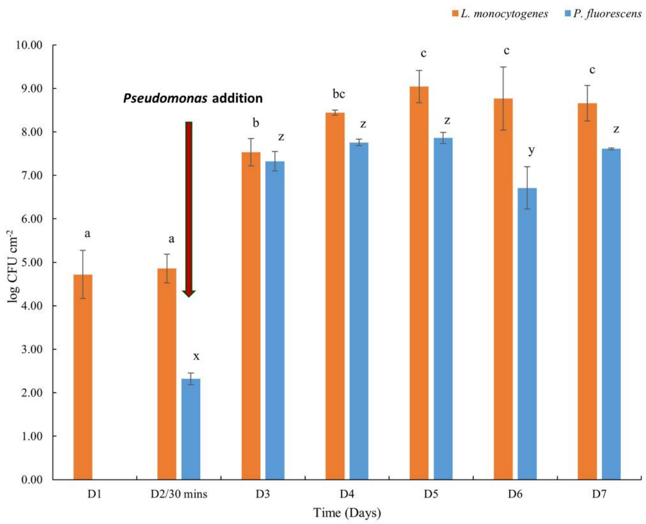


Figure 2. The cell concentrations (log CFU cm^{-2}) of *L. monocytogenes* (LM1) and *P. fluorescens* (P1) in sequentially formed dual species biofilm under turbulent flow during a seven-day incubation period. The red arrow represents the inoculation of *P. fluorescens* in *L. monocytogenes* 48 h biofilm. Note: The superscripts (a–c) and (x–z) indicate the significant differences between cell concentrations during the seven-day incubation period for *L. monocytogenes* and *P. fluorescens*, respectively.

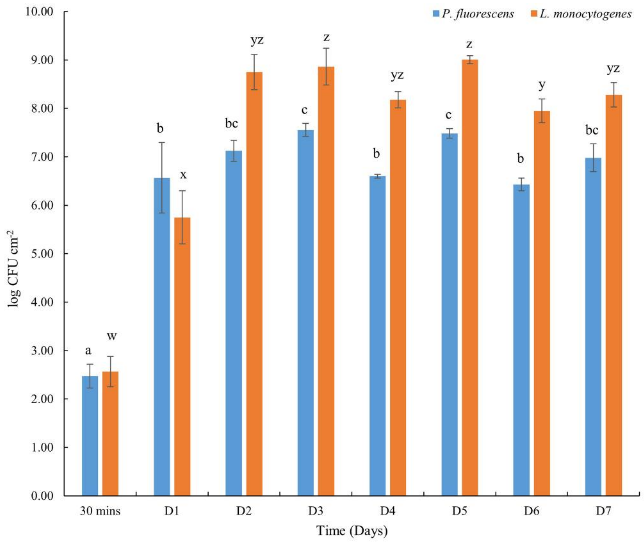


Figure 3. The cell concentrations (log CFU cm^{-2}) of *P. fluorescens* (P1) and *L. monocytogenes* (LM1) in co-inoculated dual species biofilm during a seven-day incubation period under turbulent flow. Note: The superscripts (a–c) and (w–z) indicate the significant differences between cell concentrations during the seven-day incubation period for *P. fluorescens* and *L. monocytogenes*, respectively.

Day 3 when it was inoculated into preformed *Listeria* biofilm (Figure 2).

Detachment

A periodic dispersal of cells was observed in dual-species biofilm formed under both co-inoculated (Figure 3) and pre-formed biofilm (Figures 1 and 2) after Day 3 of co-growth. In co-inoculated dual species, sloughing was observed for both bacteria; i.e. after Day 3, the cell concentrations of both *P. fluorescens* and *L. monocytogenes* dropped and rose together, showing a wave-like pattern of periodic cell dispersal (Figure 3). Similarly, when *Listeria* was inoculated in a pre-formed *Pseudomonas* biofilm, the detachment pattern showed a slow decline in cell concentration for both bacteria after Day 3 of co-growth (Figure 1). In contrast, where *Listeria* was present, only *Pseudomonas* showed the wave-like oscillation in cell concentration, while *Listeria* cell concentration remained consistent (8.6–9 log CFU cm^{-2}) throughout the incubation period (Figure 2).

In the single-species biofilm formed under flow, the shedding patterns were not observed. The cell concentrations ranged between 6.3 (Day 1) and 7.1 (Day 7) log CFU cm^{-2} for *P. fluorescens* and 4.7 (Day 1) and 6.3 (Day 7) log CFU cm^{-2} for *L.*

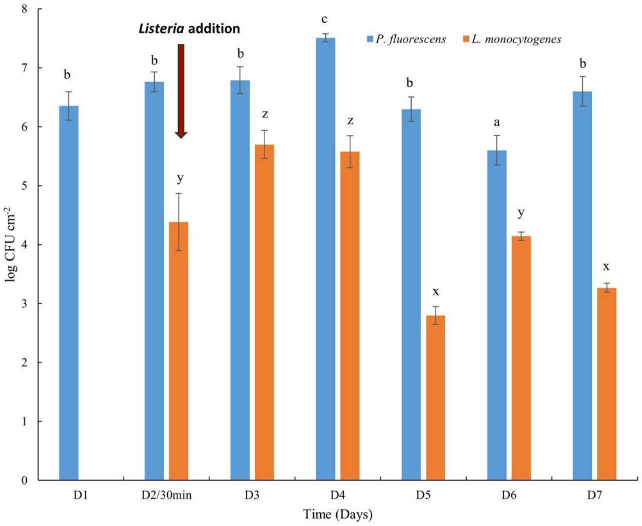


Figure 4. The cell concentrations (log CFU cm^{-2}) of *P. fluorescens* (P1) and *L. monocytogenes* (LM1) in sequentially formed dual species biofilm under static conditions during a seven-day incubation period. The red arrow represents the inoculation of *L. monocytogenes* in *P. fluorescens* 48 h mature biofilm. Note: The superscripts (a–c) and (x–z) indicate the significant differences between cell concentrations during the seven-day incubation period for *P. fluorescens* and *L. monocytogenes*, respectively.

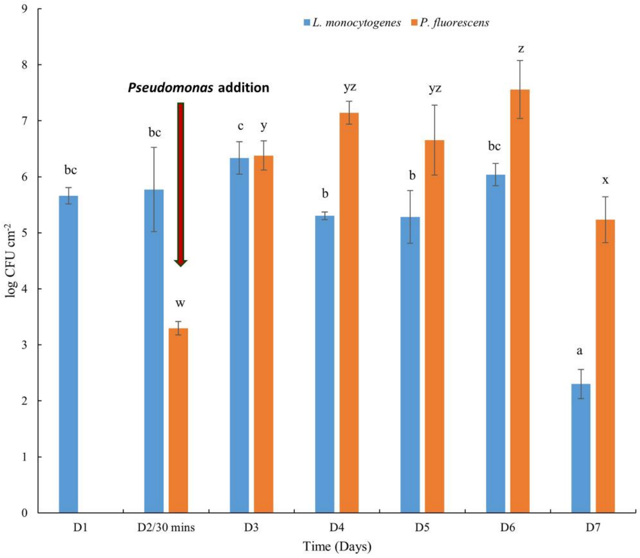


Figure 5. The cell concentrations (log CFU cm^{-2}) of *L. monocytogenes* (LM1) and *P. fluorescens* (P1) in sequentially formed dual species biofilm under static conditions during a seven-day incubation period. The red arrow represents the inoculation of *P. fluorescens* in *L. monocytogenes* 48 h biofilm. Note: The superscripts (a–c) and (w–z) indicate the significant differences between cell concentrations during the seven-day incubation period for *L. monocytogenes* and *P. fluorescens*, respectively.

monocytogenes. There was no significant reduction ($p < 0.001$) in cell concentration during seven days of biofilm formation under flow, as observed in a previous study (Pant et al. 2025).

Sequential biofilm formation under static conditions

Attachment

Under static conditions, the attachment of *L. monocytogenes* was not affected by the presence of preformed *Pseudomonas* biofilm on the surface. In the co-inoculated system, the *Listeria* attachment was 3.6 ± 0.2 log CFU cm^{-2} (Figure 6), which was comparable with *Listeria* attachment on preformed *Pseudomonas* biofilm (4.3 ± 0.1 log CFU cm^{-2}) (Figure 4) and in single species (3.6 ± 0.1 log CFU cm^{-2}). The attachment of *Pseudomonas* was significantly lower ($p < 0.05$) on *Listeria* pre-formed biofilm (3.3 ± 0.3 log CFU cm^{-2}) (Figure 5) compared to the co-inoculated (4.2 ± 0.1 log CFU cm^{-2}) (Figure 6) and in single species (4.0 ± 0.1 log CFU cm^{-2}).

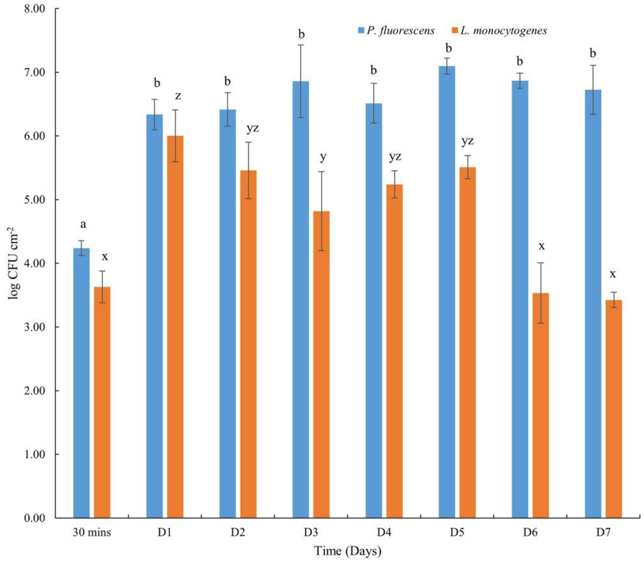


Figure 6. The cell concentrations (log CFU cm^{-2}) of *P. fluorescens* (P1) and *L. monocytogenes* (LM1) in co-inoculated dual species biofilm during a seven-day incubation period under static conditions. Note: The superscripts (a, b) and (x–z) indicate the significant differences between cell concentrations during the seven-day incubation period for *P. fluorescens* and *L. monocytogenes*, respectively.

Maturation

In all three conditions/sequences of biofilm formation, *P. fluorescens* predominated the dual species biofilm formed under static conditions. *P. fluorescens* reached its highest cell concentration on Day 2 of co-growth with 7.5 ± 0.4 log CFU cm^{-2} in *Pseudomonas* preformed (Figure 4), to 7.1 ± 0.3 log CFU cm^{-2} in *Listeria* preformed (Figure 5), and 6.8 ± 0.2 log CFU cm^{-2} in co-inoculation (Figure 6), indicating no significant ($p < 0.05$) impact of *L. monocytogenes* being introduced at any point in dual species biofilm. Similarly, the cell concentration of *L. monocytogenes* peaked on Day 1 of co-growth for all three conditions, ranging between 5.6 ± 0.2 and 6.3 ± 0.3 log CFU cm^{-2}, with limited significant differences observed for the next 3 days of co-inoculation (Figures 4–6).

Detachment

A ‘smothering’ effect of *P. fluorescens* on *L. monocytogenes* was observed for the dual species biofilm when *Listeria* was the primary attaching bacteria, with cell concentration of *L. monocytogenes* reducing to 2.3 ± 0.3 log CFU cm^{-2} (Day 5) from 6.3 ± 0.4 log CFU cm^{-2} (Day 1) (Figure 5). Under similar growth conditions (three days of co-growth), the cell concentration of *L. monocytogenes* in *Pseudomonas* preformed biofilm (3.2 ± 0.1 log CFU cm^{-2}) (Figure 4) was comparable with co-inoculated dual species biofilm (3.4 ± 0.1 log CFU cm^{-2}) after five days of co-growth (Figure 6). A periodic oscillation in cell concentration was observed for *P. fluorescens* during co-growth, but was limited to co-inoculated conditions (Figure 6) and *to Pseudomonas*-preformed conditions (Figure 4).

Table 1. The 30 min attachment of *L. monocytogenes* (LM1) on *P. fluorescens* (P1) biofilm of different ages (incubation time in hours).

	Turbulent flow (log CFUcm^{-2})		Static (log CFUcm^{-2})	
Time	P1 in biofilm	LM1 attached	P1 in biofilm	LM1 attached
24 h	6.3 ± 0.1^{a}	3.9 ± 0.1^{x}	$6.8 \pm 0.4^{*}$	5.0 ± 0.2^{A}
48 h	7.5 ± 0.1^{b}	4.6 ± 0.2^{y}	$7.0 \pm 0.3^{*}$	4.5 ± 0.1^{A}
72 h	7.8 ± 0.4^{b}	4.9 ± 0.1^{y}	$7.1 \pm 0.4^{*}$	4.6 ± 0.3^{A}

The superscripts a-b, x-y, *, and A represent the significant difference between the cell counts at 24, 48 and 72 h, for each column, respectively.

In single-species biofilms formed under flow, the cell concentration of single-species biofilm formed by *P. fluorescens* and *L. monocytogenes* was in the range 6.9–7.5 log CFU cm^{-2} and 6.4–7.3 log CFU cm^{-2}, respectively, during the first five days of biofilm formation. On the sixth day, shedding was observed for both bacteria, with cell concentrations significantly ($p < 0.05$) reduced to 5.3 ± 0.2 log CFU cm^{-2} for *P. fluorescens* and 3.8 ± 0.1 log CFU cm^{-2} for *L. monocytogenes*.

Attachment on Pseudomonas *biofilm and EPS conditioned surfaces*

A linear increase (R^2=0.98) ($p < 0.05$) was found in the attachment of *L. monocytogenes* with increasing age of *Pseudomonas* biofilm up to 72 h (Table 1). The attachment of *L. monocytogenes* on 24, 48 and 72 h preformed biofilm was 3.9 ± 0.1, 4.6 ± 0.2 and 4.9 ± 0.1

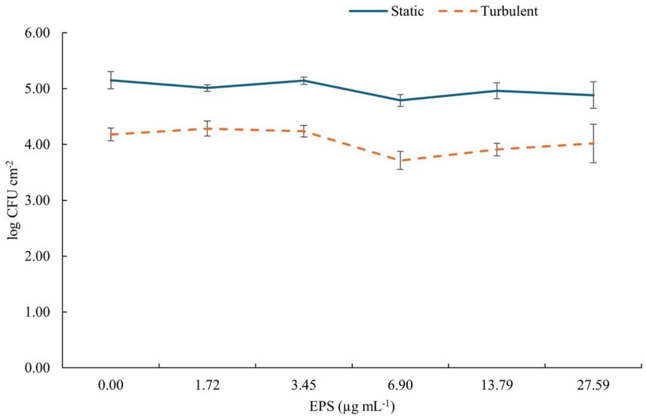


Figure 7. The attachment of *L. monocytogenes* (log CFU cm^{-2}) to exopolysaccharide (EPS) conditioned stainless-steel surfaces in the range (0–27.59 µg ml^{-1}) under static and turbulent conditions.

log CFU cm^{-2}, respectively. The cell concentration of *P. fluorescens* during this attachment for 24, 48 and 72 h was 6.3 ± 0.1, 7.5 ± 0.1 and 7.8 ± 0.4 log CFU cm^{-2}, respectively. In contrast, the *Pseudomonas* biofilm formed under static conditions did not impact the attachment of *L. monocytogenes*, with 5.0 ± 0.2, 4.5 ± 0.1 and 4.6 ± 0.3 log CFU cm^{-2} attachment for 24, 48 and 72 h, respectively, where the cell concentration of *P. fluorescens* ranged from 6.8 to 7.1 log CFU cm^{-2}. To determine whether the increasing exopolysaccharide concentration in the biofilm formed by the primary coloniser under flow is impacting the attachment of the secondary coloniser, attachment of *L. monocytogenes* to surfaces conditioned with different concentrations of EPS was then observed.

The concentration of EPS deposited on the surface for conditioning the stainless-steel surfaces had no significant ($p > 0.05$) effect on *L. monocytogenes* attachment (30 min), under both static and turbulent flow conditions. Compared to the turbulent flow system (3.7–4.2 log CFU cm^{-2}), the static system showed significantly higher attachment (30 min) ($p < 0.001$) (4.8–5.1 log CFU cm^{-2}) at all EPS concentrations (0–27.5 µg ml^{-1}) (Figure 7).

Spatial arrangement of the biofilm in co-inoculated and preformed biofilm

For all three conditions of biofilm formed under turbulent flow, the arrangement of *L. monocytogenes* (red) and *P. fluorescens* (blue) was similar, with *L. monocytogenes* predominating in the biofilm, indicating that these bacteria can rearrange themselves under turbulent flow despite the sequence of attachment (Figure 8). This correlated with a higher concentration of *L. monocytogenes* than of *P. fluorescens* under all three sequence conditions (Figures 1–3). Visually, the predominance of *L. monocytogenes* might not appear evenly throughout the surfaces (Figure 8A and C). The predominance of *L. monocytogenes* in cell counts (Figures 1–3) indicates that, in addition to the top layer of the biofilm, *L. monocytogenes* could be localized within the *P. fluorescens* layers. The circular patterns in all three conditions for biofilm formed under flow (Figure 8) show the topography of the stainless-steel coupons.

In contrast, visual differences were observed in the dual-species biofilm formed under static conditions, depending on the inoculation order. For the condition where *L. monocytogenes* was introduced to *Pseudomonas* preformed biofilm, the *L. monocytogenes* was observed on the top layer (red) (Figure 9B) (3.2 log CFU cm^{-2}) compared to *Listeria* preformed (Figure 9C) (2.3 log CFU cm^{-2}) and co-inoculated (Figure 9A) (3.4 log CFU cm^{-2}) conditions. Despite similar cell concentrations of *L. monocytogenes* in *Pseudomonas*-preformed (3.2 log CFU cm^{-2}) and co-inoculated biofilms (3.4 log CFU cm^{-2}), the visual observations representing these conditions are shown in Figure 9A and B shows a higher red intensity at the surface under *Pseudomonas*-preformed conditions. The decreased concentration of red areas under *Listeria*-preformed conditions (Figure 9C) correlated with the smothering (reduced cell concentration) of *L. monocytogenes* cells in the dual-species biofilm formed under the same conditions (Figure 5).

Discussion

In this study, *L. monocytogenes* attachment was significantly higher ($p < 0.001$) on the preformed *Pseudomonas* biofilm formed under flow (Figure 1) than during co-inoculation (Figure 3) or in single-species conditions. In contrast, the presence of

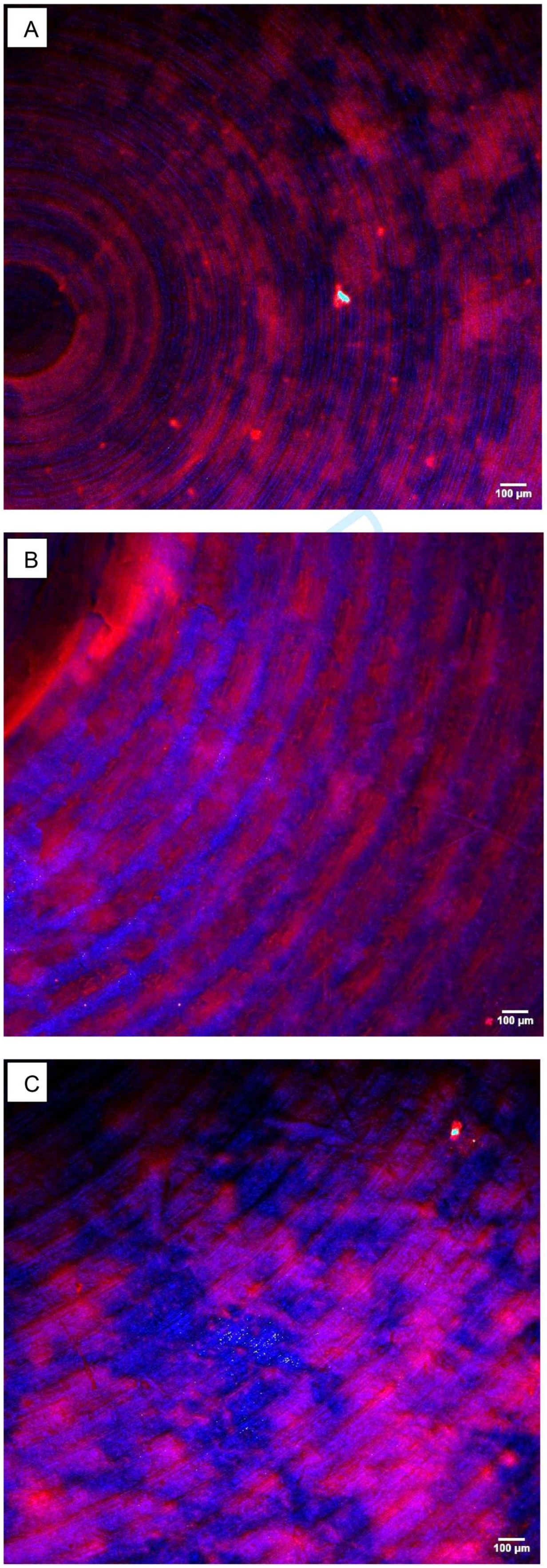


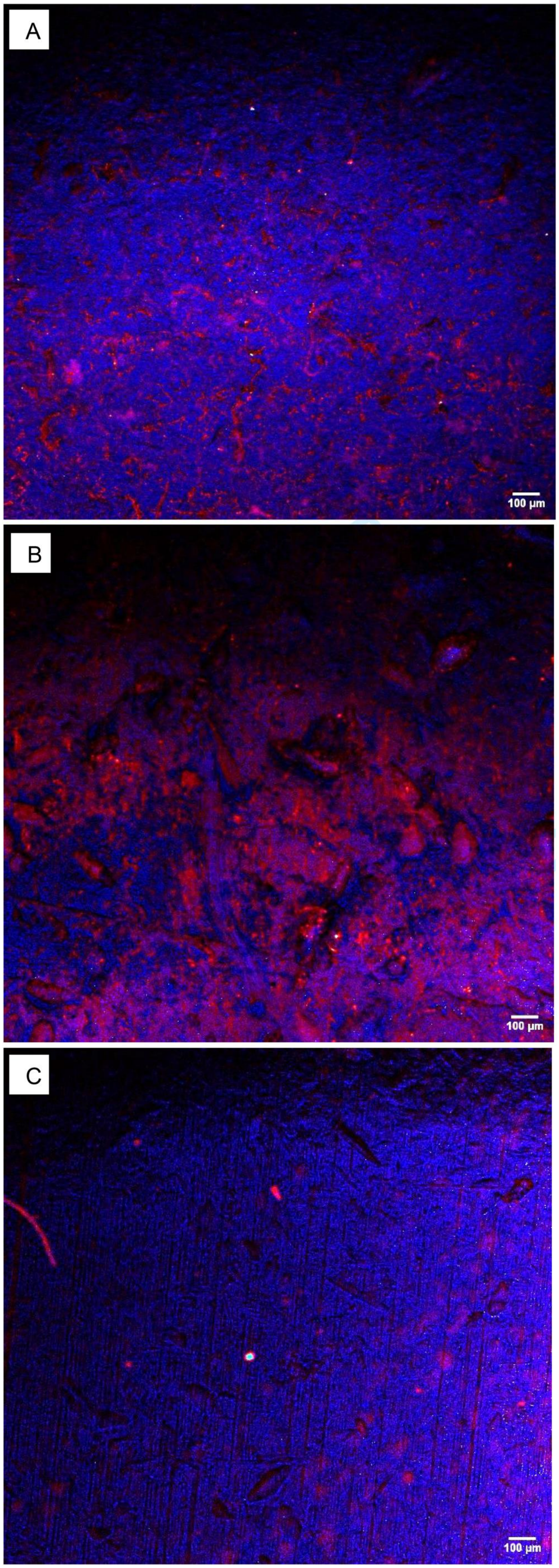
Figure 8. The images represent dual species biofilm formed under turbulent flow in (A) co-inoculation, (B) P1 (preformed biofilm) + LM1, and (C) LM1 (preformed biofilm) + P1. The red represents *L. monocytogenes* stained with WGA-Texas red conjugate, and the blue represents *P. fluorescens* stained with DAPI. The scale bar represents 100 µm.

preformed *Listeria* biofilm lowered the attachment of *P. fluorescens* (Figure 2). Various studies discuss the possibility of enhanced interaction between bacteria in the bulk liquid and the top layer when a *Pseudomonas* biofilm is already present on the surface. Sasahara and Zottola (1993) observed that *L. monocytogenes* showed enhanced attachment to glass surfaces in the presence of higher EPS-producing bacteria, such as *P. fragi. L. monocytogenes* cells were observed to adhere preferentially to microcolonies of *P. fragi*, indicating attachment and entrapment of *L. monocytogenes* cells within the EPS produced by *P. fragi* (Sasahara and Zottola 1993). Additionally, attachment of the second bacteria to the surface of the biofilm formed by the primary bacteria can be influenced by the surface roughness and the structure of the biofilm formed by the primary bacteria (Silva et al. 2024). An EPS producer that forms small aggregates with greater surface coverage has a population fitness advantage over late colonisers in a multispecies arrangement, whereas the formation of larger aggregates on the surface by the primary bacteria might result in the predominance of late-arriving colonisers (Jayathilake et al. 2017).

The bacterial attachment under different flow conditions depends on mass transport (sedimentation or diffusion), flow conditions, and topography (roughness) (Chinnaraj et al. 2021; Kreve and Dos Reis 2021). The 'sedimentation effect' has been reported to affect bacterial attachment under static and even low-flow conditions, which might explain the attachment differences observed under flow (Figures 1 and 2) and under static conditions (Figures 4 and 5) in this study. The microbes interact and settle into these surfaces using appendages (pili, flagella, curli, and fimbriae), forces (van der Waal forces for attraction, electrostatic charges for repulsion), acid-base interactions, and/or through adhesion proteins (Gordon and Wang 2019). Surface proteins, such as chaperonins, porins, and elongation Tu factors, are affected during attachment under high shear for bacteria such as *P. aeruginosa* (Hui et al. 2016). The attachment under turbulent flow is also challenged by the reversibility of unsteady bacterial attachment, reduced contact time, and shear (Wang et al. 2020). In a similar study, attachment of *Sphingomonas rubra*, *Nakamurella multipartita* and mixed bacteria was significantly reduced by shear (100 and 150 rpm) compared to static and low shear (0 and 50 rpm) conditions (Wang et al. 2020). After initial adhesion, motile bacteria can burrow into the mature, preformed biofilm through the voids and channels (Houry et al. 2012) formed by the primary bacteria, leading to a stable mixed-species biofilm.

Despite an ecological disadvantage (low cell numbers and limited space), *Listeria* not only attaches to surfaces but also outcompetes the primary bacterium (*P. fluorescens*) under turbulent flow (Figure 2). This observation was specific to biofilm formed under flow. In static conditions, while *Listeria* can form a biofilm as a secondary coloniser, it cannot outcompete the primary biofilm former (*P. fluorescens*) (Figure 4). Similar observations of *L. monocytogenes* biofilm formation on multispecies preformed biofilm (*Pseudomonas* predominating) have been observed in a model mimicking a salmon processing surface (Langsrud et al. 2016) under static conditions. In a study by Hascoët et al. (2021), the cell concentration of *L. monocytogenes* was found to be unaffected by the individual preformed biofilm (1 week) of *C. zeylanoides*, *P. luteola* and *P. fluorescens*, compared to the control (clean surface). In contrast, at shear stress (80 rpm), *L. monocytogenes* can modify the structure of the preformed *P. fluorescens* biofilm, leading to overproduction of exopolysaccharides (Puga et al. 2018). Microscopic observations show that *L. monocytogenes* embeds itself into the lower levels of the dual-species biofilm, resulting in added protection against stressors and higher cell concentration (1–2 log CFU cm^{-2}) compared to single-species *L. monocytogenes* biofilm (Puga CH, Orgaz B, et al. 2016). A sequential study on four biofilm-forming bacteria (*Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans* and *Paenibacillus amylolyticus*) conducted in a drip flow reactor (DFR) showed that prior attachment of a good biofilm former results in a population advantage during sequential attachment (Olsen et al. 2019). Overall, the EPS producing primary coloniser usually has a competitive advantage in the mixed species biofilm (Jayathilake et al. 2017), but the observations varied when shear is present, as noted above.

A spike in the cell concentration of the subsequent coloniser (*Listeria*) was observed under turbulent flow. In contrast, the cell concentration of the resident bacteria (*Pseudomonas*) remained unaffected (Figure 1), which could result from the synergistic interactions also observed during co-inoculated mixed-species biofilm formation (Figure 3). In a mixed-species biofilm containing *Pseudomonas*, *Pseudomonas* was observed to migrate to the top of the biofilm (Silva et al. 2024), resulting in overproduction of EPS and a predominance of *Pseudomonas* cells (Xavier and Foster 2007; Puga et al. 2018). These


A
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observations have been made for mixed-species biofilm, which also explains the sanitiser tolerance and the protection conferred by *Pseudomonas* (Puga et al. 2018).

Under turbulent flow, the cell concentration of *L. monocytogenes* reached 9 log CFU cm^{-2} in mixed species biofilm in all three conditions of sequential attachment (Figures 1–3), which is significantly higher ($p < 0.001$) compared to *L. monocytogenes* in single species biofilm (6.5 log CFU cm^{-2}) under flow. The following hypotheses may explain the rapid increase in *Listeria* cell concentration in multispecies under flow. Biofilm formed under low shear stress shows relatively patchy surface coverage, whereas turbulent flow allows the formation of complex structures (filamentous streamers) (Stoodley et al. 1999). These complex structures could facilitate the entrapment of secondary bacteria and promote better proliferation during longer incubation times and under stressful conditions. In single-species biofilms, over time, *Listeria* cells shed from the biofilm, revealing its oscillatory dynamics, but in dual-species biofilms with *Pseudomonas*, cells are retained in the EPS matrix, resulting in significantly higher cell concentrations in dual-species biofilms compared to single-species biofilms (Puga et al. 2018). Flow also helps transport nutrients into the deeper layers of the biofilm and enhances bacterial proliferation within the biofilm, resulting in a higher cell concentration than in the static system without flow (Tsai 2005).

Under static conditions, the dual species biofilm where *P. fluorescens* was later inoculated on *Listeria* preformed biofilm resulted in the 'smothering' of *Listeria* with reductions in cell concentrations from 6.3 log CFU cm^{-2} (Day 1) to 2.3 log CFU cm^{-2} (Day 5) over the period of five days of co-growth (Figure 5). In a similar study, *P. aeruginosa* predominated in the dual-species biofilm with *L. monocytogenes* and inhibited its growth (Dong et al. 2022). Structurally, in a mixed-species biofilm, bacteria at the top have improved access to nutrients and oxygen (Lee et al. 2014). The predominance of *Pseudomonas* in the dual-species biofilm (Figure 5) could be favoured by conditions in which *Pseudomonas* attached later, leading to settlement on the top layers of the biofilm, as observed visually (Figure 9C).

After attachment and maturation, detachment of the multispecies biofilm was also influenced by the colonisation sequence, as observed under both flow and static conditions. After three days of co-growth under flow, biofilm started shedding for both *Pseudomonas*-preformed biofilm (Figure 1) and *Listeria*-preformed biofilm (Figure 2). In *Pseudomonas*, preformed biofilm detachment resulted in a significant reduction in both *P. fluorescens* and *L. monocytogenes* cell concentrations (Figure 1). Meanwhile, in the *Listeria*-preformed biofilm, a significant reduction was observed only for *Pseudomonas*, with *Listeria* cell concentration remaining constant throughout the incubation period (Figure 2). This might be explained by how each bacterium attaches to the surface under flow conditions. In a previous study, we observed that *L. monocytogenes* undergoes stress adaptations to attach and form biofilms under turbulent flow (Pant et al. 2025), which might have resulted in stronger attachment than that of *P. fluorescens* and, consequently, more stable biofilm formation. In a similar study, single- and multispecies biofilms formed by *Sphingomonas rubra* and *Nakamurella multipartite* under high shear stress were more stable, as indicated by lower detachment observed after 9 h of incubation (Wang et al. 2020). The sloughed biofilm matrix and cells, under shear stress, retain the biofilm phenotype, whereas the released cells under non-stress conditions exhibit a planktonic phenotype (Donlan 2002). The biofilm matrix, consisting of both cells and EPS, after detachment may be protected from environmental and chemical stresses, resulting in a high bacterial load and a fast-tracked process for colonisation of a clean surface (Ghadakpour et al. 2014).

A linear relationship was found between the *L. monocytogenes* attachment and the biofilm age of *Pseudomonas* (24, 48 and 72 h) on preformed *Pseudomonas* attachment under flow. This was absent in biofilm formed under static conditions. In both cases, the cell concentration of *Pseudomonas* in preformed biofilm is comparable, indicating either the concentration, composition or architecture of the extracellular matrix formed by the primary biofilm former (*P. fluorescens*) is impacting the subsequent attachment of the secondary coloniser (*L. monocytogenes*). The manually conditioned

Figure 9. The images represent dual species biofilm formed under static conditions in (A) co-inoculation, (B) P1 (preformed biofilm) + LM1, and (C) LM1 (preformed biofilm) + P1. The red represents *L. monocytogenes* stained with WGA-Texas red conjugate, and the blue represents *P. fluorescens* stained with DAPI. The scale bar represents 100 µm.

exopolysaccharide on the stainless-steel surface did not impact the attachment of *L. monocytogenes* (Figure 7), despite the concentration range covering five times the EPS concentration at which significantly higher attachment was observed under flow (Figure 1). This could be interpreted in various ways: (a) the presence of *Pseudomonas* cells is required in the biofilm for improved attachment of *Listeria*; (b) the freeze-drying and rehydration of EPS to prepare the conditioning film for the stainless-steel surface changes the properties of the exopolysaccharide; and (c) the architecture of the biofilm produced is more important than the concentration of the EPS for the attachment of secondary bacteria. Contrasting observations have been made regarding the attachment of poor biofilm-forming bacteria, such as *Listeria*, to the extracellular matrix produced by higher EPS-producing bacteria, such as *Pseudomonas*. The matrix can act as a conditioning film, enhancing the attachment of bacteria from the bulk fluid, especially under flow. As far as the initial attachment is concerned, in the context of multispecies bacteria, the presence of exopolysaccharide-producing bacteria was found to be more important than surface properties, such as hydrophobicity, surface charge, and flagellar movement (Sasahara and Zottola 1993). The extracellular matrix consists of polysaccharides, proteins and nucleic acids. The coating of these compounds on stainless-steel surfaces modifies surface properties, such as hydrophobicity, surface charge, roughness and surface functional groups (Gubner and Beech 2000; Tsuneda et al. 2003), which can affect subsequent bacterial attachment. Studies have shown both enhancement and inhibition of subsequent attachment depending on the EPS composition and environmental conditions (Hwang et al. 2012). The conditioning of the surface and the metabolic interactions with preformed bacteria are the most common hypotheses put forward to explain the increased attachment and growth of subsequently attaching bacteria (Olsen et al. 2019). Hwang et al. (2012) observed that the presence of EPS on the surface negatively impacted the initial adhesion of *Burkholderia cepacia* and *Pseudomonas aeruginosa*, owing to the protein components in the coating.

Conclusion

In conclusion, pathogens like *L. monocytogenes* can integrate into, survive within, and even proliferate in the existing biofilm of ubiquitous bacteria such as *P. fluorescens*. In fact, the presence of preformed *Pseudomonas* biofilm improved the attachment of *L. monocytogenes*. In contrast, the presence of preformed *Listeria* biofilm reduced the attachment of *P. fluorescens*, indicating the diverse impact of conditioning layer on the attachment patterns of secondary coloniser under turbulent flow. The increased attachment of *L. monocytogenes* led to faster biofilm formation, with the biofilm reaching a plateau within 24 h of co-growth, suggesting that the biofilm matrix produced by a primary bacterium can serve as anchoring and breeding sites for pathogens such as *L. monocytogenes*. The extracted EPS and manual surface conditioning did not affect *L. monocytogenes* attachment under either static or flow conditions, suggesting that factors other than exopolysaccharide concentration are at play in the enhanced attachment. The flow conditions favoured higher attachment, quicker maturation to higher cell counts, and predominance of serious food pathogens such as *L. monocytogenes*. This indicates that understanding the impact of the flow is vital to understanding the dynamics of multispecies biofilm relevant to food production environments. Further research on the architecture and composition of the biofilm formed by higher EPS-producing bacteria, such as *P. fluorescens*, and its role in the attachment and biofilm formation of subsequently attaching bacteria would be significant for understanding the role of primary biofilm formers in the synergistic multispecies biofilm.

Author contributions

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

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