

## Review Article: Multispecies Biofilms an Overview

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### ABSTRACT

Microorganisms frequently form structured communities enclosed within copious amounts of extracellular polymeric substance (EPS), known as biofilms. These biofilm formations are widespread and can be found on surfaces such as plants, soil, the human body, and medical implants. In natural settings, biofilms consist of multiple species, and these multi-species biofilms (MSBs) are known to enhance resistance against physical and chemical challenges. Additionally, MSBs exhibit greater biomass due to synergistic interactions compared to their single-species counterparts. The development of MSBs involves several stages, including initial colonization, incorporation into EPS, attachment of subsequent species to pioneers, and eventual biofilm dispersal as the biofilm matures. Cooperation and competition within MSBs play pivotal roles in shaping the overall structure of these microbial communities. Various mechanisms have been proposed to elucidate how MSBs achieve significantly higher antimicrobial resistance levels, often exceeding those of planktonic counterparts by orders of magnitude.

**Keywords-** Biofilm, Multispecies, Coexistence, Cooperation.

## I. INTRODUCTION

Microorganisms typically have a tendency to create organized groups encased within abundant extracellular polymeric substance (EPS) known as biofilms. These formations are widespread and can be found on various surfaces, including plants, soil, the human body, and medical implants, as indicated by (Yannarell et al., 2019). The extracellular polymeric substance (EPS) within biofilms consists of a mixture of components, including polysaccharides, lipids, proteins, and nucleic acids. The EPS serves multiple functions, including adhesion, storing nutrients, and safeguarding against environmental challenges, predators, and antimicrobial substances, as outlined by (Lee et al., 2017). In natural environments, bacteria exist within widespread and intricate biological communities known as biofilms, as highlighted by (Flemming and Wurtz, 2019). It is noteworthy that these biofilms often consist of multiple

species, each possessing distinct characteristics and playing unique physiological roles when compared to their respective single-species biofilms, as discussed by (Peters et al., 2012, Flemming and Wurtz, 2019). It is probable that these multi-species biofilms (MSB) are present across a range of medical implants, potentially giving rise to significant health-related issues and posing economic difficulties, as indicated by (Kvich et al., 2020).

The formation of a multi-species biofilm is a complex but tightly regulated process driven by the interactions among the resident species. These interactions occur in a specific and sequential manner, leading to diverse changes in both the structure and function of the entire biofilm. Ultimately, these interactions enhance the pathogenicity of the species involved, as outlined by (Joshi et al., 2021). As a result of these interactions, the biofilm becomes extremely resilient to traditional therapeutic approaches, calling for innovative and more potent strategies. To develop these

new treatment methods, it is essential to have a deep understanding of how the resident species communicate with each other. Infections involving multi-species biofilms have generated significant concerns, making the treatment of such biofilms a formidable and challenging task, as emphasized by (Karygianni et al., 2020).

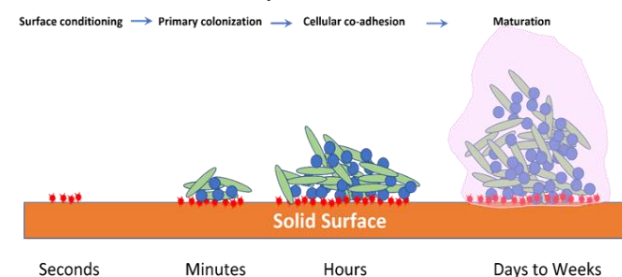
The primary objective of this review is to provide an up-to-date perspective on multi-species biofilms. In subsequent sections of the review, we will offer a concise examination of various aspects, including the phases of multi-species biofilm formation, interactions among different species, the dynamics of competition and collaboration among coexisting species, Infections characterized by multi-species biofilm formations, with a particular emphasis on elucidating the mechanisms that underlie antimicrobial resistance.

### 1.1 Stages of Multispecies Biofilm Formation

Interestingly, the interactions between cells, such as co-adhesion and co-aggregation, play a pivotal role in enhancing cell-cell communication among proximate cells within the identical biofilm, with these interactions effectively influencing the recruitment of planktonic cells into the developing biofilm, as elucidated by (Vinod Kumar et al., 2019). While the intricate process of multi-species biofilm formation is challenging, it can be accurately delineated into three distinct stages, as explicated by (Chugal and Lin, 2016):

1. In the initial stage, pioneers attach themselves to a specific surface, which can be either living (biotic) or non-living (abiotic), and begin to envelop themselves with extracellular polymeric substances (EPS). These EPS consist of various components, including exopolysaccharides, nucleic acids, proteins, and lipids. The colonizers, on the other hand, form microcolonies by utilizing their own EPS. During this phase, highly specific physio-chemical interactions play a pivotal role. It is important to emphasize that pioneer cells have the capacity to exclusively co-aggregate with other pioneer cells, and not with secondary arrivals. Consequently, co-adhesion emerges as a pivotal factor in the initial stages of colonization. Furthermore, the augmentation of extracellular polymeric substance (EPS) production is imperative for the formation of microcolonies, given the role of EPS as a binding agent, holding the newly formed cells together as the biofilm matures. This process was elucidated by (Rickard et al., 2003).
2. Following this, the succeeding colonizers will adhere to the already formed microcolonies and initiate their reproduction.
3. When the biofilm reaches maturity, it undergoes dispersal in response to diverse environmental cues. Consequently, the cells will be released and disperse,

seeking new locations to initiate the formation of fresh biofilms, as discussed by (Salinas and et al., 2020).



**Figure 1: Temporal Evolution of Multi-Species Biofilm Development (Chugal and Lin, 2016)**

### 1.2 Interspecies Interactions in Multispecies Biofilm

Notably, there are four primary methods used by the constituents of multi-species biofilms to maintain communication among themselves:

- 1- Cell-to-cell communication involves physical interactions mediated by ligand-receptor binding, facilitating the exchange of genetic material among distinct bacterial species. These interactions foster synergistic relationships that bolster the stability of the biofilm community, rendering it less susceptible to anti-biofilm agents. Moreover, such cell-to-cell communication assumes a prominent role in the formation of cellular aggregates through collaborative mechanisms, as expounded upon by (Wicaksono et al., 2022).
- 2- Genetic transfer occurs through horizontal gene transfer, utilizing mobile genetic elements, as discussed by (Ma et al., 2021).
- 3- Metabolic collaboration is accomplished through the improvement of adhesion by the matrix secreted by collaborating organisms, or through cross-feeding, where one species utilizes the byproducts of another as a source of nutrients, as outlined by (Yuan et al., 2020).
- 4- Signaling molecules, specifically autoinducers, play a crucial role in regulating the configuration and physiological aspects of the biofilm community, as well as in modulating interactions between different species, as highlighted by (Subramoni et al., 2021).

Through intricate molecular processes, the mentioned interactions and communications between different species can give rise to social dynamics that may be competitive, cooperative, or neutral in nature. However, it is important to note that competitive and cooperative behaviors can significantly shape the structure and physiological aspects of the evolving multi-species community, as explained by (Jara et al., 2022).

### 1.3 Coexistence and Cooperation

During the growth and maturation of multi-species biofilms, numerous intricate interactions take place, as observed by (Fan and et al., 2020). A noteworthy facet of these interactions resides in the competition for resources and physical space. Microbial cells within the biofilm consortium partake in a dynamic interplay of both cooperative and competitive behaviors, driven by their pursuit of enhanced overall fitness. Some bacterial

species produce organic acids, H<sub>2</sub>O<sub>2</sub>, and bacteriocins, gaining a competitive advantage over other species within the multi-species biofilm, as discussed by (Jara et al., 2022).

In multi-species biofilms, the collaborative behavior of bacterial species is facilitated through synergistic interactions. These interactions govern the modulation of gene expression and cellular responses, affording each species the capacity to adeptly adapt to the prevailing conditions inherent to the biofilm environment, as elaborated by (Joshi and et al., 2021).

Due to the widespread occurrence of multi-species biofilms (MSBs), it can be inferred that synergistic interactions are more prevalent than antagonistic interactions in terms of coexistence, as suggested by (Zupancic et al., 2018). Interestingly, some bacterial species lack the capability to form biofilms when alone; however, they can participate in the creation of multi-species biofilms. Notably, oral bacteria are known to form multi-species biofilms through interspecies communication, with each species having its specific role. As an illustration, *Enterococcus faecalis* plays a role in safeguarding and fostering the growth of *Porphyromonas gingivalis* within a multispecies biofilm (MSB) model, culminating in a notably increased biofilm thickness irrespective of oxygen availability. Nevertheless, the elevation in protein content is solely observed when oxygen is present, as documented by (Tan and et al., 2022). The capacity for co-aggregation is notably contingent upon cell surface attributes, whereby certain oral bacterial species exhibit an ability to recognize the cell wall polysaccharides of streptococci. This recognition event subsequently engenders the formation of co-aggregates, thereby playing a pivotal role in the eventual development of plaque, as elucidated in the discourse presented by (Choo et al., 2021).

It's noteworthy that certain factors are exclusively activated within multi-species biofilms, whereas their expression is reduced in single-species biofilms. Cope et al. (2011), conducted research on rhinosinusitis and found that *Haemophilus influenzae* highly expresses type IV pili when coexisting with *Streptococcus pneumoniae* in a multi-species biofilm. The authors pointed out that these two species collaborate to enhance their adhesion to host epithelial cells, thereby facilitating the formation of a multi-species biofilm community.

When *Pseudomonas fluorescens* and *Lactococcus lactis* coexist within a single biofilm, their adhesion increases substantially, by approximately 100-fold and 20,000-fold, respectively. Notably, *L. lactis*, which is not a strong biofilm producer on its own, leverages the capabilities of *P. fluorescens*, a robust biofilm former, to enhance its own adhesion. *P. fluorescens* quickly produces the biofilm polymeric matrix, boosting *L. lactis*'s adhesive capacity. Concurrently, certain metabolic substances generated by *L. lactis* may serve as nutrients for *P. fluorescens*, as

elucidated by (Yuan and et al., 2020). In a similar vein, *Ralstonia insidiosa* establishes an environment abundant in nutrients within a polymicrobial biofilm, thereby conferring advantages to a microbial consortium comprised of *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella enterica*. Multi-species biofilms frequently attain significantly higher levels of biomass than monospecies biofilms, even in the absence of additional nutrient inputs, as discussed by (Jara et al., 2022).

When *Pseudomonas aeruginosa* releases its virulence factors in close proximity to *Candida albicans*, it can impede the formation of the *Candida* biofilm. Similarly, the substances produced by *Lactobacillus* exhibit bactericidal properties against *Listeria monocytogenes* within multi-species biofilms, as noted by (Rao and et al., 2020).

#### 1.4 Multibacterial Biofilm-Associated Infections

Multispecies biofilms are accountable for approximately 80% of bacterial infections, as reported by (Tytgat et al., 2019). Infections resulting from multi-species biofilms tend to have more severe outcomes compared to those caused by single-species biofilms, as observed by (Jorge et al., 2018). For example, in cystic fibrosis patients, lung deterioration is significantly more pronounced when the lung is infected with a multi-species biofilm consisting of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, as opposed to a single-species biofilm, as indicated by (Limoli and Hoffman, 2019).

Within healthcare facilities, multi-species biofilms (MSBs) serve as the predominant habitat for numerous disease-causing bacteria. In susceptible individuals, the microbial community can be disrupted and overtaken by invading pathogens, transforming the formerly commensal community into a pathogenic multi-species biofilm, as noted by (Tytgat et al., 2019). Importantly, indwelling medical devices are frequently colonized by MSBs, posing significant risks to patients' lives, as highlighted by (Julak et al., 2018).

The overall physiological condition of multi-species biofilms is significantly influenced by interactions between different species, and this, in turn, enhances their virulence. For example, the presence of *C. albicans* in conjunction with *S. aureus* within a shared biofilm elevates the infectivity of *S. aureus*. Notably, the peptidoglycan component present in Gram-positive bacteria serves as a signaling molecule for *P. aeruginosa*, triggering the production of additional virulence factors that both harm the host and exert an influence on the microbial community's composition. Furthermore, co-infection with *E. faecalis* exacerbates pyelonephritis initially triggered by *P. aeruginosa*, as described by (Lee et al., 2017).

#### 1.5 Antimicrobial Resistance of Multispecies Biofilms

A particularly significant and challenging aspect of biofilm management is its increased resistance to antimicrobial agents and the rapid physiological adaptations that occur due to the hostile environment in

and around the biofilm, as highlighted by (Gupta and Ayan, 2019).. Within the biofilm structure, microbial cells are enveloped by extracellular polymeric substance (EPS), which constitutes a substantial portion, typically ranging from 70% to 90%, of the biofilm's overall mass. This EPS plays a crucial role in shielding the enclosed cells from potential eradication by various factors, including external environmental elements such as UV radiation, disinfectants, antibiotics, and other antimicrobial agents, as well as the host's defense mechanisms, as discussed by (Lee et al., 2017).

The cooperative behavior significantly regulates the resistance of multi-species consortia to antimicrobial agents. In addition to the protective function of extracellular polymeric substance (EPS), communication among microbes within multi-species communities plays a crucial role in this resistance. The arrangement of microbial cells from different species in multi-species biofilms is meticulously organized and influenced by the overall fitness of the diverse microbial community. These synergistic interactions are not present in single-species biofilms; however, multi-species communities can establish a mixed structure, as explained by (Yuan et al., 2020).

In multi-species biofilms (MSBs), the arrangement of species tends to follow a layered pattern, wherein one species is located in the lower strata while the other resides in the upper strata. The observed distribution pattern seems to be shaped by the survival strategies adopted by individual species within the multispecies community, a result of competitive or cooperative interactions, as documented by (Nadell and et al., 2016). In a similar Lee and et al. (2017), observed a distinctive spatial distribution within a dual-species biofilm, wherein *Enterococcus faecalis* predominantly inhabited the lower stratum, while *Pseudomonas aeruginosa* was primarily situated in the upper region. The authors elucidated that *E. faecalis* played a stimulating role in prompting the increased production of biofilm matrix polysaccharides (psl and pel) by *P. aeruginosa*, ultimately leading to a thicker extracellular polymeric substance (EPS) when compared to single-species biofilms. This increased thickness contributes to the enhancement of virulence in multi-species biofilms and provides protection against various anti-biofilm agents and external stressors.

## II. MECHANISMS OF RESISTANCE OF MSB

The complete understanding of how multi-species biofilms (MSBs) resist antimicrobial agents remains elusive due to the ongoing changes in the composition of biofilm EPS and microbial communication, as pointed out by (Rao et al., 2020). Nevertheless, some hypotheses have been proposed:

1. Some species within the intricate structure of multi-species biofilms can achieve a form of spatial protection

through their co-aggregation with other species, as suggested by (Yuan et al., 2020). For instance, due to the competitive advantage of *Vibrio parahaemolyticus*, it consistently occupies the upper regions of multi-species biofilms. Consequently, its resistance to antibiotics decreases when it coexists with *Listeria monocytogenes*, as observed by (Chen et al., 2016).

2. The thickness of the extracellular polymeric substance (EPS) is suggested to play a crucial role in the resistance of multi-species biofilms by acting as a barrier that hinders the penetration of antimicrobial agents to the lower layers, as proposed by (Guillonnet al., 2018). Additionally, antimicrobial agents with positive charges can become entrapped within the EPS components, as noted by (Ermolaeva et al., 2015). For example, in a multi-species biofilm involving *S. epidermidis*, the EPS traps fluconazole, thereby shielding *C. albicans* from the drug's action, as described by (Delben et al., 2016)

3. Species residing in the lower regions of multi-species biofilms can impact the overall biofilm physiology through interactions between different species. These changes are demonstrated by the exchange of genetic material related to antimicrobial resistance, as discussed by (Orazi and O'Toole, 2019). For example, in multi-species biofilms, A plasmid bearing a carbapenemase enzyme has the capability to undergo horizontal transfer from *Escherichia coli* to either *Acinetobacter baumannii* or *Pseudomonas aeruginosa*. Importantly, this transfer phenomenon does not manifest when these species exist in a planktonic state, as documented by (Tanner et al., 2017). Similarly, *Pseudomonas aeruginosa* augments the resistance of *Staphylococcus aureus* to *vancomycin*, aminoglycosides, and chloroxylenol through the secretion of 2-heptyl-4-hydroxyquinolone N-oxide, as observed in the study conducted by (Orazi and O'Toole, 2019).

Any of these proposed mechanisms possess the capacity to substantially increase antimicrobial resistance within multispecies biofilms, ranging from 100 to 1000 times greater than their planktonic counterparts, as indicated by (Hoiby et al., 2010). Therefore, disrupting these proposed mechanisms could hold promise as an effective approach for eliminating biofilms in various environmental and disease-related contexts.

## III. CONCLUSION

Multispecies biofilms found in nature play a significant role in enhancing resistance against physical and chemical factors. These biofilms, consisting of multiple species, exhibit increased biomass compared to their corresponding monospecies biofilms due to synergistic communications among the different species. Cooperation and competition within MSBs contribute to shaping the overall structure of the community. Numerous mechanisms have been proposed to explain the exceptionally high levels of antimicrobial resistance observed in MSBs. These findings emphasize the significance of studying and understanding the complex

interactions and dynamics of multispecies biofilms in nature.

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