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Multispecies biofilm cities and the importance of the order of colonization

Krisha Pant *, Jon Palmer, Steve Flint

School of Natural Sciences and Food Technology, Massey University, Palmerston North 4442, New Zealand

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ABSTRACT

Biofilms are sessile communities of cells embedded in the extracellular matrix of polysaccharides formed to protect themselves from adverse conditions and are attached to the biotic/abiotic surfaces. In the natural environment, the probability of bacteria existing in multispecies is higher than the bacteria existing in isolation. The first step to a stable multispecies biofilm formation is the attachment and colonization of the surface by one or more bacteria. This review aimed to understand the impact of sequential attachment in overall multispecies biofilm formation, its role in defining biofilm properties, and the possible challenges it could present during the removal and disinfection process. In several cases, the highest biofilm former attached to the surface first resulting in a stronger biofilm which explains the enhanced resistance to removal in multispecies biofilm. Following the formation of a stable biofilm, environmental variables (e.g. temperature, surface, nutrient availability), and metabolic exchange between the bacteria drive the properties of biofilm, finally resulting in sequential detachment, driven by the predominant bacteria. These insights are vital in understanding biofilm formation and spatial layering of pathogenic bacteria for efficient biocontrol and removal.

1. Introduction

In food, environment, and industrial settings, the probability of bacteria existing in multispecies co-aggregation is higher than bacteria existing by themselves (Lee et al., 2014; Liu et al., 2018; Wang et al., 2021). By existing in a mixed population, the bacteria gain the advantage of maximum exploitation of the current environment they are attached to as well as a competitive advantage over the co-existing bacteria in the biofilm (Natan et al., 2022). This is possible because of interspecies communication between bacteria via multiple pathways, including but not limited to genetic material transfer, quorum sensing, metabolic pathways, and physical interactions (Joshi et al., 2021). Understanding the interactions and communications between bacteria is vital to developing biocidal agents with increased effectiveness against biofilms (Kirisits & Parsek, 2006).

In mixed species biofilms, the bacteria that attach to the surface first and influence the properties of the biofilms and interactions with the secondary bacteria are also referred to as resident microbiota (Hascoët et al., 2019), founder bacteria (Clarke, 2016; Eigentler et al., 2022), pioneer bacterium (Lapointe et al., 2019) or promoter bacteria (Prabhukhot et al., 2024). The impact of early colonizers on later colonizers can be either inhibitory/prior residence advantage or facilitative termed as 'priority effects' (Fukami, 2015; Olsen et al., 2019). These priority effects define the composition and the interaction between the bacteria in the biofilm (Fukami, 2015). In facilitative conditions, the resident microbiota forms a microenvironment from which the second colonizer acquires nutrients/metabolites and increases resistance to antimicrobials. In food, several resident bacteria such as *Pseudomonas* spp, *Serratia*, *Staphylococcus*, *Stenotrophomonas* spp, *Bacillus licheniformis* have been noted to thrive in the niches of industry surfaces, where the pathogens can attach themselves (biofilm) and enhance the chances of contamination through food (Cleto et al., 2012). *Listeria monocytogenes*, one of the most important pathogens in food safety, has been isolated together with bacteria such as *Pseudomonas fluorescens* from several surfaces (Fagerlund et al., 2021). The use of a non-pathogen beneficial bacteria as a primary colonizer has also been explored (Angoshtari et al., 2023). The application of guided microbial ecology has been practiced in livestock areas (Guéneau, Plateau-Gonthier et al., 2022), Iberian pig processing plant (Hascoët et al., 2021), and commercial broiler chicken houses (Guéneau, Rodiles, et al., 2022) as the replacement for chemical biocides to control pathogens.

The impacts of sequential colonization have been studied extensively in the human gut (Debray et al., 2022; Gensollen et al., 2016; Gibbons et al., 2017; Lee et al., 2013), and nature (soil/plants) (Eng et al., 2020; von Gillhaussen et al., 2014) but we must acknowledge the fact that microbes on food surfaces also follow similar patterns of biofilm

* Corresponding author.
E-mail address: kpant@massey.ac.nz (K. Pant).

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assembly (Pang & Yuk, 2019). With the added complexities of biotic (multiple strains/species/kingdom) and abiotic (food industry surfaces, wastewater treatment surfaces, wounds in clinical settings) variables, the traditional biofilm formation process, spatial arrangements, and the interactions between the bacteria discussed in the literature will vary case to case (Sauer et al., 2022). Biofilms have been described as similar to cities (Clarke, 2016; Paula et al., 2020) but does it matter which bacterium establishes itself first? This review examines the impact of sequential colonization on the formation, spatial arrangement, interspecies interactions, and removal of multispecies biofilm from industrially relevant surfaces (Fig. 1).

2. Biofilm formation

There are five stages of biofilm formation according to the traditional single species model:

A) Reversible attachment b) Irreversible attachment c) Maturation d) Microcolony formation and e) Dispersion (Sauer et al., 2022). In multispecies biofilm formation, changes in the formation steps can be observed with the assumption of sequential colonization as represented in Fig. 2, a) primary colonizer attaching on the surface b) microcolony formation by the primary colonizer c) attachment of secondary bacteria on the microcolonies formed by the primary colonizer d) maturation, and d) sequential dispersion of the biofilm (Cheah & Bae, 2023). In the presence of multispecies bacteria, higher biofilm-forming bacteria/fast colonizer becomes the primary colonizers, often easing the attachment of the low biofilm forming bacteria. In dual species biofilm containing *Pseudomonas*, early attachment of *Pseudomonads* is observed frequently, owing to its ability to form higher biofilm (exopolysaccharide production) (Periasamy et al., 2015).

2.1. Attachment and microcolony formation

The first stage of biofilm formation requires the contact between abiotic surface and bacteria, facilitated by the flow of the media and Brownian motion of the particles in the media in addition to the structural characteristics of bacteria such as flagella and pili, proteins, and surface polysaccharides (Floyd et al., 2017; Luo et al., 2022). This step is reversible and can be affected by the insufficient adhesive compounds produced with the flow patterns combined. There are two crucial steps for a microcolony formation in a multispecies setup. Co-adhesion (attachment of secondary bacteria to the surface with primary bacteria) and co-aggregation (binding of bacteria in the suspension) (Foster & Kolenbrander, 2004; Joshi et al., 2021; Kolenbrander et al., 2010; Yao et al., 2022). Similar to a single species, the attachment of a second bacteria to the primary microcolony also requires co-aggregation between the bacteria (Clarke, 2016). This coaggregation was observed to be higher for multispecies biofilm in the case of *L. monocytogenes* and *Myroides odoratus* compared to their single species counterpart auto aggregation (Jacobs & Chenia, 2009). Sequential attachment and ecological succession of the bacteria were observed in dual-species biofilm of *E. coli* and *P. aeruginosa* with *E.coli* attaching first (fast colonizer) but being overpowered (larger coverage area) by *P. aeruginosa* which attached itself to the *E. coli* biofilm in the later stages (slow colonizer) (Wetherington et al., 2022). After surface attachment, the accumulation of loosely adhered exopolysaccharides is required around the polar region of the cell, to form a stable arrangement and an irreversible structure (Kaur & Dey, 2023). The EPS produced by primary colonizer can act like 'cement' for the attachment of secondary bacteria (Cheah & Bae, 2023). The bacterial composition of the biofilm at the time of attachment can look quite different compared to the bacterial composition of the mature multispecies biofilm depending on the microbes involved, and internal and external stimuli conditions (Clarke, 2016).

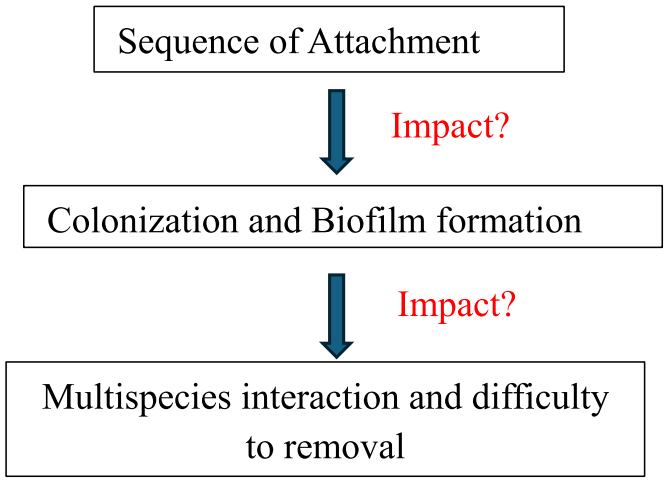

Fig. 1. Flow chart of factors that are discussed in this literature review.

2.2. Maturation

A mature biofilm consists of compact layers of multiple species of bacteria embedded in the extracellular matrix with microenvironments suitable for each bacterium and linked with interstitial channels throughout the layers providing access to nutrients to cells under the layers and outlet of waste metabolites (Yao et al., 2022). The properties and constituents of a mature biofilm can be vastly different depending on the complexities of bacterial species in the biofilm (Eigentler et al., 2022). The relative abundance of the bacteria in a mature biofilm is determined by the ability of the bacterium to replace itself (growth rate) while encountering stress, nutrient gradients, environmental factors (temperature, oxygen, pH, shear stress), and other metabolic challenges. Due to these variables, the question of how the founding cell impacts the mature biofilm and the intercellular interactions remains a mystery on many levels (Eigentler et al., 2022). In a 79-day biofilm maturation study, six out of nine of the founder bacteria abundant (>5 %) at 8 days were replaced by minority species when analysed at the endpoint of 79 days. Additionally, the bacteria that dominated the overall multispecies biofilm were observed in high concentrations only from the 56-day mark of the 79-day study (Brislawn et al., 2019). Hence, the higher abundance of founder bacteria is not guaranteed and has been reported to fluctuate as the biofilm matures. The presence of multispecies also impacts the properties of a mature biofilm. The time taken for the maturation of dual species biofilm of *Shewanella baltica* and *Pseudomonas fluorescens* was found to be longer despite the decreased thickness, biomass, and polysaccharide content compared to their single species counterparts (Zhu et al., 2019).

In a mature biofilm, communication between bacteria is facilitated by the secretion and reception of specific molecules (4 classes) also known as quorum sensing molecules (Elias & Banin, 2012). The first class is Acyl Homoserine lactones, also known as AI-1, responsible to regulate population density in gram negative bacteria (Das, 2022). The second class is autoinducer-2 (AI-2), a universal signalling molecule for gram-positive and gram-negative bacterial populations responsible for interspecies communication. The third class is autoinducer-3 which has responsible for communication between the bacteria and their host (mammalian) (Yiyang et al., 2025). Finally, the fourth class, autoinducer peptide compounds (AIP) is known as AI-4, responsible for communication between gram-positive bacteria (Mohana Sheela et al., 2018). The concentration of these autoinducers is a deciding factor in the expression of certain genes responsible for EPS production and biofilm formation (Das, 2022).

2.3. Dispersal

Dispersion of the biofilm is often triggered by external stimuli such as

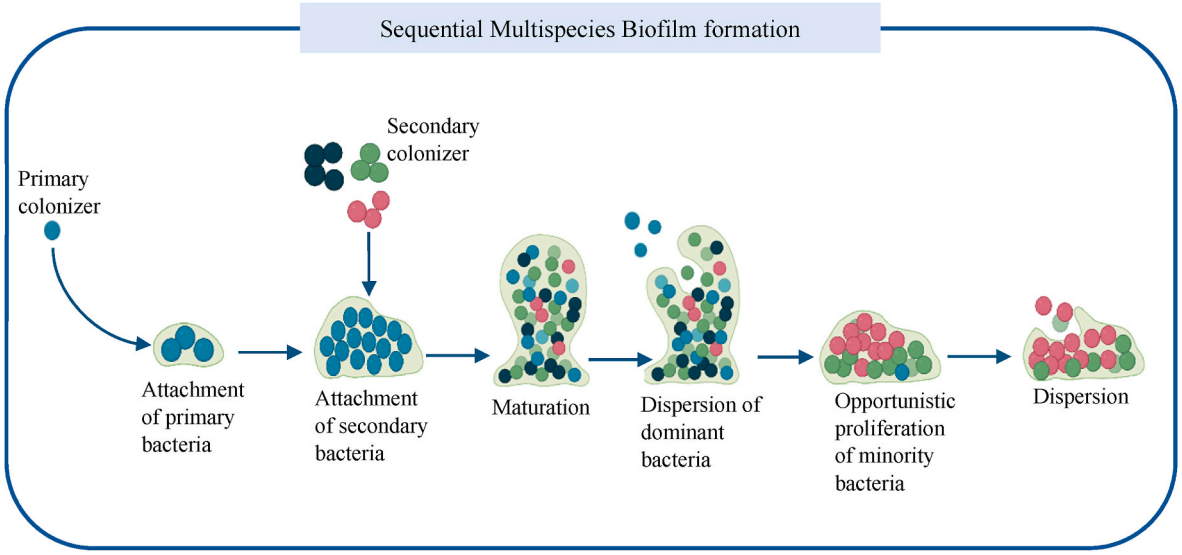

Fig. 2. Multispecies biofilm formation steps representing one of the conditions of sequential colonization and sequential detachment (Developed using BioRender).

nutrients, pH, and temperature (Roy et al., 2021), and supported by the release of dispersal enzymes produced by the bacteria in the biofilm (Rumbaugh & Sauer, 2020). Depending upon these variables, sequential detachment can be observed where the cells with high cell density are expelled from the multispecies biofilm (Fig. 2) (Culotti & Packman, 2014). In such conditions, the dispersion of one bacteria from the biofilm can present itself as an opportunity for other bacteria to attach themselves to the hollow voids left behind by that bacteria and colonize the space (Culotti & Packman, 2014). In a three-species biofilm formed by *P. aeruginosa*, *E. coli*, and *E. faecalis,* the dispersal of *P. aeruginosa* bacteria was higher in biovolume compared to the other two species present in the biofilm, allowing all three bacteria to maintain its population and coexist in the biofilm in cyclic periods. In the lack of dispersion, *P. aeruginosa* dominated the biofilm, displacing both *E. coli* and *E. faecalis* from the biofilm. The periodic dispersal of *P. aeruginosa* cells allowed the coexistence and population boom of other bacteria in the multispecies biofilm (Holt et al., 2024). While there are assumptions that the dispersal of higher EPS-producing bacteria-producing surfactants such as rhamnolipid is also capable of removing other bacteria from the biofilm (Wood et al., 2018), this is highly dependent on the nature of the spatial arrangement and location of the bacteria in the biofilm. It can also be assumed that the bacteria embedded in the *Pseudomonas* biofilm layers might be removed during the dispersal of *Pseudomonas* biofilm, allowing the rare species on the outskirts an opportunity to multiply and flourish (Holt et al., 2024). In addition to the natural biofilm cycle, the removal of predominating bacteria (*Stenotrophomonas maltophilia*) through the antimicrobial treatment (commercial alcohol-based disinfectant- Bacillol) increased the abundance of antimicrobial resistant smaller bacterial community (*E. faecium* and *Stenotrophomonas haemolyticus*) in the multispecies biofilm (Wicaksono et al., 2022). The attachment of new bacteria to the remnants of EPS produced by the primary after dispersion or cleaning is very much a possibility (Castonguay et al., 2006; Puga et al., 2018). Either way, the dispersal of bacteria in multispecies biofilm is closely related to the concept of competition-colonization trade-off in multispecies bacteria competing for space and resources in the biofilm (Wetherington et al., 2022).

3. Regulation of biofilm through the sequence of attachment

In multispecies biofilm arrangement, the co-attachment of multiple species of bacteria to the abiotic surface simultaneously would be rare. If the continuous influence of new colonizers in a multispecies biofilm is taken into account, significant changes in the biofilm composition and properties and interactions can be observed (Sauer et al., 2022) also shown in Table 1. This is referred to as bacterial conditioning and impacts the attachment of the sequential bacteria that comes after the first bacteria (primary colonizer/resident microbiota) (Pang & Yuk, 2019). The resident bacteria in the biofilm can prevent or enhance the

Table 1
Sequence of colonization and its impact on biofilm composition in multispecies biofilm system.

Bacteria	Sequence of Colonization and Observation	Reference
Escherichia coli (STEC) O157: H7 and *Escherichia coli* O111:H8-	Co-inoculation: Serotype O157:H7 outcompetes the O111:H8 Preformed condition: Pre-colonizer dominates the biofilm	Wang et al. (2015)
Bacillus safensis and *Listeria monocytogenes*	Co-inoculation: Inhibition of L. monocytogenes *B. safensis* preformed: Inhibition of L. *monocytogenes*	(Ripolles-Avila et al., 2022) Hascoët et al. (2021)
L. monocytogenes and *L. sakei*	Co-cultured: Inhibition of L. monocytogenes biofilm formation *L sakei* preformed: Prevention of L. *monocytogenes* biofilm formation	Pérez-Ibarreche et al. (2016)
L. monocytogenes and *Pseudomonas fluorescens*-	Co-cultured: Reduced growth of *L. monocytogenes* *Pseudomonas* preformed: Higher cell concentration overall and matrix overproduction	Puga et al. (2016) (Puga et al., 2018)
B. thuringiensis and *S, aureus*	*S. aureus* preformed: complete replacement by *B. thuringiensis* and eradication of *S. aureus*	(Houry et al., 2012)
P. fluorescens, L. plantarum, and *Leuconostoc pseudomesenteroides*	*P. fluorescens* or *L pseudomesenteroides* preformed: Enhanced attachment of *L. plantarum*	Lapointe et al. (2019)
Stenotrophomonas rhizophila, Xanthomonas retroflexus, Microbacterium oxydans and Paenibacillus amylolyticus	Co-inoculation: Synergistic interaction-higher biofilm *S. rhizophila* and *X. retroflexus* preformed: Higher biofilm formation *M. oxydans* and *P. amylolyticus* preformed: Lowered biofilm formation	Olsen et al. (2019)

attachment and growth of subsequently attaching bacteria (different species/genera) depending on the resident bacteria present (Hascoët et al., 2021), through niche pre-emption (depletion of resources by early colonizers), niche facilitative modification (early colonizers produces metabolites beneficial for later arriving microbes), and niche inhibitory modification (competition that prevents the attachment of later arriving microbes) (Debray et al., 2022). In synergistic interactions, the presence of one bacteria on the surface reportedly enhances the attachment of consecutive bacteria (Lapointe et al., 2019).

In some cases, the predominance of specific bacteria (in a multi-species biofilm) or isolates (in a multi-strain biofilm) can be observed regardless of the stage of biofilm where the new colonizer was introduced (co-culture vs sequential). *E. coli* virulent serotypes can dominate the biofilm through competition regardless of the order of colonization, which explains the frequent isolation of this specific serotype from food outbreaks (Wang et al., 2015). On the other hand, the production of biosurfactant by primary colonizer *Bacillus safensis* has been associated with the reduced attachment of secondary colonizers such as *L. monocytogenes* and *S. epidermidis* (Table 1) (Abdelli et al., 2019; Hascoët et al., 2021). Similar reduction in biofilm formation was observed for *L. monocytogenes* when inoculated to preformed *Lactobacillus sakei* biofilms (bacteriocin formers) (Pérez-Ibarreche et al., 2016). In addition to the inhibitory compounds, the exopolysaccharide produced by *L. sakei* have been assumed to inhibit the adhesion of *L. monocytogenes* on the preformed *Lactobacillus* biofilm surface (Pérez-Ibarreche et al., 2016). Exposure of bacteriocin carrier *B. thuringiensis* to preformed biofilm of *S. aureus* successfully eradiated the 24h *S. aureus* biofilm and re-established the biofilm consisting only of *B. thuringiensis*. This process was accelerated by the motility of *Bacillus* on the biofilm which created channels within the preformed *S. aureus* biofilm leading to easier delivery of bacteriocin molecules (Houry et al., 2012). Three-species biofilm formed under shear stress (Centre for Disease Control Bioreactor) showed that primary colonization by both *P. fluorescens* and *Leuconostoc pseudomesenteroides* resulted in enhanced attachment of *L. plantarum* indicating the synergistic interaction between the three bacteria enhanced by the amphiphilic nature of EPS produced by *Pseudomonas* (Lapointe et al., 2019). Another interesting observation was made by (Olsen et al., 2019) where the pre-colonization of single species (good biofilm formers) had no effect on subsequent colonizers and overall biofilm formation. But when the surface was pre-colonized with dual species (good biofilm formers), the subsequent biofilm formation was higher compared to the four species biofilm formed through co-inoculation (via drip flow reactor) comprising of *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans,* and *Paenibacillus amylolyticus* (Table 1). The bacteria with a higher growth rate would be expected to have higher abundance in the biofilm through competition during co-attachment. But when a bacterium is introduced to an already established biofilm, the one with co-operative behaviour would have more chances of establishing itself, regardless of its growth rate (Valiei et al., 2024).

4. Spatial arrangement of bacteria in multispecies biofilm

The limited mobility of the multiple bacterial colonies inside the biofilms results in a specific spatial arrangement commonly observed through microscopic observations (Guéneau et al., 2023), and modelling (Gordon et al., 2019). The spatial arrangement of the bacteria in a multispecies biofilm setup is regulated by the metabolic function, the overall role of each bacterium, and nutrient gradient across the biofilm (Lee et al., 2014). The spatial location and density of the primary colonizers were significant in the competitive interactions between the bacteria in a multispecies biofilm (Eigentler et al., 2022). In dual species biofilm containing *P. aeruginosa* and *E. coli*, the arrangement resulted with *P. aeruginosa* on the top which were efficient at obtaining nutrients from the media and *E. coli* on the bottom providing essential structural support (Cheah & Bae, 2023). In addition, facultative anaerobes can be found in the bottom layers, covered by the species that require oxygen for growth and EPS production (Yao et al., 2022). The spatial arrangement of the biofilm can be categorized as microcolonies, co-growth by the layering of the microbes, and specific mixing patterns depending on the genotypic, phenotypic trials and nutritional distribution in the media (Liu et al., 2018). Bacteria in relatively heterogenous multispecies biofilm benefit through access to the 'public goods' which are the EPS produced by surviving bacteria or metabolites secreted during cell death (da Silva Fernandes et al., 2017). Much of the spatial structure is dependent on the extracellular matrix, directly affecting the resilience, intercellular communication, nutrient distribution, and genetic material exchange between the cells (Lee et al., 2014; Nadell et al., 2017). In dual-species biofilm formed by *Pseudomonas* spp. and *L. monocytogenes*, the latter was found in the bottom layers of biofilm, providing higher survival during surface sanitizer treatment (Puga et al., 2018; Ripolles-Avila et al., 2022). In addition to the co-culture, the sequentially added *L. monocytogenes* was able to incorporate itself deep into the bottom layers of preformed *Pseudomonas* biofilm, with the latter imitating a blanket layer of bacteria on top (Puga et al., 2018; Thomassen et al., 2023). In dual species biofilm of *E. coli* and *P. aeruginosa*, *E. coli* was able to outcompete *P. aeruginosa* in both co-culture and *P. aeruginosa* preformed conditions. Overgrowth of *E. coli* was observed inside the big clusters pre-formed by *P. aeruginosa* resulting in reduced *P. aeruginosa* growth under nutrition-limited conditions (Culotti & Packman, 2014). When a higher EPS-producing spoilage microbe such as *P. fluorescens* is introduced in multi-strain biofilm environments, it has been known to over produce EPS and migrate to the top of the biotic and abiotic layer for the increased access to oxygen (Kim et al., 2014). In addition to the layering effect, the formation of individual microcolonies on the surface where the primary colonizer is attached to the inside and the second bacteria attached to the outside in layers has also been observed. This spatial arrangement was observed for bacteria with high oxygen requirement, where one microcolony maintains a safe distance from another and each microcolony has two layers of bacteria as observed in dual species biofilm of *P. putida* and *Acinetobacter* sp. C6 (Haagensen et al., 2015). The migration of bacteria on the preformed biofilms and the overall bacterial population is driven by the gradients over the surface created by high cell density (centre of biofilm), oxygen access, waste products across the plane, and access to nutrients (edges and surface of the biofilm) (Serra & Hengge, 2014; Xavier et al., 2009). The pre-colonization of any one bacterium in a subsequent four-species biofilm formed by *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans*, and *Paenibacillus amylolyticus* did not affect the spatial arrangement of the biofilm compared to co-inoculation. Whereas, the pre-colonization condition was noted to cause variation in biofilm biomass of the four species biofilm (Olsen et al., 2019). These strain-specific observations show that the spatial distribution of the bacteria in the biofilm depends on the inherent properties and metabolic requirements of the bacteria and is independent of the sequence of colonization.

5. Interaction between the bacteria in the multispecies biofilm

The high-density bacterial cell nature of the biofilms ensures both inter and intra-species interactions within the biofilms (Kreth et al., 2005). The inter species interactions in multi-species biofilms are so intensely linked that these biofilms have often been referred to as multicellular individuals (Yao et al., 2022). This coalescing can lead to various interactions, cooperative, antagonistic, and neutral (Burmølle et al., 2014). The coaggregation leading to cooperative interactions has been studied extensively in dental biofilms (Kim et al., 2017; Kreth et al., 2005). One commonly undertaken basic step to broadly categorise the interactions between the bacteria is to study biofilm biomass under similar incubation and growth conditions and compare the single species and multispecies biomass. For example, the biomass of multiple bacteria will be higher than the sum of single species involved in

synergistic interaction. Similarly, for neutral and antagonistic the sum of single species will be the same or lower than the multiple species respectively (Ren et al., 2015). A 1.5 fold increase in biofilm was observed for a four species combination of dairy bacteria, *Stenotrophomonas rhizophila*, *Bacillus licheniformis*, *Microbacterium lacticum*, and *Calidifontibacter indicus* compared to their single species counterpart as the result of synergistic interactions (Sadiq et al., 2023). In addition to the interaction between bacteria in a mature biofilm, the first interaction between the bacteria takes place during the attachment to the surface/biofilm also known as 'priority effects' and can be categorized as follows.

5.1. Synergistic interactions

When the bacteria attach to the surface in an order, the early colonizers get the opportunity to modify the environment, also known as 'niche modification' which could ease or discourage the attachment and growth of secondary bacteria (Debray et al., 2022; Fukami, 2015). The enhanced attachment of the second bacteria is also known as the facilitative priority effect and could lead to synergistic interactions once the bacteria successfully attach to the surface.

Protective effects against the stressor for one or more bacteria in the biofilm are one of the advantages of synergistic multispecies interactions (Bottery et al., 2022). The presence of multiple species allows the use of available nutrients to produce a simplified carbon source termed as cross-feeding which normally is not possible for single species communities (Tan et al., 2017). For e.g. when the interaction between *Acinetobacter* strain C6 and *P. putida* R1 was observed in the presence of benzyl alcohol in dual species biofilm, *P. putida* was found to overgrow *Acinetobacter* through the metabolism of benzoate, an intermediary product formed by *Acinetobacter* during benzyl alcohol degradation (Christensen et al., 2002). In addition to the improved survival of bacteria in the mixed population, the overproduction of EPS and increased resistance to the antimicrobial compounds are some of the factors associated with synergistic interactions (Table 2) (Bottery et al., 2022). Multiple species of bacteria present in the same biofilm are also known to alter the resistivity of select bacteria to antibiotic treatments. Streptococcal protein A produced by *S. aureus* improved the biofilm formation and increased the tobramycin resistance of *P. aeruginosa* (Beaudoin et al., 2017). In exchange, 4-hydroxy-2-heptylquinoline-*N*-oxide and siderophores produced by *P. aeruginosa* increases the vancomycin resistivity of *S. aureus* (Orazi & O'Toole, 2017). The higher biofilm biomass in the synergistic biofilm can also be the result of production of limited metabolites (e.g. Vitamin B6 and iron) by multiple species of bacteria in the biofilm (Wicaksono et al., 2022).

5.2. Antagonistic interactions

In sequential colonization, the attachment prevention of the late colonizers can take place through 'niche pre-emption', where the resources available (nutrients/space) has already been utilized by the early colonizers hence preventing the attachment of late-arriving species (Debray et al., 2022; Fukami, 2015). In cases where co-inoculation is presumed, this interaction has been studied as antagonistic interactions as the result of competition in the multispecies biofilm.

Nutrient competition and negative metabolic interaction can result in the negative effect of one bacterium on other bacteria present, leading to survival of dominant bacteria more suited to the growth conditions (Burmølle et al., 2014). Competitive interactions are reoccurring when bacteria from different sources are introduced together (Madsen et al., 2016). The antagonistic interaction results in the reduction of chlorine tolerance, with the exception of the presence of bacteria which produces specific structure that resists chemical stress (chlorine) (Z. Zhu, Shan, et al., 2020). The antagonistic interactions are commonly followed by the production of antagonistic metabolites which are used to outcompete other bacteria present in the biofilm. For example, when *B. cereus* and *L. monocytogenes* were introduced on the stainless-steel surface, *B. cereus* produced metabolites that inhibited the growth and adherence of the *L. monocytogenes* to the stainless-steel surface (Alonso et al., 2020). A similar observation was made in a dual species biofilm of *B. safensis* and *L. monocytogenes*, where a preformed biofilm of *B. safensis* was able to reduce the cell count of *L. monocytogenes* through competition for nutrient, attachment space, and production of biosurfactant (Hascoët et al., 2021). Furthermore, the negative interaction between *Lactococcus piscium* and *Listeria monocytogenes* was found to require cell-to-cell contact, the mechanism of inhibition assumed as horizontal gene transfer via secretion pathways (types IV and VI) and nanotubes (Saraoui et al., 2016). In addition to the transfer of DNA and protein molecules, under nutrient-limiting conditions, the production of surface-inhibiting compounds (Bacteriocins CibA and CIbB) also results in the inhibition of secondary bacteria upon cell to cell contact with *Streptococcus pneumoniae* (García-Curiel et al., 2021). The inhibition of bacteria in multispecies species environment is also dependent on the physio-chemical changes of the media like pH and temperature. In a

Table 2
Multispecies and the result of their interaction in multispecies biofilms relevant to the food industry.

Microbiological Niches	Microbes Involved	Interaction	Result of interaction	References
Meat processing conditions	*Leuconostoc pseudomesenteroides, Lactobacillus plantarum* and *Pseudomonas fluorescens*	Synergistic	-Enhanced attachment of *L. plantarum* -Increased resistance of *Leuconostoc*	Lapointe et al. (2019)
Poultry processing environment	*S. Enteritidis, C. jejuni* and *C. perfringens*	Synergistic	-Increased cell concentration of *C. jejuni*	Kim and Oh (2024b)
Dairy surface	*Stenotrophomonas rhizophila, Bacillus licheniformis, Microbacterium lacticum, and Calidifontibacter indicus*	Synergistic	-Elevation in biofilm formation (1.5 fold)	Sadiq et al. (2023)
Medical surfaces	*Acinetobacter baumanii, Enterococcus faecium, Escherichia coli, Staphylococcus haemolyticus and Stenotrophomonas maltophilia*	Synergistic	Higher biofilm formation- Higher resistance to Bacillol (commercial disinfectant)	Wicaksono et al. (2022)
Stainless steel surfaces of food industry	*Pseudomonas aeruginosa* and *Salmonella*	Antagonistic	Reduced biofilm formation	Pang et al. (2017)
Fish processing industry	*Shewanella baltica* and *Pseudomonas fluorescens*	Antagonistic	-Lowered biomass and polysaccharides -Longer time taken for biofilm cycle -Less compact biofilm in dual species biofilms	Zhu et al. (2019)
Food processing industry	*Staphylococcus aureus* and *Salmonella enterica, Raoultella planticola*	Antagonistic	-Decreased biofilm biomass in dual species biofilm containing *S. aureus*	Makovcova et al. (2017)
Fish juice	*Pseudomonas fluorescens* and *Staphylococcus aureus*	Antagonistic	-Dominance of *P. fluorescens* -Increased sensitivity to carvacrol	Wang et al. (2020)
Food processing facilities	*Listeria monocytogenes* and *Salmonella Typhimurium*	Neutral	-No changes in cell concentration	Tadielo et al. (2022)
Industrial juice filtration membranes	*Rhodotorula mucilaginosa, C. tropicalis, Candida krusei* and *Candida kefyr*	Neutral	-No change in resistance to farnesol, 2-phenyl ethanol	Agustín et al. (2019)

complex four-species biofilm formed in microfiltered milk, the inhibition of *Staphylococcus aureus* by the combination of *Lactococcus garvieae*, *Lactococcus lactis,* and *Enterococcus faecalis* co-incubation was found to be because of pH reduction (Alomar et al., 2008).

Another type of interaction are neutral interactions, which can be observed when the presence of one or more bacteria does not have any significant effect on the bacterial population consisting of other bacteria and vice-versa. Depending upon the availability of nutrients, neutral interactions can lead to an antagonistic one with time, where the competition for nutrients leads to domination of one or more bacteria compared to others in the population. This type of interaction has also been observed in the interaction in the biofilm (*Sphingomonas* + *Microbacterium*) when compared 24h (neutral) with 48 and 72h (competitive) (Z. Zhu, Shan, et al., 2020).

6. Environmental factors affecting the multispecies biofilm formation

6.1. Substrate and growth medium

The type of substrate has been known to have an impact on the formation of both single and multiple species biofilm. Depending upon what has been defined as natural (pebble/wood) and artificial (carbon fibre and polyvinyl chloride) substrate, the formation of biofilm was found to be more intricate with higher diversity and stability in the artificial substrate with the increased ability to metabolise nitrogen, carbon and arsenic sources in addition to resistance to extrinsic factors (Miao et al., 2021). The modification of stainless-steel surfaces to inhibit the attachment of multiple species of bacteria has been researched extensively. For e.g: superhydrophobic coatings against *E. coli* and *L. innocua* (DeFlorio et al., 2023), plasma polymerised coating against *L. monocytogenes* (Fernández-Gómez et al., 2023), peptide modification against *S. aureus* (Cao et al., 2018).

The higher synergy in the multispecies biofilms was found in the minimal nutrient media (M9), and media containing disinfectant (2.5 % Bacillol-sublethal concentration of commercial disinfectant) compared to nutrient rich media such as tryptone soy broth, indicating positive correlation with stress (Wicaksono et al., 2022). The interaction between bacteria in the multispecies is affected by the nutrient availability and the presence of antimicrobials during the formation of the biofilm (Parijs & Steenackers, 2018). The presence of meat exudate favoured the dual species biofilm of *E. coli* and *S. aureus* (compared to MH broth) with *E. coli* colonizing the surface primarily at 24h (Dutra et al., 2018). This has been attributed to the conditioning layer formed by the concentrated food constituents (nutrient layers) on the surface for enhanced biofilm formation (Mazaheri et al., 2023). The presence of *L. lactis* improved the biofilm counts (15–100-fold) of *P. fluorescens* in UHT milk in the dual species biofilm conditions through the uptake of lactic acid produced by *L. lactis*. In return, *P. fluorescens* provides a stable EPS matrix, and anaerobic conditions which improves the biofilm counts of *L. lactis* (15–40 fold) in the dual species compared to their single species counterparts (Kives et al., 2005). The presence of chicken juice significantly impacted the proportion of *P. aeruginosa* and *L. monocytogenes* cells in the dual species biofilm compared to TSB. In dual species biofilm, the cell concentration of *L. monocytogenes* was significantly higher in TSB whereas *P. fluorescens* was favoured by the chicken juice media (Dong et al., 2022).

6.2. Temperature and pH

Studies on the effect of temperature on the arrangement of bacteria embedded in the biofilm show that the biofilm formation in mixed species culture is dependent on the temperature. In the specific case of *L. monocytogenes* and *Pseudomonas* biofilm, low temperature stress was found to be unfavourable to synergistic biofilm formation as compared with higher temperatures (Puga et al., 2016). Additionally, the thickness of the higher temperature biofilm was found to be double as compared with the lower temperature one (Puga et al., 2016). In the biofilm formed by multispecies microorganisms like *Enterococcus* and L. *monoctyogenes* over the period of 8 days, the reduction of pH (7–4.4) was found to be favourable for growth of *Enterococcus* compared with *L. monocytogenes* (da Silva Fernandes et al., 2015).

6.3. Shear stress

The presence of shear stress (high flow velocities) promotes denser biofilm formation (Araújo et al., 2016) and resistivity to chemical and mechanical removal of the biofilm from the surface (Simões et al., 2022). This is the result of either a continuous supply of nutrients to the cells or the adaptation of the cells during the biofilm formation itself, which is overlapping in experimental studies. In addition to the number of cells, the dominating population in the polymicrobial biofilm, the spatial arrangement, and the biofilm properties such as thickness, and composition can also be impacted by the shear stress (Chawla et al., 2020). A study on the multispecies biofilm (*Streptococcus* spp., *Bacillus* spp., *Micrococcus* spp., *P. fragi*) in the dairy processing environments observed that the laminar flow resulted in higher biomass and cell counts in the polymicrobial biofilm compared to turbulent flow (Fysun et al., 2019). The cell counts of *E. coli O157:H7* increased with the shear stress in multispecies biofilm formed with *L. monocytogenes*, and *R. insidiosa* on the stainless steel surface (Prabhukhot et al., 2024). After the formation of biofilm, the sloughing off of the bacteria from the surface (Chawla et al., 2020), 'washing' away of the quorum sensing molecules are some of the other factors impacted by the flow system. The sloughing off of the biofilm from the surface in the flow system should reduce the biofilm biomass but research shows that it increases instead indicating that the higher the biofilm formation rate the greater the resistance to sloughing off (Rickard et al., 2004). Signalling molecules such as cyclic-di-GMP are expressed under shear stress for *P. aeruginosa* and this is indicated by the over production of the EPS matrix in the biofilm (Rodesney et al., 2017). This over production of EPS acts as a physical barrier against stresses and protects the bacteria within, as noted in dual species biofilm of *P. aeruginosa* and *E. coli* (Cheah & Bae, 2023).

7. Resistance to disinfections in multispecies biofilm

Chemical (antimicrobials), physical (UV, cold plasma), and biological (bacteriophages) measures have been targeted towards dismantling the biofilm matrix and inhibiting the bacteria embedded in the matrix, and so further attachment of the bacteria into new biotic and abiotic surfaces does not take place (Sadekuzzaman et al., 2015; Y. Zhu, Shan, et al., 2020). The removal of mature biofilm is dependent on various factors including but not limited to the attachment to abiotic/biotic surfaces, surface roughness, the composition and properties (thickness) of the biofilm (Y. Zhu, Shan, et al., 2020).

Sodium hypochlorite, peracetic acid, and quaternary ammonium compounds are some of the commonly used sanitizers in the food industry for high efficiency and low costs (Table 3). Sodium hypochlorite affects the cell through oxidation of sulfhydryl groups of certain enzymes inside the cellular membrane efficiently but have limited effect on mature biofilm due to its interaction with organic compounds, which are major biofilm components (Rodríguez-Melcón et al., 2019). In contrast, peracetic acid has minimal effect on other biofilm components and works by oxidizing cellular compounds (Castro et al., 2021). Even though the peracetic was more effective post application (lower biofilm formation after re-inoculation with fresh media on the treated surface), higher growth control was achieved with sodium hypochlorite in multispecies studies (Fernández-Gómez et al., 2023). Owing to the rise of multidrug-resistant bacteria, numerous studies have examined the antibiofilm effect of bioactive compounds such as monoterpenes (carvacrol, thymol, linalool), sesquiterpenes and phenylpropanoids for

Table 3
Effect of antibiofilm agents in the multispecies biofilm.

Microbiological Niches	Microbes Involved	Treatment	Observation	References
Food processing surfaces- stainless steel	*Escherichia coli* O45 and *Salmonella enterica* serovar Typhimurium	100 mg/l sodium hypochlorite for 1 min	Reduced log reduction in multispecies compared to single species- increased chlorine tolerance	Lin et al. (2022)
Food contact surfaces	*P. fluorescens* and S. Enteritidis	Quaternary ammonium compounds (20 ppm/5 days)	Enhanced survival of S. Enteritidis	Pang et al. (2020)
Food industry surfaces	*Escherichia coli*, *Salmonella Typhimurium*, and *Listeria monocytogenes*	Grapefruit seed extract	Enhanced resistance in multi-species biofilm	Kim and Oh (2024a)
Dairy pasteurizer	*Stenotrophomonas rhizophila, Bacillus licheniformis,* and *Microbacterium lacticum*	Standard CIP[a] regime	Protected against disinfection in multi-species	Sadiq et al. (2024)
Fruit packing facilities	*Listeria monocytoegens, Pseudomonadaceae, Xanthomonadaceae, Microbacteriaceae,* and *Flavobacteriaceae*	Benzalkonium chloride (12.5 ppm/2h)	Enhanced survival of *L. monocytogenes*	Rolon et al. (2024)

[a] CIP: water rinse-1.5 % (w/v) sodium hydroxide at 60 °C/6.45 min-another water rinse-1.0 % nitric acid at 60 °C/4.45 min-water rinse-0.01 % peracetic acid/7.6 min-water rinse.

single and multispecies biofilms (Kim & Oh, 2024a). The adhesion and growth of *P. aeruginosa*, *L. monocytogenes*, and S. Typhimurium in multispecies biofilm decreased in the presence of piperine and black pepper essential oil (dos Santos et al., 2023). The mechanism of inhibition of sessile bacteria in the biofilm by essential oils was commonly found to be membrane injury (Klančnik et al., 2021). Multispecies biofilm formed by *Escherichia coli*, *Salmonella* Typhimurium, and *L. monocytogenes* showed enhanced resistance to grapefruit seed extract compared to their mono-species counterpart (Kim & Oh, 2024a). More recently, combinations of essential oils have been introduced to overcome limitations with the application of single bioactive compounds.

The sequence of attachment has been reported to impact the sanitizer resistivity of the bacteria through compositional (both bacterial abundance and EPS) changes. The early colonization of *P. fluorescens* resulted in higher production of EPS in the dual species biofilm, better attachment of *L. monocytogenes* and higher resistance of the later bacteria against desiccation or disinfectant treatment (Pang & Yuk, 2019). Similar protective observation was made for three species biofilm of *Stenotrophomonas rhizophila*, *Bacillus licheniformis*, and *Microbacterium lacticum* in co-inoculation where the increased EPS produced by *B. licheniformis* protected the whole consortium against cleaning and disinfection processes (Table 3) (Sadiq et al., 2024). When countering multispecies biofilm, increased EPS production, genetic characteristics (genes related to oxidative stress resistance), biofilm age, and metabolite interference are some of the measures taken by bacteria for the enhanced resistance (Li et al., 2021). In addition to the interspecific interactions between the bacteria, the resistance to the chemical stressors have also been explained by the structural protection (hidden in viscous EPS layers) and spatial distribution in multispecies bacteria (Z. Zhu, Shan, et al., 2020).

8. Conclusion

In many multispecies biofilm formations, the pre-conditioning of the surface by the primary colonizer is a reality which has rarely been considered in interaction studies. In conclusion, the colonization sequence impacts the attachment and biofilm formation ultimately affecting the interspecies interactions and its response to antimicrobial agents. On the other hand, the spatial arrangement of multi-species bacteria was found to be independent of the order of colonization and is mostly dependent on the motility, and the metabolic requirements of the bacteria. This finding allows us to explore the biocontrol strategies that can keep the pathogens at bay as well as the interference that needs to be done before the keystone antimicrobial-resistant bacteria are cemented on the surface. Further research needs to be done to understand how this priority-based assembly of the biofilm impacts functional properties of multispecies biofilm relevant to food surfaces.

CRediT authorship contribution statement

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

Data availability

No data was used for the research described in the article.

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