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Review

Overview of multi-species biofilms in different ecosystems: Wastewater treatment, soil and oral cavity

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ABSTRACT

In various natural ecosystems, bacteria most often live in a sessile state enchased in a self-produced extracellular matrix forming biofilms. Due to their either negative or positive impact on different aspects of our daily life, the number of studies devoted to biofilms is increasing. Most research is based on biofilms formed by a single bacterial species. These simple models allowed the understanding of the mechanisms involved in biofilms formation and regulation. This likewise helped the development of several means to control the biofilms formation. However, these models do not closely mimic the natural biofilms known as biochemically and microbiologically heterogeneous and dynamic structures. For this reason, current studies focus more on multispecies biofilms using complex models to best approximate the natural environment. In this review, we addressed on available examples of multispecies biofilms in different domains to illustrate the complexity and organization of life within a consortium. Finally, we review the most used analytical techniques to study multispecies biofilms highlighting the need of multi-scale strategies to better decipher this complex lifestyle.

1. Introduction

Biofilms are typically defined as a community of microorganisms attached on biotic or abiotic surfaces coated with a self-produced exopolymeric substances (EPS) (Flemming and Wuertz, 2019; Hall-Stoodley et al., 2004). However, some microorganisms do not specially require the physical support for a community lifestyle. Certain can colonize the air-liquid interface and form pellicle (Kovács and Dragoš, 2019). Others are able to aggregate and form granules or flocs particularly in wastewater treatment systems (Kim et al., 2020), but in both cases, EPS are still present. The EPS bind with cells through complex interactions and form a powerful scaffold for microbial communities (More et al., 2014; Sheng et al., 2010). Mostly, EPS consist of polysaccharides, proteins, and smaller quantities of extracellular DNA and lipids. Biofilms are bacteria's privileged lifestyle reflecting their ubiquity (Flemming and Wuertz, 2019). The diversity of microorganisms forming biofilms and EPS composition heterogeneity provides biofilms different properties. Thus, EPS are a protective sheathing against antimicrobial molecules and host immune system, generating thus serious problems in both clinical (Stoica et al., 2017) and industrial fields (Mattila-Sandholm and Wirtanen, 1992; Vishwakarma, 2020). On the other hand, many biofilms are beneficial and they are positively used in bioremediation (Singh et al., 2006), water treatment (Boltz et al., 2017; More et al., 2014) and food field (Turhan et al., 2019). Furthermore, the use of biofilms in industrial applications such as sustainable energy production is emerging as a promising future technology. Indeed, electroactive biofilms are the key players in bioelectrochemical systems involving electrocatalytic reactions mediated by microorganisms (Angelaalincy et al., 2018).

Because of double biofilms influence on our daily life, many studies have been devoted to them. In fact, a NCBI PubMed search using the keywords "biofilm" returns more than 60,000 publications. Most investigations were focused on single-species populations biofilm models facilitating the understanding of the mechanisms of biofilm formation and regulation. It is now accepted that the formation of biofilms involves a series of steps including adhesion of cell to surface, micro-colony formation, EPS production and later biofilm dispersion, releasing planktonic cells to restart the cycle (Beloin and McDougald, 2021; Costerton et al., 1987). The establishment and coordination of all these events are governed by complex regulatory systems. Numerous studies have shown that quorum sensing (QS), a cell–cell communication mechanism that synchronizes genes expression in response to population cell density, is strongly involved in the control of biofilm

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installation, development and dispersion (Li and Tian, 2012; Nadell et al., 2008; Solano et al., 2014). On the other hand, the preponderant role of two-component systems (TCS) in the formation of biofilms was severely highlighted (C. Liu et al., 2019; Stubbendieck and Straight, 2017). TCS are the transduction pathways, based mainly on a phospho-relay between a histidine kinase and a response regulator, allowing bacteria to respond to environment changes (Papon and Stock, 2019). Cross-regulation between different TCS is another strategy used to integrate and coordinate various stimuli to control biofilm formation (C. Liu et al., 2019). More recently, functional relationship between protein post-translational modifications (e. g., Ser-Thr-Tyr phosphorylation (García et al., 2018; Poh et al., 2020), bacterial Lys-acetylation (Reverdy et al., 2018; VanDrisse and Escalante-Semerena, 2019) and biofilm regulation was reported. All this knowledge allowed to characterize and to develop a variety of molecules and strategies to control biofilm formation (e.g., natural biofilm inhibitors, modified biomaterials, interruption of the QS system) (Shahid et al., 2019).

In their natural environment, biofilms are overly diversified microbial consortia which are subjected to complex interactions impacting their spatial organization and biomass production (Burmølle et al., 2014; Rendueles and Ghigo, 2012; Røder et al., 2020; Stewart and Franklin, 2008). Moreover, the development of the natural biofilms is closely affected by versatile environmental stress (Hall-Stoodley et al., 2004). Thus, individual component species models, developed under well-controlled laboratory conditions cannot mimic natural biofilm. Now, focus is increasingly on studying multispecies biofilm communities using a variety of population study tools, including meta-omics approaches and high-resolution imaging (Beloin and McDougald, 2021; Joshi et al., 2021). Consequently, current studies now look at biofilms as multi-species consortia with different levels of microbial diversity (from two to a hundred species) (Tan et al., 2017a). Due to all these specificities, the question of reproducibility for biofilm models must also be asked (Azevedo et al., 2021).

Here, we present a global overview of the available data on the diversity and dynamics of the microbial population within complex biofilm consortia as well as the composition of the EPS matrix. We rely on examples of multi-species biofilms from various ecosystems including wastewater treatment systems, soil and oral cavity. We emphasize the multi-scale technical means and methods for investigating these structurally, biologically, and chemically complex biofilms.

1.1. *Multispecies biofilms in wastewater treatment systems*

The potential of multispecies biofilms is an effective strategy for environmental biotechnology since work is distributed across different individuals in a consortium through mutualistic interactions in a well-defined spatial arrangement. This task partition improves a specialized function by reducing the metabolic load of each population (Mukherjee and Cao, 2021; Røder et al., 2020). Thus, fixed biofilms, suspended sludge flocs and aerobic granular sludges are the most used technologies for wastewater treatment (Boltz et al., 2017; Kim et al., 2020). These distinctive microbial aggregate structures differ on their performance according to the diversity of microbial communities (Winkler et al., 2013; Zhang et al., 2014), EPS composition and their physicochemical characteristics (Adav and Lee, 2008; Seviour et al., 2019; Zhang et al., 2014). However, the molecular mechanisms governing these proprieties are not yet well elucidated and so, the engineering of multispecies biofilms is still in its infancy. Its deployment requires the maintenance of long-term and stable interactions among community members (Johns et al., 2016; Mukherjee and Cao, 2021). Moreover, due to their microbiological diversity and biochemical complexity, the characterization of these biofilms is very laborious and requires the combination of several adapted multi-scale strategies (e.g., meta-omics, microscopy, microfluidic devices).

Mahendran et al., 2012 examined the flocs and biofilms from integrated fixed film activated sludge systems. The distribution of cells and composition of EPS, determined with confocal imaging using different fluorescent labelings, showed a difference in the surface characteristics between the studied aggregates (Mahendran et al., 2012). Thus, the EPS content of flocs was significantly higher and richer on protein fraction and more hydrophobic than in the biofilm counterpart. Regarding the microbial distribution, nitrifiers and denitrifiers were predominantly associated with the biofilms, and the latter were found to be dispersed throughout the biofilm suggesting partial oxygen penetration. Recently, Zang et al., 2015, conducted an overall comparative investigation on fixed-biofilm and suspended sludge aggregate (S-sludge) in an integrated fixed-film and suspended growth sequencing batch reactor. The evaluation of morphologies, microbial communities, extracellular proteins and polysaccharides variation between the two lifestyles provided crucial elements for elucidate the diversity of aggregated architectures (Zhang et al., 2015). The environmental scanning electron microscope imaging showed that the microorganisms had much stronger aggregation capacity in the biofilm. In suspended sludge flocs, the EPS network presented abundant porosity. These observations reveal the flexible and loose aggregate structure of the suspended sludge. The 16 S rRNA gene amplification and sequencing investigation showed the dissimilar microbial communities: the dominant genera were *Gemmatimonas*, *Nitrosomonas, Thermomonas* and *Truepera* in the suspended sludge, and were *Nitrosomonas*, *Opitutus*, *Nitrospira* and *Truepera* in the biofilm. The authors highlighted the extensive denitrification in S-sludge compared to biofilm. Interestingly, the large-scale analysis of EPS proteins produced by dominant phyla of microbial communities reveals the presence of adhesion proteins in biofilm, and catalytic proteins in suspended sludge which could decrease their compactness. Concerning the saccharide EPS fraction, the qualitative Fourier Transform Infrared (FT-IR) spectra of exopolysaccharides from the two samples were similar, whereas their monosaccharide compositions were various.

The microbial aggregation is strongly influenced by environmental conditions. For example, several studies reveal the dynamics of microbial population, proprieties and performance of aerobic granular sludge under salt stress (Bassin et al., 2011; Pronk et al., 2014). Metagenomic analysis showed that *Sphingobium*, *Cythophaga* and *Comamona* were the most abundant genera at low salinity, while *Devosia*, *Sphingomonas*, and *Muricauda* were the most abundant genera at high salinity (Ramos et al., 2015). Nevertheless, the response mechanism of aerobic granular sludges to salt stress has not been fully elucidated. Wang et al., 2018 (Wang et al., 2017), performed, for the first time, a metaproteomic analysis for investigating proteomic profiles of aerobic granules sludges under salt stress. Specifically, the authors highlighted the importance of porin and periplasmic-binding protein in high-salt tolerance indicating high activity of transmembrane transporters under salt stress.

Membrane bioreactor technology, combining activated sludge process and membrane filtration, is a promising method for wastewater treatment (Le-Clech et al., 2006). Despite the performance of the system (e.g., less sludge production, improved nitrogen nitrification, reduced requirement for disinfection of the treated wastewater), membrane fouling is a serious problem hindering their universal application (Banti et al., 2017; Maddela et al., 2019). Membrane fouling is principally caused by microbial deposition/growth and accumulation of the microbial soluble products or EPS on membranes (Kampouris et al., 2018; Wu and Fane, 2012). Consequently, several antifouling strategies using physical cleaning or antibacterial compounds were developed, but their ecotoxicity hampered their application (Grandclément et al., 2015). Since the importance of QS was reported in wastewater treatment systems, its disruption (Quorum Quenching system (QQ) may be a powerful way to control membrane biofouling (Feng et al., 2013; Paluch et al., 2020). Thus, various QQ associated-molecules inhibiting, or degrading QS mediators were recently characterized (Delago et al., 2016; Kim et al., 2014; Lade et al., 2014; Rampioni et al., 2014). However, this strategy is confronted to the purification cost and instability of QQ molecules (Ergön-Can et al., 2017). Interestingly, several indigenous bacteria which naturally exerted QQ (bacteria-QQ) were isolated from

wastewater treatment-associated plants and seems to be promising tool for biofouling control (Kampouris et al., 2018; Kim et al., 2014). For instance, Kim et al. (2013) (Kim et al., 2013), reported that the use of the entrapping bacteria- QQ (*Rhodococcus* sp. BH4) into alginate beads shown to be efficient for biofouling control in membrane bioreactor. In fact, microbial cells generated fewer EPS and thus formed a loosely bound and easily removed biofilm from the membrane surface. Therefore, the bacterial QQ worth investigating further concerning the to reach a full-scale application stage in the wastewater treatment sector in nearest future (Ahmed et al., 2020).

1.2. Multispecies biofilms in soil

The soil is an environment that harbours a myriad of microorganisms, most of which living in biofilm mode (Flemming and Wuertz, 2019). Soil biofilms are composed by complex microbial consortia attached to soil particles, roots, fungal hyphae or decomposing organic materials and secreted EPS to form aggregates (Burmølle et al., 2012; Cai et al., 2019; Wu et al., 2019). In the soil, biofilms represent a hotspot where robust biogeochemical processes and intensive interactions take place (Kuzyakov and Blagodatskaya, 2015). Otherwise, microbial EPS are known for their importance on improving soil aggregation, moisture maintenance, and nutrient trapping (Costa et al., 2018; Zethof et al., 2020). Thus, plant or soil inoculation with EPS producers (i.e., *Pseudomonas* species) (Cipriano et al., 2016), addition of pure EPS to soil (Chang and Cho, 2012), modulating of EPS production in soil (Wu et al., 2019) are promising strategies for agricultural practices aimed to improving soil quality (Bhardwaj et al., 2021).

Despite their undoubted importance, current knowledge of soil biofilms is incredibly limited. Indeed, their characterization is a real challenge owing to (i) spatial heterogeneity within any single soil aggregate (ii) the soil matrix opacity making microscopy imaging almost impossible (iii) the complex biochemical composition of EPS (Cai et al., 2019; Costa et al., 2018). Accordingly, it is therefore advantageous to build biofilm models from sampled soil consortia (Aleklett et al., 2017; Ren et al., 2014). Recently, a soil biofilm model composed on consortium of four species (i. e., Sttenotrophomonas *rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans* and *Paenibacillus amylolyticus*) was used to investigate the prevalence of synergistic effects into the bacterial community in batch or continuous flow cultivation systems (Herschend et al., 2017; Liu et al., 2017; Ren et al., 2015). Ren et al. (2015) revealed a greater biofilm biomass production in the four species co-culture than in biofilm formed by a single species culture. This study reveals that *X. retroflexus* is the only specie out of the four, able to form abundant monospecies biofilm and in accordance, was predominant within the four-species consortium. However, in the multispecies biofilm, the number of all strains were enhanced when compared with those of single-species biofilms (Ren et al., 2015). The authors reported that each species benefits from inclusion in the multispecies community and each is indispensable for the synergistic interaction within the biofilm. Similar results have also been reported using continuous flow systems (Liu et al., 2017), suggesting the key role of the low abundant species in the spatial organization and stabilization of the function and composition of complex biofilms. Herschend et al. (2017), used a metaproteomic approach to bring molecular elements to better elucidate these complex network regulations including competitive and/or facilitative interactions (Herschend et al., 2017). Thus, metaproteomic profiling in community and single-species biofilms were compared. For instance, the opposite pathway regulation of branched-chain amino acids metabolism in *P. amylolyticus* and *X. retroflexus* was highlighted. Moreover, *X. retroflexus* showed to decrease this specific amino-acid metabolism when cultivated in consortium biofilm, suggesting that it can cross-feed on the branched-chain amino acids produced by *P. amylolyticus* (Herschend et al., 2017). These studies reflect the overly complex processes and interactions governing the development of simple model multispecies biofilm. Therefore, there is no doubt on the complexity level of the common life organisation in soil aggregate containing a hundred microbial species. Currently, the investigation of soil biofilms is only in an early stage, especially the composition, functions and genetic regulation of EPS, keep staying overly broad.

1.3. Multispecies biofilm in clinical area

Biofilms are a major public health challenge because of the increased resistance of biofilm-associated microorganisms to antimicrobial agents and their ability to modulate the host immune response (Bryers, 2008; Lindsay and von Holy, 2006; Srivastava et al., 2019; Vazquez-Munoz et al., 2020). Microbial biofilm eradication being challenging, promising strategies against microbial biofilms are now developed. while remaining to be clinically evaluated (Zhang et al., 2020). Although most of the available data on clinical biofilms is based on simple monospecies biofilm models, it is widely accepted that biofilms associated with contamination of medical devices and chronic infections are formed with multispecies population. The medical multispecies biofilms that have often been studied are those occurring in oral cavity, wounds, gut, inner ear, ventilator-associated pneumonia and catheter-associated urinary tract infections (Bispo et al., 2015; Eick, 2021; Gabrilska and Rumbaugh, 2015; Paula et al., 2020).

We discuss here the oral biofilms which are complex, dynamic and structurally heterogeneous environments closely related to periodontal diseases (Socransky and Haffajee, 2005) and peri-implant infections (Berger et al., 2018; Koldsland et al., 2009). So far, they are the mostly investigated multispecies biofilms associated with the human body. However, the direct studying of the natural oral biofilms is hampered by several difficulties including i) species variability of human subjects ii) continuous access to samples over time iii) small sample sizes and complicated ethical issues, imposing the use of in vitro consortia biofilm models (Edlund et al., 2013). Different models are currently available (Thurnheer and Paqué, 2021) and each one takes care to get as close as possible to oral cavity environment: Model combining 2–10 bacteria species (Dong et al., 2020; Foster and Kolenbrander, 2004; Guggenheim et al., 2001; Kreth et al., 2005; Sánchez et al., 2011), model using of saliva-conditioned flow cell and or serum supplement (Foster and Kolenbrander, 2004), model containing dental implant material such as titanium (Kommerein et al., 2018, 2017) or hydroxyapatite as mimicking dental tissues (Bao et al., 2015), model under static (Ammann et al., 2012) or dynamic conditions (Paramonova et al., 2009; Schlafer et al., 2012), mathematical model modelling biofilm dynamics (Jing et al., 2019). Breakthroughs of the omics methods (i.e., genomics, transcriptomics, and proteomics) and microscopy have certainly advanced the deciphering of cooperation, competition interactions and cross talk between the community individuals and their spatiotemporal organisation. Edlund et al. (2018), combined metagenomics and metatranscriptomics approaches to address the dynamic of the population and its functional evolution within a saliva derived biofilm model. They reveal that community taxonomy changed according to the pH decreasing due to fermentation of carbohydrates. In this condition, thousands of genes were differentially expressed affecting various metabolic pathways and revealed several mechanisms behind the growth of cariogenic species (i.e., *Lactobacillus fermentum*) (Edlund et al., 2018). Bao et al. (2015) used the quantitative metaproteomic strategy to elucidate the impact of the pathogenic *Aggregatibacter actinomycetemcomitans* on a 10-species biofilm model. They reported the differential expression for 483 proteins, principally involved in metabolic rate, the ferric iron-binding, and the 5S RNA binding capacities, in the community in presence or absence of the pathogen. The impact of this pathogen is accentuated on *Prevotella intermedia* since all its quantified proteins were up regulated. However, *A. actinomycetemcomitans* did not affect the numbers of the other 9 species in the biofilm. A similar study was also conducted to understand the effect of another pathogenic bacteria (*Anaeroglobus geminatus*) on the same biofilm model (Bao et al., 2015). The authors supported that *A. geminatus* caused a significant

increase in *P. intermedia* numbers and causes quantitative proteomic shifts commensurate with increased virulence properties.

1.4. multispecies biofilms investigation methods and associated challenges

The diversity and dynamics of microbial populations, the complexity of their interactions and the heterogeneity of the biochemical composition of EPS on the other hand, makes the study particularly challenging (Beloin and McDougald, 2021).

2. Multispecies biofilms modelling

Although the study of multispecies biofilms directly in their natural environment is crucial, it is confronted to many difficulties including access to the sample (i.e., sampling in the human body or water treatment-associated plants), the low quantity of the sample and its intrinsic properties (i.e., presence of contaminants, soil opacity preventing direct imaging) (Cai et al., 2019; Edlund et al., 2013; Fish et al., 2015). Therefore, most of the fundamental available data on multispecies biofilms are based on models designed under laboratory or engineering conditions, which have advantages and limitations specifically (Azeredo et al., 2017). These models are designed considering (i) the reproducibility and low-cost of the system (ii) the ease in repeatability and flexibility to modify conditions (iii) the maintenance of stable and enduring interactions within the community iv) the faithful simulation of the natural environment (Gabrilska and Rumbaugh, 2015; Mukherjee and Cao, 2021; Tan et al., 2017b). However, this latter is the most difficult parameter to control, since it is not always easy to reproduce what is happening in nature. For example, it is incredibly challenging to establish growth media and conditions to satisfy all the community members (Gabrilska and Rumbaugh, 2015). Their co-culture is therefore almost impossible. The obvious drawback of biofilm models is perfectly illustrated in the medical field by their lack of the host immune response, hence the obligation to develop in vivo models (Dalton et al., 2011; Rumbaugh and Carty, 2011).

3. Microbial community investigation

The advances in imaging approaches helped the establishment of the spatial cartography of complex biofilms. Fluorescence in situ hybridization (FISH) combined with epifluorescence and confocal laser scanning microscopy (CLSM) or with Combinatorial Labeling and Spectral Imaging (CLASI) is a widely used technique to locate and trace individual cells within the multispecies biofilm (Almeida et al., 2011; Karygianni et al., 2012; Lopes et al., 2018; Shi et al., 2020; Valm et al., 2012). In addition, with multiplex FISH strategy it is possible to visualise many different species simultaneously (Allkja and Azevedo, 2021; Kreth et al., 2020). This strategy can be also combined with microfluidic systems to follow the dynamics of the population over time within dynamic multispecies biofilm model (Massalha et al., 2017; Pousti et al., 2018). The Environmental Scanning electron image (ESEM) is another tool allowing biofilm analysis with preservation of the integrity of biofilm in its natural state without any pre-treatment. Therefore, it provides information about the spatial structure and detects the presence of EPS (Bridier et al., 2013; Zhang et al., 2015). Thus, all these imaging-based strategies provide both elements of organization and the evolution of consortia members (Letham and Bharat, 2020; Relucenti et al., 2021). However, the deciphering of these complex microbial maps required supplementary information on the physiological states and activities of community members.

Different molecular techniques have been used for the identification of bacterial members of biofilm consortia. The 16S rRNA gene amplicon sequencing has provided great insight into diversity and phylogenetic affiliation of species in multispecies biofilms (Armougom et al., 2009; Faust and Raes, 2012; Urakawa et al., 1999; Zhang et al., 2015). The denaturing gradient gel electrophoresis (DGGE) method was often employed to separate PCR-amplified community 16S rRNA (Araya et al., 2003; Welsh and McLean, 2007). The real-time PCR (qPCR) assays has been frequently applied to enumerate biofilm's viable organisms and quantify individual members in mixed-species biofilms (Azeredo et al., 2017; Khan et al., 2013; Kommerein et al., 2018; Ren et al., 2015).

Owing to the microbial diversity of multispecies biofilms, the use of the large-scale meta-omics approach including meta-genomic, meta-transcriptomic, and meta-proteomic is certainly valuable. The meta-genomic description of several multispecies biofilms is currently known thanks to analysis of the extracting DNA from biofilm's community (Schmeisser et al., 2007). Although the meta-genomic approach is a powerful tool for studying and exploiting the microbial diversity (Hemdan et al., 2021), it is mainly limited for the lack of correlation between the presence of a functional gene and the actual activity of that gene (Terrón-González et al., 2014). Thus, alone, this approach cannot provide complete information on the functional involvement of community's individuals.

Unlike meta-genomic approach, meta-transcriptomic method can provide information about differences in the active functions of microbial communities. Thus, it is severally used, singly or coupled with meta-genomic analysis, to study the diversity of active genes within multispecies biofilms and to quantify their expression under different conditions (Edlund et al., 2018; Hua et al., 2014; Ishii et al., 2015; Nakamura et al., 2016). Interestingly, combinatorial mRNA labeling and sequential FISH (seqFISH) allowed to highlight the microscale organization of microbial populations and communities (Chen et al., 2015; Dar et al., 2021; Eng et al., 2019). Indeed, this targeted transcriptome-imaging approach records both gene-expression and spatial context within microscale assemblies at a single-cell and molecule resolution (Dar et al., 2021). To investigate the bacterial profiles of communities in biofilm, next generation sequencing (NGS) can be used. In fact, this approach, which targets the amplicon of the bacterial 16 S rRNA gene, whose sequencing of different 16 S rRNA variable regions provides a sufficiently clear insight into the composition of the community and the acquisition of complete genetic sequences from uncultured microorganisms (Garza and Dutilh, 2015; Hemdan et al., 2019; Luo et al., 2022; Rawat and Joshi, 2019).

Comparing to genes and the corresponding mRNAs, proteins reflect the real functionality with respect to metabolic reactions and regulatory cascades and give more direct information about microbial activity (Z. Liu et al., 2019; Wilmes and Bond, 2006). Thus, meta-proteomic is a widely used tool for exploring the biological functions of the population within multispecies biofilms (Barr et al., 2016; Herschend et al., 2017; Leary et al., 2014; Ram et al., 2005). In addition, it can help identify the metabolic dynamics between and within species (Tartaglia et al., 2020). Quantitative meta-proteomics approach allows comparison between protein profiles under different conditions and thus emphasizing new molecular actors involved in biofilm formation (Bao et al., 2018, 2015; Wang et al., 2017). However, it still faces technical and computational obstacles at different levels of analysis. In fact, the protein identification is limited by the lack of complete protein databases. Ideally, the meta-proteomic investigation may be based on the in-house databases provided on metagenomics data previously obtained from the same environment (Chiapello et al., 2020). In addition, the least abundant proteins are mostly drowned among the most abundant one, making the analysis of the proteins from the sub-populations exceedingly difficult (Herschend et al., 2017; VerBerkmoes et al., 2009).

4. Characterization of EPS in multispecies biofilms

The exploration of the multispecies biofilm matrix remains marginal. This is surely due to the biochemical complexity of the EPS and the difficulty of its extraction (Azeredo et al., 2017; Karygianni et al., 2020; Sheng et al., 2010). Indeed, despite the diversity of chemical (i.e., ethanol, formaldehyde, formamide, NaOH, EDTA) and physical (i.e., ultrasound, high speed centrifugation, cation exchange resin) extraction

techniques, there is no universal extraction protocol of EPS (Cheah and Chan, 2022; Pan et al., 2016; Sheng et al., 2010). There is also no known strategy to be the better and as a consequence, combined multidisciplinary techniques are recommended to study EPS. It is therefore advisable to choose a technique or to combine several one according to the properties of the considered EPS (Azeredo et al., 2017; Pan et al., 2016). However, the cation exchange resin method has become the most popular approach for EPS extraction in multispecies biofilms (Frølund et al., 1996; Wang et al., 2019; Zhang et al., 2015). Its high effectiveness is reflected by the low cell lysis, the maintaining of EPS chemical structure and the high extraction efficiency for proteins (Redmile-Gordon et al., 2014).

The matrix proteins could be characterized by a proteomic approach (Zhang et al., 2015). This enables to bring additional elements to apprehend the mechanism multispecies biofilm formation, the better deciphering of community member interactions and the characterization of interest molecules such as enzymes. The carbohydrate fraction could be analyzed using multiple qualitative and quantitative analytical techniques, most often gas chromatography–mass spectrometry (GC-MS) and Fourier transform infrared spectroscopy (FT-IR), from hydrolyzed polysaccharides to obtain a carbohydrate fingerprint of biofilm EPS (Allen et al., 2004; Dignac et al., 1998; Ortega-Morales et al., 2007; Zhang et al., 2015). The FISH-CLSM is a powerful tool for the in-situ characterization of EPS. Indeed, it provide the strong information about spatial distribution of EPS and its content (exoproteins, exopolysaccharides, eDNA) within the not destroyed biofilm (Andersson et al., 2008; Fish et al., 2015; Karygianni et al., 2020; Schlafer and Meyer, 2017).

5. Conclusion

Since the awareness of the multispecies biofilm's omnipresence, the investigations aimed to unravel the mystery of microbial community life keep increasing in different fields. Technological advances have multiplied and refined study tools of biofilms. This enabled to deepen the knowledge concerning the diversity of microorganisms, the dynamics of their evolution, their spatial distribution and their interactions within multispecies biofilms. Current data allow to understand that ultimately life within a multispecies biofilm is like human life, with births, deaths, friends, neighbors, enemies, moves, communication, cooperation and competition. However, the study of multispecies biofilms is in its infancy and there is still a long way to go to answer some questions. It is not enough to know what microorganisms are present within multispecies biofilms, and which are dominant, but it is essential to know which ones arrived first? Which and how ones invite, help, or kill the others? How are the tasks distributed? Which metabolic pathways are turned on, and how are they regulated? To address these questions, future works must combine several multidisciplinary strategies including high resolution microscopy, molecular biology, biochemical and physical classical techniques, and large-scale omics methods. Particularly, proteomics is a powerful tool to investigate post-translational modifications which will highlight the regulatory pathways involved in multispecies biofilms regulation. New technologies targeting single cell within community will provide valuable elements to better understand the microbial behavior within multispecies biofilms. The in-situ investigation of biofilms, without prior destruction, has to be encouraged since it provides a global view of both microorganisms and the matrix. Further investigations should focus on chemical composition, mechanical properties, structural organization, dynamics of regulation, and remodeling of matrix formation during biofilm cycle life. Current studies of multispecies biofilms are often focused on bacteria, so a further look at the role of fungi, yeasts, or algae would be needed. To obtain a real image, it would be interesting to emphasize, when possible, the exploration of biofilms directly coming from their natural environments. When biofilm modeling is required, the choice of the model is essential to get as close as possible to real natural conditions. In this sense, microfluidic systems, bioengineering reactors and in vivo models are promising tools. Each of these strategies brings an additional piece to the puzzle. The complementarity of these techniques will finally make it possible to reconstruct the entire complex multispecies biofilms map and to decipher the molecular mechanisms involved in their formation and regulation which will offer more means to control their formation.

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.

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