

Review

Mimicking biofilm formation and development: Recent progress in *in vitro* and *in vivo* biofilm models

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SUMMARY

Biofilm formation in living organisms is associated to tissue and implant infections, and it has also been linked to the contribution of antibiotic resistance. Thus, understanding biofilm development and being able to mimic such processes is vital for the successful development of antibiofilm treatments and therapies. Several decades of research have contributed to building the foundation for developing *in vitro* and *in vivo* biofilm models. However, no such thing as an “all fit” *in vitro* or *in vivo* biofilm models is currently available. In this review, in addition to presenting an updated overview of biofilm formation, we critically revise recent approaches for the improvement of *in vitro* and *in vivo* biofilm models.

INTRODUCTION

Bacteria may seem simple entities when compared with eukaryotic organisms. However, their molecular machinery allows them to become rapidly adapted to a variety of environmental conditions. For instance, the ability of bacteria to modulate gene expression allows them to finely tune their growth rate depending on nutrient availability (Planson et al., 2020). Bacteria can also shift from an individual and free-floating (planktonic) state to a community-like condition where they improve their ability to survive in harsh environments. This self-organized arrangement, known as biofilm, consists of a three-dimensional (3D) microbial structure with cells enclosed within a self-produced extracellular matrix that may be attached to a substratum (Costerton et al., 1999). Bacteria may also autoaggregate or coaggregate to form biofilms in pure or mixed cultures, respectively, if no solid substrate is available for their attachment (Bjarnsholt et al., 2009; Alhede et al., 2011). Biofilm bacteria exhibit distinct metabolism and gene expression than their planktonic counterparts and present an altered phenotype with increased tolerance to host immune defense mechanisms and exogenously administered antimicrobial substances (Nickel et al., 1985). For instance, sessile bacteria are between 10 and 10,000 times more resistant to antimicrobial agents in comparison to bacteria in planktonic state (Olson et al., 2002a; Luppens et al., 2002; Mah and O'Toole, 2001; Nickel et al., 1985).

Biofilms have been present on earth for around 3.4 billion years (Hall-Stoodley et al., 2004), performing important roles in biogeochemical cycling processes (Paerl and Pinckney, 1996). Biofilms can colonize a great variety of abiotic and biotic surfaces (Yin et al., 2019; Hall-Stoodley et al., 2004). The ideal biotic environment for bacteria to thrive must include a reserve of nutrients, humidity, and an appropriate temperature. Thus, humans are a perfect source of biotic microenvironments for bacterial colonization and biofilm formation, which in most of the cases lead to infectious diseases. The first scientist to describe microbial aggregates in his own dental plaque was Anthony van Leeuwenhoek (1683), and approximately 180 years after, Louis Pasteur (1864) reported bacterial aggregates in wine (Høiby, 2014). Although the term biofilm had been previously used in microbiological and environmental reports, it was in 1985 that J.W. Costerton introduced this term to the field of medical microbiology (Høiby, 2017). Since then, thousands of reports trying to explain the nature, behavior, and potential methods of biofilm eradication have been published. Hence, one may question what has delayed a full understanding of biofilms after more than three centuries of study.

According to the National Institutes of Health, approximately 80% of microbial infections have a biofilm-related etiology (NIH, 2002). In general, biofilm microbial infections can be classified as:

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- (1) Intrinsic to host tissues, and
- (2) Associated with indwelling medical devices (Sun et al., 2013).

The former are mainly chronic opportunistic infections that develop in different tissues and organs, including cystic fibrosis, osteomyelitis, conjunctivitis, vaginitis, urethritis, nonhealing wounds, native valve infectious endocarditis, some pediatric respiratory infections such as otitis media and rhinosinusitis, as well as diverse oral diseases, including caries, periodontitis, halitosis, and gingivitis (Filardo et al., 2019; Lebeaux et al., 2014; Hamilos, 2019). The second type of biofilm-associated infections is characterized by the development of biofilms on medical devices, such as intravascular and urinary catheters, pacemakers, heart valves, contact lenses, breast implants, endotracheal tubes, and orthopedic implants (Zimmerli and Sendi, 2017; Donlan, 2001; Lebeaux et al., 2014; Beloin et al., 2017).

The chronic nature of biofilm-related diseases and their high resistance to antimicrobial substances and antibiotics represent an economical burden for health care systems worldwide. In this comprehensive review, we have highlighted the importance of acknowledging the complexity of biofilm formation and development. This will allow the reader to consider a more conscious selection or creation of the appropriate models to better mimic the native conditions under which biofilms are formed. Such complexity can only be tackled using scientific interdisciplinarity. Thus, this review has been structured for welcoming scientists from different fields of expertise to the field of biofilms. Schematics and brief explanations of fundamental concepts are included in this work to help understand abstract concepts and mechanisms. In the first section, we offer the reader an overall picture of biofilm development by presenting a general overview of the cellular, molecular, mechanical, and physicochemical mechanisms and events involved. One of our goals is to discuss the wide variety of special considerations affecting biofilm formation and development, which are usually underestimated when selecting the experimental models for the study of biofilms and the evaluation of potential antibiofilm strategies. Moreover, we consider that once having a better understanding of such, the reader will become more critical when evaluating the previous and more recently developed *in vitro* and *in vivo* biofilm models (see Sections “*in vitro* models for biofilm assessment” and “*in vivo* models for biofilm assessment”). Finally, a brief discussion of the general advantages and limitations of biofilm models/studies is presented along with a road map that may serve as a guide for the selection of biofilm models.

FUNDAMENTAL CONCEPTS ON BIOFILM DEVELOPMENT

The outer and inner surfaces of the human body are ideal hosts for microbial colonization. However, depending on the site of entry, microbes face important environmental challenges that culminate in stress of these cells. Some of such stressors include cellular and humoral immune defense mechanisms, forces exerted by body fluids, and variability in the access to oxygen and nutrients. To withstand these challenges, bacteria modulate their genetic makeup (for review, see Beitelshes et al., 2018). Hence, biofilm formation arises as a microbial mechanism of defense to ensure survival of bacteria. There is not a single type of biofilm; rather than that, biofilms will depend on the type of microbes forming them, interactions with host immune effectors, and the physicochemical and mechanical properties of the microenvironment. This reflects the level of complexity for the study of biofilms and the development of effective antimicrobials for their eradication.

Biofilms are varied in microbial composition; they can be formed solely by single microorganisms (monospecies biofilms) (Cole et al., 2004; Tolker-Nielsen et al., 2000; Su et al., 2012), by a combination of two or more microbes (multispecies biofilms) from the same and/or different species and strains (Gibbons and Nygaard, 1970; Tolker-Nielsen et al., 2000; Price et al., 2020; Burmølle et al., 2006), or even by the integration of microbes from different taxonomic levels (interkingdom biofilms) (Tchekmedyian et al., 1986; El-Azizi et al., 2004; Adam et al., 2002). The term “polymicrobial biofilm” is often employed to refer to any combination of microorganisms into the biofilm, independently of their phylogeny. Both bacteria and fungi (yeast and filamentous) have the ability to produce biofilms (Costerton et al., 1999; Chandra et al., 2001; Mowat et al., 2009). Despite this high variability, common processes and mechanisms of biofilm development have been found to be shared between species. Hence, although biofilm morphology and physiology vary depending on the initial microbe(s) involved, the microenvironment and interactions occurring (or not) between the microorganisms and a potential surface, the complex series of events that take place during biofilm development are usually broadly grouped to allow a better understanding.

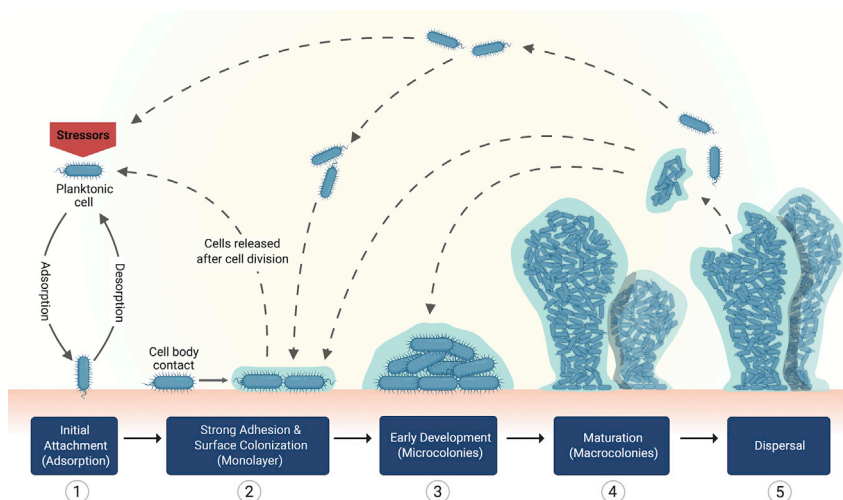


Figure 1. Schematic representation for single bacterial species biofilm formation on a solid surface

The schematic depicts the five main steps for the formation and spreading of biofilms.

It is important to make clear that these are not, by any means, single events following a straight and unique line. A variety of different processes will occur and potentially overlap during biofilm development, where some of them may be exclusive for particular microorganisms and microenvironment conditions. Because of this complexity, and in order to provide an overview for interdisciplinary scientists, we have decided to present biofilm development by introducing such series of events following the traditional model of five main stages. Despite the use of clearly identified and labeled stages of biofilm formation and development, the actual processes occurring under native conditions are far more complex, dynamic, and varied.

Hence, in this review, we use this structure as an overall picture and provide a more detailed discussion in the following subsections to show some of the events and mechanisms involved, as well as the potential consequences of such, to highlight the multifactorial nature of biofilm formation and development. We consider that in order to develop relevant biofilm models, it is important to understand the molecular and cellular events that influence biofilm formation and heterogeneity at each stage, which as a consequence influence their susceptibility to potential antimicrobial strategies.

Since the current knowledge on biofilms has been mostly derived from *in vitro* studies and surface-attached biofilms, where *Pseudomonas aeruginosa* has served as a model microorganism over several years of research, we will mainly focus on surface-related biofilm development. As previously mentioned, and later discussed, biofilms of clinical relevance are also found to be not necessarily attached to a surface. Because of their significance, several research studies on this matter are also briefly discussed throughout this section.

The main stages of bacterial biofilm formation may include the following: (1) adsorption, (2) adhesion, (3) formation of microcolonies, (4) maturation, and (5) dispersal (Figure 1). In general, these stages apply for both bacterial and yeast biofilms (Costerton et al., 1987; Stoodley et al., 2002; Chandra et al., 2001; Blankenship and Mitchell, 2006; Harding et al., 2009; O'Toole et al., 2000). Some authors have proposed to subdivide them to explain biofilm formation by filamentous fungi. Specifically, in this case, the formation of microcolonies considers the germling and/or formation of a monolayer, which leads to mycelial development, hyphal layering, and hyphal bundling (Harding et al., 2009).

Adsorption of bacterial cells to the surface: reversible attachment

Planktonic bacteria move toward a surface by the effect of physical and gravitational forces and by sensing changes in physicochemical properties (Xu et al., 1998; Kimkes and Heinemann, 2020). Biofilms can be formed onto abiotic or biotic surfaces, differing in some of the mechanisms for their anchorage (discussed later). Initially, bacterial cells become adsorbed to a substrate through nonspecific interactions in both abiotic and biotic surfaces (Bos et al., 1999) (Figure 2). These involve a series of attractive and repulsive physicochemical interactions between bacteria and the surface, where Lifshitz-van der Waals forces,

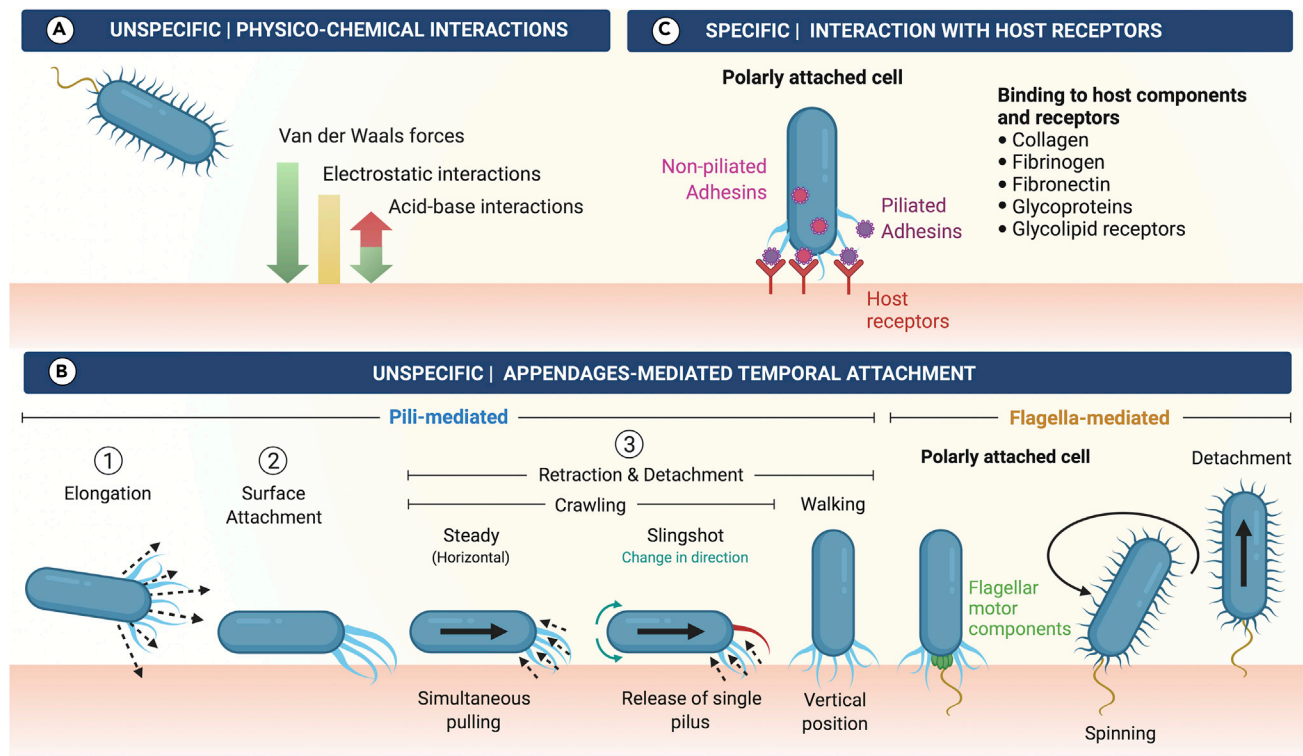


Figure 2. Schematic representation for the main nonspecific and specific interactions between bacteria and surfaces

(A) Some physicochemical interactions include the attractive van der Waals forces; attractive or repulsive electrostatic interactions, which depend on the microenvironment conditions, where the presence of a conditioning film may contribute to reducing repulsion; and the attractive/repulsive acid-base interactions (Kimkes and Heinemann, 2020).

(B: Left side) Pili-mediated temporal attachment. Pili elongation allows attachment to the surface, whereas pili retraction may cause the bacterium to be tugged toward the surface, reach different directions, change from horizontal to vertical (and vice versa) orientations by using different types of motility, or it may be released back.

(B: Right side) Flagella-mediated temporal attachment may be caused because of their hydrophobic nature, as well as by some of the flagellar motor components. When flagella become anchored to the surface, the polarly attached cells spin around, often leading to detachment of bacteria.

(C) Specific temporal attachment may be mediated by binding of adhesins, expressed onto the bacterial surface or at the tip of certain pili appendages, to particular host receptors.

electrostatic interactions, and Lewis acid-base hydrophobic forces are the first to participate (Ren et al., 2018; Bos et al., 1999). Steric forces given by bacterial structures, such as the polymeric brush layer in *P. aeruginosa*, *P. putida*, and *Escherichia coli*, also influence surface interactions (Berne et al., 2018). The net result between attractive and repulsive forces dictates the strength of bacterial adhesion, which is variable depending on the surface, microbial species, and surrounding medium (An and Friedman, 1998). Furthermore, most bacteria have extracellular projections of varied size, structure, and function generally known as bacterial appendages. Apart from influencing cell morphology, these surface-associated filamentous structures gear bacterial cells up to promote locomotion, survival, niche acquisition, and modulation of immune response in the host (Yang et al., 2016). Flagella and pili are two families of bacterial appendages that play main roles during the initial interactions with the target surface.

Flagella can either be found as long helical filament(s) located outside the cell, like in *P. aeruginosa* (O'Toole and Kolter, 1998) or, residing within the periplasmic space (Nakamura and Minamino, 2019) such as in spirochetes like *Borrelia burgdorferi* (Kumar et al., 2017; Sapi et al., 2012). These structures allow bacterial motion toward a gradient of nutrients (chemotaxis-directed motility) (Yang et al., 2016) and other types of motility to achieve surface migration. Pili, on the other hand, are hair-like structures varied in composition, which surround the bacterial cell body to serve as virulence factors during infection (Proft and Baker, 2008). Pili are proteinaceous polymers composed by "pilin" subunits and are also involved in a bacterial locomotion style known as twitching motility (O'Toole and Kolter, 1998; Yang et al., 2016). Pili can be found both in Gram-negative and Gram-positive bacteria (Proft and Baker, 2008; Dramsi et al.,

2006; Abbot et al., 2007). Gram-negative bacteria have five different types of pili where two members of the chaperone-usher pili family, type I and P pili, have an adhesin protein assembled at the tip: FimH and PapG, respectively (Hospenthal et al., 2017; Fronzes et al., 2008; Dodson et al., 2001; Choudhury et al., 1999). The amino-terminal lectin domain of these adhesins serves for the specific interaction of bacteria with host proteins (Hospenthal et al., 2017). Recently, type IV pili, a well-characterized bacterial structure in Gram-negative bacteria (Craig et al., 2004; Craig et al., 2006; O'Toole and Kolter, 1998), has been reported to be also present in a diversity of Gram-positive bacteria having motility and adherence functions as well as a role in biofilm formation (Imam et al., 2011; Varga et al., 2006; Varga et al., 2008).

Apart from the initial nonspecific physicochemical interactions (Figure 2A) and once surpassing the repulsive forces between bacteria and the surface, an unspecific or specific appendages-mediated temporal attachment occurs. The first (Figure 2B) may be mediated by pili polymerization to favor pili extension and attachment, as well as pili depolymerization events, for retraction and detachment from the surface (Merz et al., 2000; O'Toole and Kolter, 1998; Skerker and Berg, 2001; Sun et al., 2000). These mechanisms allow surface exploration by bacteria in horizontal (crawling motility) (Conrad et al., 2011; Merz et al., 2000) and/or vertical positions (walking motility) (Conrad et al., 2011; Gibiansky et al., 2010). It has also been shown that bacteria can change their direction by the release of single pilus (slingshot) while crawling (Jin et al., 2011) or alter their orientation with respect to the surface as a consequence of pili retraction where the cell is reoriented from a horizontal to an upright position, being able to switch between both of them (Conrad et al., 2011; Sangermani et al., 2019).

Flagella are also involved in surface exploration through swimming motility (Conrad et al., 2011), and as these are hydrophobic structures, they tend to adhere to surfaces of the same nature (Friedlander et al., 2015). Moreover, flagellar motor components have been reported to be involved in both the initial attachment and strong attachment stages of biofilm development (Schniederberend et al., 2019; Toutain et al., 2007). Some bacterial cells have been found spinning in a polar position, which suggests attachment of flagella to the substrate (Conrad et al., 2011; Sauer et al., 2002; Toutain et al., 2007), and it generally precedes cell detachment (Conrad et al., 2011). Depending on the function that needs to be achieved, any or a combination of the previous processes is carried out by the bacterium. For instance, in smooth and dry inert materials, such as glass and quartz, an unspecific appendage-mediated temporal attachment would require type IV pili-mediated extension, attachment, and retraction mechanisms, as observed in *P. aeruginosa* (Piepenbrink and Sundberg, 2016; Skerker and Berg, 2001). In the case of abiotic surfaces, like implants and medical devices, the initial bacterial adherence takes place within the formed conditioning film, which may be used by the attached bacteria as a source of nutrients (for review, see Khatoon et al., 2018). Specifically, conditioning films are thin biopolymeric coatings formed at the interface of a material surface and the aqueous medium provided by body fluids and secretions (Donlan, 2002). They can be also formed onto living tissues such as tooth enamel surfaces in the oral cavity (Marsh and Bradshaw, 1995). The presence of conditioning films consequently modifies the physicochemical properties of the surface influencing bacterial adherence (Herrmann et al., 1988; Lorite et al., 2011; Wang et al., 1993). Other factors including roughness, porosity, pore topology, charge, and hydrophobicity/hydrophilicity of the surface also influence bacterial adherence (Feng et al., 2015; Fletcher, 1988; Fletcher and Loeb, 1979; Friedlander et al., 2013; Singh et al., 2011).

Regarding the specific temporal attachment at this stage, bacteria utilize piliated and nonpiliated bacterial adhesins to mediate binding to host components, including extracellular matrix (ECM) proteins, such as collagen, fibrinogen, and fibronectin, as well as glycoproteins and glycolipid receptors (Gries et al., 2020; Lee et al., 1994; Pouttu et al., 1999; Tielker et al., 2005). The initial specific binding is a reversible attachment mechanism employed by bacteria to sense the surface microenvironment. In order to achieve this, bacterial cells use the swimming and twitching motilities provided by flagella and pili to explore and sense the local microenvironment (Berne et al., 2018; Ellison et al., 2017; Merz et al., 2000), transmit signals, and modulate gene expression (Li et al., 2012). For instance, the flagellar rotary motor of *Caulobacter crescentus* has been reported to be involved in surface adherence, where it functions as a mechanosensitive device to promote surface adaptation and anchoring (Hug et al., 2017). Bacteria at this stage are found attached by the cell pole (Agladze et al., 2005; Li et al., 2012), which enables them to be easily detached by retraction and spinning along their axis (Gibiansky et al., 2010; Merz et al., 2000).

Bacterial interactions with surfaces allow them not only to sense the surface but also to become progressively adapted to it by successive attachment-retraction events (Lee et al., 2018; Li et al., 2012). Bacterial

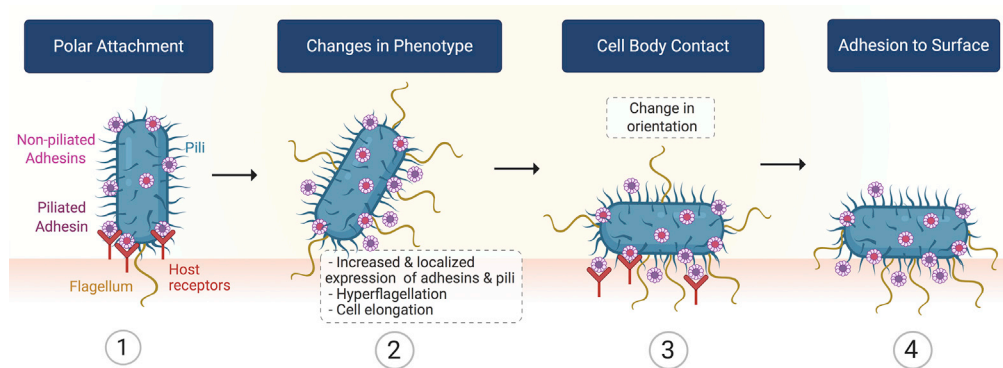


Figure 3. Schematic representation for the functional steps involved in bacteria adhesion to a solid surface

sensing through structural elements (mechanosensing) includes adherence via appendages (Hoffman et al., 2015; Hug et al., 2017), surface proteins (Siriyaporn et al., 2014), piliated adhesins (Thomas et al., 2002), cell wall deformation (Otto and Silhavy, 2002), and stimuli-receptor interactions (Lower et al., 2010).

Bacterial adhesion to the surface: increased adhesion strength

The mechanical cues experienced during surface sensing cause molecular alterations at the biochemical level (mechanotransduction), which gradually increase the attachment strength of the loosely adhered bacterial cells (Hug et al., 2017) (Figure 3). The intracellular signaling driven by mechanotransduction contributes to the production/expression of more adhesins to reinforce adhesion (Hug et al., 2017; Li et al., 2012).

Participation of bacterial appendages can be observed throughout the biofilm development stages. The interplay between pili and flagellum rotation has been reported to encourage the transition from a temporal bacterial attachment toward a stable, often called “irreversible,” state (Li et al., 2012). Particularly, some components of the bacteria flagellar motor contribute to reaching a stronger bacterial adhesion to the surface (Toutain et al., 2007). For recent reviews on bacteria-surface interactions, please refer to Berne et al. (2018) and Kimkes and Heinemann (2020). Furthermore, biofilm-forming bacteria experience changes in phenotype during biofilm development (Sauer et al., 2002; Southey-Pillig et al., 2005). For instance, it has been shown that upon surface sensing, some bacteria such as *Vibrio parahaemolyticus* switch from using a single polar flagellum to mixed flagellation by expressing multiple lateral flagella (Belas and Colwell, 1982). *Proteus mirabilis* bacteria, one of the main species responsible for catheter-associated urinary tract infections (Jacobsen et al., 2008), also show changes in phenotype after contact with solid surfaces such as an increased expression of lateral flagella and bacterial elongation (Fusco et al., 2017; Jones et al., 2004). Lateral flagella play important roles in cell adhesion, invasion, and biofilm formation as reported for *Aeromonas* species biofilms (Gavin et al., 2003; Gavin et al., 2002).

Moreover, together with the bacterial cell wall lipopolysaccharides (Chao and Zhang, 2011) and *de novo* production of proteins (Hinsa et al., 2003), flagella contribute to changes in the orientation of bacterial cells (Gu et al., 2016; Toutain et al., 2007) from a polar attachment to a flat position where the bacterial cell body directly contacts the surface enhancing adhesion strength. However, there are some bacterial species that do not require this change in orientation to reach a strong surface adhesion, as the case of *C. crescentus* which irreversibly attaches in a polar orientation by producing polarly localized adhesins (Li et al., 2012).

Consequences of bacterial adhesion to the surface

The first two stages of biofilm development serve bacteria to sense and evaluate the microenvironment of a potential target for colonization. Depending on the nature of the initial bacterial interactions with the surface, the subsequent mechanotransductive mechanisms determine whether planktonic bacteria will continue into the next stages of biofilm development or not (for review, see Stones and Krachler, 2016). If bacteria become adapted to the surface and microenvironmental conditions, other important alterations at the biochemical level take place. Some of these involve the activation of metabolic processes (Sauer and Camper, 2001), including protective metabolic pathways (Bhomer et al., 2010; Sauer and Camper, 2001),

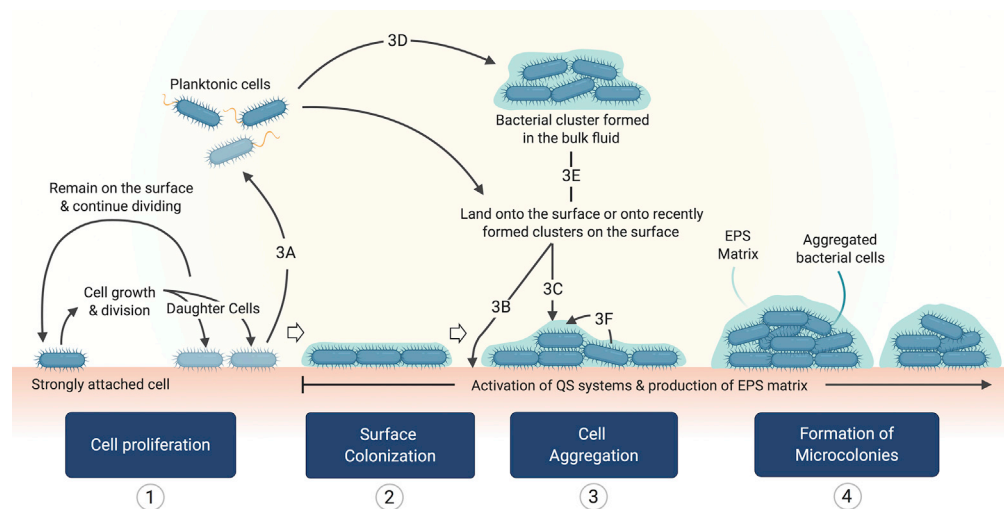


Figure 4. Potential routes for the formation of microcolonies

Microcolony formation arises from the accumulation of cells in continuous growth and division and may be enhanced by the incorporation of planktonic cells from the bulk fluid or product of cell division and the integration of bacterial clusters. (1) Once bacteria become strongly attached, the colonization of the surface takes place by means of cell growth and division. One of the daughter cells may remain attached, and the other may be released from the surface (3A), where it becomes free to colonize other sites (2) by landing onto target surfaces (3B) or, it may become part of recently formed bacterial clusters either on the surface (3C) or the bulk fluid (3D). Bacterial clusters formed in the absence of a solid substrate may colonize new surfaces or land onto biofilms under development (3E). During cell proliferation and biofilm formation, QS signals and production of EPS matrix occur. As cell density increases, some of the bacteria slide along each other (3F) leading to the formation of small bacterial aggregates, which correspond to “immature” biofilms known as microcolonies (4).

regulation of two-component systems (Persat et al., 2015), and upregulation of virulence factors (Gode-Potratz et al., 2011; Persat et al., 2015) to evade and modulate host immune response, being bacterial adhesion a virulence factor per se (Busscher and van der Mei, 2012).

Implicit in the mechanisms required for surface recognition/interactions and acquired properties is the fact that these involve bacterial genetic expression of target genes for better responding to all the challenges and stressors that bacteria are subject to. Part of these events is mediated by the second messenger molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP). This signaling molecule stimulates the biosynthesis of adhesins and extracellular polymeric substance (EPS) matrix components while reducing/inhibiting various forms of motility, contributing to the strong adhesion reached at this stage (for review, see Hengge, 2009). Moreover, at this point, bacteria not only become prepared to mount an adequate response for themselves or neighboring cells during the adsorption and adhesion stages but also has been reported that bacteria are able to “keep track” of these events retaining a multigenerational memory through the oscillations in the levels of the second messenger molecule cyclic adenosine 3',5'-monophosphate (cAMP) and type IV pili activity (Lee et al., 2018). Finally, it has been recently reported that the biofilm forming environmental bacteria *C. crescentus* experience cell differentiation and speed up cell cycle progression after surface sensing (Snyder et al., 2020).

Cell growth and division: formation of microcolonies

Once a strong bacteria-to-surface adhesion has been achieved, attached bacteria grow and divide either by binary fission (Costerton et al., 1995; Read et al., 1989) or asymmetric division (Conrad et al., 2011; Laventie et al., 2019) (Figure 4). This implies cell proliferation and colonization of the surface, which leads to activation of second messengers, intercellular communication, and initial secretion of EPS matrix (Sauer and Camper, 2001). When the “monolayer biofilm” has been formed, any or a combination of three potential mechanisms may follow:

- (1) Additional planktonic bacteria are recruited from the bulk solution via agglutinins, such as *Staphylococcus aureus* surface protein G (Geoghegan et al., 2010) and the aggregative adherence fimbriae in *E. coli* (Nataro et al., 1992);

- (2) One of the planktonic bacteria that resulted from cell division detaches/moves away from the surface and potentially follows any of two pathways: land onto (or become attracted to) a recently formed layer of cells or stay free to initiate the colonization of other sites (Conrad et al., 2011; Laventie et al., 2019);
- (3) The initiation of biofilm formation occurs in the absence of a substrate, and the aggregated bacterial cells then land directly onto the monolayer biofilm or onto a noncolonized surface for further biofilm development (Kragh et al., 2016). The terms “flocks” and “clusters” are used to refer to autoaggregated (same species) or coaggregated (different species) bacteria in suspension. Hence, biofilm formation does not necessarily require a solid surface in order to succeed, and this is observed in infections with biofilm-related etiology, including cystic fibrosis (Bjarnsholt et al., 2009) and rhinosinusitis (Cryer et al., 2004).

Moreover, some reports suggest that cell aggregation mediated by either substrate attachment or occurring substrate free do not exclude each other, and both can occur simultaneously as shown for joint implant-associated infections (Stoodley et al., 2011; Stoodley et al., 2008). A number of agglutinins contribute to cell aggregation depending on the bacterial species, and although they are mainly proteinaceous, such as pili, flagella, M proteins, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and β -barrel proteins; some other molecules such as carbohydrates (for review, see Trunk et al., 2018) and even the extracellular DNA (eDNA) act as agglutinin (Das et al., 2010).

Cellular aggregation by any of the aforementioned mechanisms leads to the formation of bacterial clusters known as microcolonies. As microcolonies are being formed, the initial single layer of attached cells become multilayered by the accumulation of cells, causing an increase in thickness, which leads to the transition from a two-dimensional to a 3D arrangement (Su et al., 2012). In order to reach this spatial distribution, bacteria must communicate chemically with each other and express particular genes for the secretion of an EPS matrix, which will serve as a structural support while also allowing cellular communication for the stabilization of microcolonies and further biofilm network (Dominiak et al., 2011; Koo et al., 2010; Xiao and Koo, 2010).

Furthermore, a role in the formation of microcolonies has been attributed to type IV pili-mediated twitching motility (O'Toole and Kolter, 1998) and the poly-N-acetyl-glucosamine (PNAG) produced by some species, including *S. epidermis* (Gerke et al., 1998) and *E. coli* (Amini et al., 2009). At this stage, microcolonies may be able to move as a whole entity across the surface, as shown by *P. aeruginosa* (O'Toole and Kolter, 1998). The ability of small clusters to displace themselves onto a surface and fuse with each other to form microcolonies has been described by Haagenzen et al. (2015) for *Acinetobacter* sp. strain C6. These representative examples invite us to be cautious and avoid labeling this, and the previous strong adhesion stages as “irreversible attachment” of bacterial cells as this may not be accurate.

Production of EPS matrix

Following adhesion, bacteria secrete an EPS matrix, which contributes in different ways to biofilm development, being particularly important for the formation of microcolonies where it serves as a structural support by filling the space between cells and covering them all over. Hence, the EPS matrix assists in the multilayer arrangement of bacterial cells, acting as an interphase with the surrounding microenvironment (Allison and Sutherland, 1987; Yang et al., 2011). Although the biofilm composition is variable depending on the microorganism(s) involved, biofilm maturation stage, and microenvironmental conditions, most biofilms are constituted by approximately 50%–90% EPS matrix (Frølund et al., 1996; Nielsen et al., 1997). The remaining percentage corresponds to the embedded microorganisms, which, although reduced compared with the EPS matrix, have a cell density that can be considered as high given that biofilm development usually starts from single or few cells and reaches cell numbers of a few thousands, as shown in studies of single-cell tracking (Drescher et al., 2016; Paula et al., 2020).

Molecular components of the EPS matrix. Chemical composition of the EPS matrix varies along with the biofilm development process (Chao and Zhang, 2012), and it is influenced by the composition of the environment, temperature, physical and mechanical stress, and cellular interactions (Flemming et al., 2016). Biofilm EPS matrix is mainly composed of water, which accounts for up to 97% of its content (Zhang et al., 1998). The molecular elements that confer the structural and functional properties of the EPS matrix

are contained in the remaining 3%. Among these, polysaccharides usually represent the major component (Wingender et al., 2001), including exopolysaccharides (such as PNAG (Roux et al., 2015)), lipo-associated and wall-associated teichoic acids (Gross et al., 2001), and cellulose (Zogaj et al., 2001). Proteins are the second major EPS matrix component, although in some cases they have been reported to be present in larger proportion than polysaccharides (Jahn et al., 1999). Other EPS matrix components include eDNA (Whitchurch et al., 2002) and ions (Wang et al., 2019). Also, extracellular proteinaceous bacterial structures such as pili and flagella (van Schaik et al., 2005), together with eDNA (Jennings et al., 2015) and curli (amyloid structures) (Romero et al., 2010), serve as structural elements that contribute to stabilize and strengthen the EPS matrix.

EPS matrix functions. The EPS matrix fills in the space between the microbes forming the biofilm, facilitating cell-cell adhesion (Romero et al., 2010) and bringing cells in close proximity to allow intercellular interactions and horizontal gene transfer (Hausner and Wuerzt, 1999). The rich and heterogeneous infrastructure of the EPS matrix provides the required elements to confer the architecture and mechanical stability of biofilms. Moreover, it shields and protects the inner bacteria from (1) the external microenvironment (Izano et al., 2007; Mulcahy et al., 2008), (2) the host immune response (discussed in the next sections), and (3) protozoan grazing (Matz et al., 2004).

Cell-to-cell communication enhances biofilm development

The effective communication between bacterial cells at this stage is a key factor for the transition from a fairly simple aggregated state into the initial bacterial arrangements, which will further develop into a complex and well-structured community style of living: the biofilm. For this purpose, a cell-to-cell communication mechanism known as quorum sensing (QS) is utilized by bacteria to collectively adapt through the activation/repression of target genes (Fuqua et al., 1994). This communication is mediated by small diffusible or transported molecules, collectively known as autoinducers (AIs), which are continuously being produced by bacteria (Pearson et al., 1999). As cell density increases, AIs accumulate in the surroundings (Figure 5). Once this increase reaches a threshold concentration, often referred to as quorum level, response regulator proteins part of the QS systems become activated by binding to AI molecules, leading to the modulation of gene transcription (for review, see Haque et al., 2019). As a consequence of this intercellular communication mechanism, bacteria become synchronized to express particular QS phenotypic profiles. There are several QS systems that may be shared between different bacterial species or may be specific for certain species in particular (for review, see Haque et al., 2019 and Waters and Bassler, 2005). It has been widely reported that QS contributes to biofilm development, from the formation of microcolonies to biofilm maturation and dispersal (Boles and Horswill, 2008; Davies et al., 1998; de Kievit et al., 2001), mainly by influencing its architecture and promoting a series of beneficial phenotypic traits. Some of the biofilm-associated collective behaviors influenced by QS include the spread of beneficial mutations such as the regulation of EPS matrix production (Sakuragi and Kolter, 2007), modulation of nutrient utilization (An et al., 2014), expression of virulence factors (Erickson et al., 2002; O'Loughlin et al., 2013), synthesis of biosurfactants (Pearson et al., 1997), and regulation of antimicrobial resistance (Shih and Huang, 2002; Zhao et al., 2020).

Biofilm maturation

The regulation of biofilm EPS matrix production by second messenger molecules and QS systems at this stage contributes to reaching a stable biofilm structure (Colvin et al., 2012). After the formation of microcolonies, cell proliferation and aggregation of bacteria continues increasing along with the synthesis of EPS until reaching an optimal cell density, as regulated by QS. At this point, multiple layers form the microcolonies, and these acquire complex structural features through adhesive and disruptive processes (O'Toole et al., 2000). Here, bacterial microcolonies spread out forming larger aggregates that are referred to as "macrocolonies" or "towers" (Gupta et al., 2016; Ha and O'Toole, 2015), causing the biofilm to thicken (Figure 6). Biofilm thickness is variable depending on the species, substrate, time required for maturation, microenvironment conditions such as nutrient availability and shear flow, as well as the methods applied for its determination (Heydorn et al., 2000a; Heydorn et al., 2000b; Janakiraman et al., 2009; Jean et al., 2004; Meyer et al., 2011; Suarez et al., 2019). Most biofilms are found to be multispecies, where each microorganism influences the morphology and architecture of the resulting biofilm; which may be different from monospecies biofilms. For instance, the mean thickness of *in vitro* monospecies biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* was found to be 29 and 100 μm , respectively; whereas a multispecies biofilm composed of both microorganisms reached a mean thickness of 400 μm (Murga et al., 1995). Furthermore,

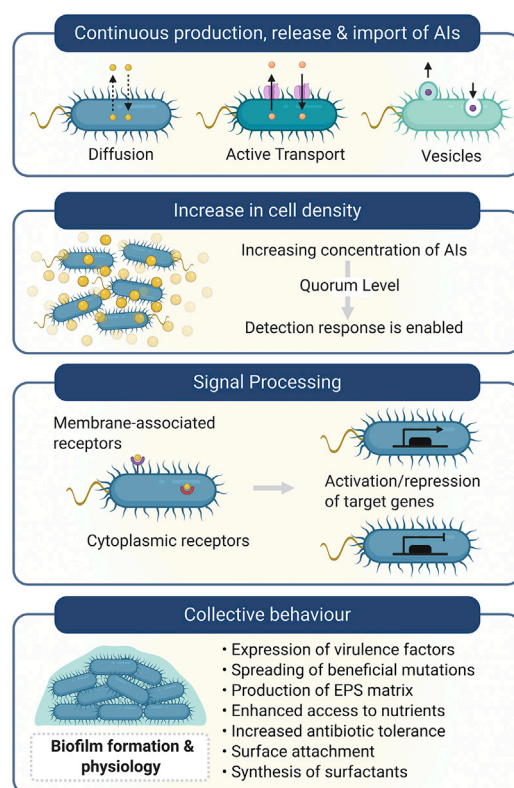


Figure 5. QS influences biofilm formation and physiology

AIs are continuously being produced by bacteria; they constantly move in and out of the cell by diffusion, active transport, or into vesicles, depending on their chemical nature. When the concentration of AIs reaches a threshold level as consequence of increased cell density, the detection response is enabled, and they bound to the corresponding membrane-associated or intracellular receptors, activating these response regulator proteins. As a consequence, signal processing leads to collective transcription of target genes to produce a communal behavior of the bacterial population. One of the potential responses is the expression of a series of beneficial traits involved in biofilm physiology.

most of the time, a mature biofilm is characterized by sessile EPS matrix-enclosed macrocolonies that are separated by a well-defined network of channels.

Biofilm morphology and architecture

Biofilms are highly heterogeneous in their 3D structure, morphology, and physiology (for review, see [Evans et al., 2020](#) and [Stewart and Franklin, 2008](#)). Depending on their chemical and structural composition, their morphology may be smooth and flat, rough, fluffy, or filamentous ([Flemming and Wingender, 2010](#)). Pores, channels for gas and nutrient exchange, and dense areas of packed cells are the main components of the mature biofilm structure ([Lawrence et al., 1991](#); [Robinson et al., 1984](#); [Stoodley et al., 1994](#)). It has been reported that channels within the biofilm are formed by substances secreted by bacteria to degrade and remodel the biofilm EPS matrix. Some of them include nucleases ([Seper et al., 2011](#)) and detergent-like molecules ([Periasamy et al., 2012](#)). Moreover, specialized bacterial subpopulations known as “swimmers” have been reported as capable to swim at high speed within the biofilm leading to the formation of transient pores to increase nutrient flow ([Houry et al., 2012](#)). Biofilm morphology and structural components and arrangements contribute to the formation of mature biofilms in a variety of architectures, from flat and undifferentiated biofilms ([Chung et al., 2014](#)) to the classic tall mushroom-like macrocolonies found in the biofilm model microorganism *P. aeruginosa* ([Klausen et al., 2003](#)). For a recent review on biofilm architecture, please refer to the study by [Kassing and van Hoek \(2020\)](#).

Gradients within the biofilm

Bacterial cells occupy different regions within the biofilm architecture, and as a consequence, their proximity to the external microenvironment, access to nutrients, interactions with the host immune defenses,

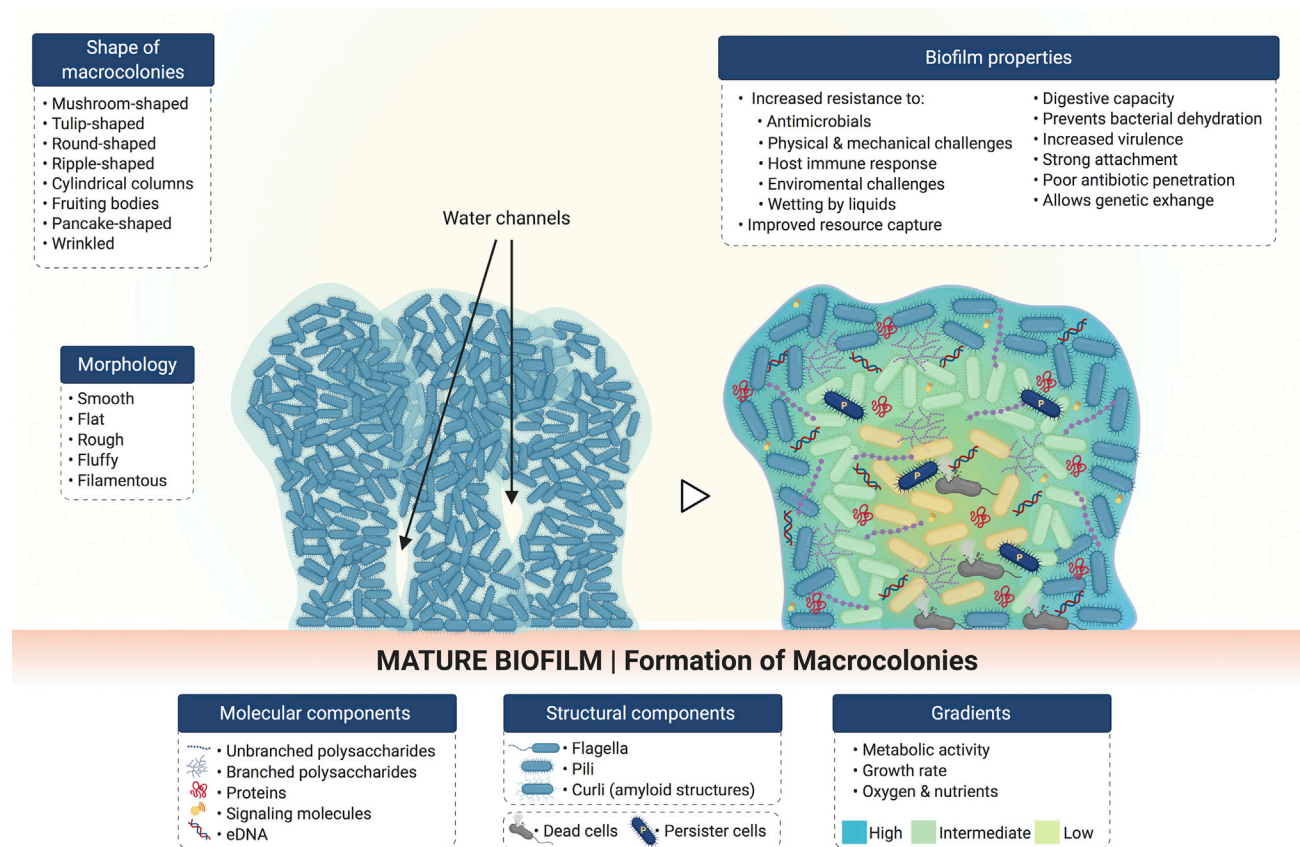


Figure 6. Morphology and architecture of mature biofilms

Molecular and structural components contribute to the 3D spatial organization and physiology of biofilms. Phenotypically different bacteria are found along the gradients observed in mature biofilms.

and the forces they are subject to are variable. Hence, as the microenvironment and requirements are different for bacteria in different regions, the presence of gradients is observed within the biofilm (Figure 6). Some of them include gradients in terms of growth rate (Wentland et al., 1996), metabolic activity (Walters et al., 2003), oxygen concentration (de Beer et al., 1994; Walters et al., 2003), and pH (Hunter and Beveridge, 2005). For instance, bacterial cells in the inner biofilm regions show reduced metabolic activity (Walters et al., 2003), which provides them with the beneficial property of being resistant to conventional antibiotics (for review, see Høiby et al., 2010). Moreover, genetic diversity is found among biofilm-living bacteria, which is observed by the presence of bacterial subpopulations showing different phenotypes (Boles et al., 2004; Vlamakis et al., 2008). A cell type known as “persister cells,” was reported two decades ago in *P. aeruginosa* biofilms (Brooun et al., 2000; Spoering and Lewis, 2001). Persister cells were in the stationary phase of the cell life cycle, assuming a dormant phenotype where they neither grow nor divide, exhibiting a high antibiotic tolerance. The current thought is that such cells may be ubiquitous among bacterial species (Fisher et al., 2017), being able to “survive treatment by all known antimicrobials” (Lewis, 2007). Although persisters show a reduced metabolic activity, they have the ability of becoming active and restarting growth once the stressor has been removed (Roberts and Stewart, 2005). This represents a great advantage not only for antimicrobial resistance and infection chronicity but also further spread and colonization. Furthermore, a second type of dormant cells with increased resistance to antimicrobials has been reported for a wide range of bacteria, with *Staphylococcus* being one of the most studied microorganisms (for review, see Kahl et al., 2016). These cells are referred to as “small colony variants” because of their reduced size: one-tenth of the size of the colonies formed by their wild-type counterparts (Proctor et al., 2006). Small colony variants have been reported to be strong biofilm producers (Malone, 2015) in chronic and implant-related infections (Baddour and Christensen, 1987; Fauvart et al., 2011; Häußler, et al., 2003).

Living in a biofilm community: microbial interactions

Most biofilms are composed by more than one species living in close proximity. As consequence, social interactions arise among them in order to succeed and ensure biofilm formation as a strategy to withstand the effects of different stressors. Microbial interactions in multispecies and interkingdom biofilms have been widely reviewed (Krüger et al., 2019; Shirtliff et al., 2009; Wargo and Hogan, 2006). Here, we present a general classification of microbial interactions and their spatial arrangement within the biofilm rather than discussing particular examples of each one. Microbial interactions in mixed biofilms occur through competition (Fazli et al., 2009), cooperation (Castonguay et al., 2006; Paula et al., 2020), and chemical communication (Riedel et al., 2001), being not mutually exclusive. Taking into consideration that multispecies and interkingdom biofilms are found colonizing a wide variety of environments, it is inviting to assume that although antagonistic interactions occur to some degree during biofilm formation for certain species, synergistic cooperative interactions may predominate (for review, see Elias and Banin, 2012). As previously mentioned, both host microenvironment-bacteria and bacteria-bacteria interactions result in genetic modifications to adapt and respond as required. It has been reported for environmental (Gao et al., 2016) and health-related biofilms (Jakubovics, 2015) that intercellular interactions are responsible for the regulation of genetic expression in a coordinated fashion to modulate not only cell density, antimicrobial resistance, and virulence factors but also the spatial distribution of the species within the biofilm.

Microbial spatial distribution can be explained as three different modes:

- (1) Spatial segregation, where each microorganism produces its own microcolony because of competitive or weakly cooperative interactions resulting in formation of monospecies microcolonies (Kara et al., 2007);
- (2) Spatial intermixing, where both species are found coaggregated as result of strong cooperation, or one microorganism being exploited by the other (Kim et al., 2020); and
- (3) Spatial stratification, where the microorganisms are found forming different layers within the biofilm as a consequence of either strongly cooperative or strongly competitive interactions (Fazli et al., 2009; Kim et al., 2020). Liu et al. (2016) reviewed the influence of microbial interactions on spatial distribution in multispecies biofilms (Liu et al., 2016).

Moreover, the nature of microbial interactions is also dependant on the bacterial timing of colonization. For instance, in a model of dental biofilm, the interactions between *Streptococcus mutans* and *Streptococcus sanguinis* were competitive if one species colonized the surface before the other; whereas both species were able to coexist when there was a simultaneous colonization (Kreth et al., 2005).

Bacterial cells and clusters are dispersed from the biofilm

At some point after reaching maturity, biofilms suffer a partial structural loss that may occur by detachment and/or dispersion (Figure 7). Detachment involves four mechanisms that imply the release or loss of biofilm portions as a consequence of mechanical and shear stress (abrasion, erosion, and sloughing) as well as the impact of immune attack from the host (grazing) (for review, see Petrova and Sauer, 2016). Dispersion, on the other hand, implies more than being torn away from the biofilm by external stressors; it requires the sensing and processing of particular signals, which culminate in the expression of the corresponding physiological alterations (for review, see Rumbaugh and Sauer, 2020).

The inputs that prompt biofilm dispersal (Figure 7) may arise within the biofilm or may be induced in response to alterations in the microenvironment, such as variations in nitric oxide levels (Barraud et al., 2009), oxygen tension (Thormann et al., 2005), temperature (Kaplan and Fine, 2002), and changes in the availability of nutrients (Sauer et al., 2004). Furthermore, the gradients that were initially useful during biofilm differentiation to allow bacterial survival and optimization of available resources now become dispersion cues from within the biofilm. These gradients cause bacteria in the deepest zones to become exhausted because of the stress generated by starvation, hypoxia, scarce signaling molecules, and slow growth. As a consequence, these stressors cause the activation of regulatory mechanisms, including QS (Boles and Horswill, 2008), c-di-GMP (An et al., 2010; Barraud et al., 2009; Gjermansen et al., 2010), small regulatory RNAs (sRNAs) (Chua et al., 2014), cis-2-decenoic acid (Davies and Marques, 2009), and the stringent response mediators guanosine tetraphosphate and guanosine pentaphosphate (collectively known

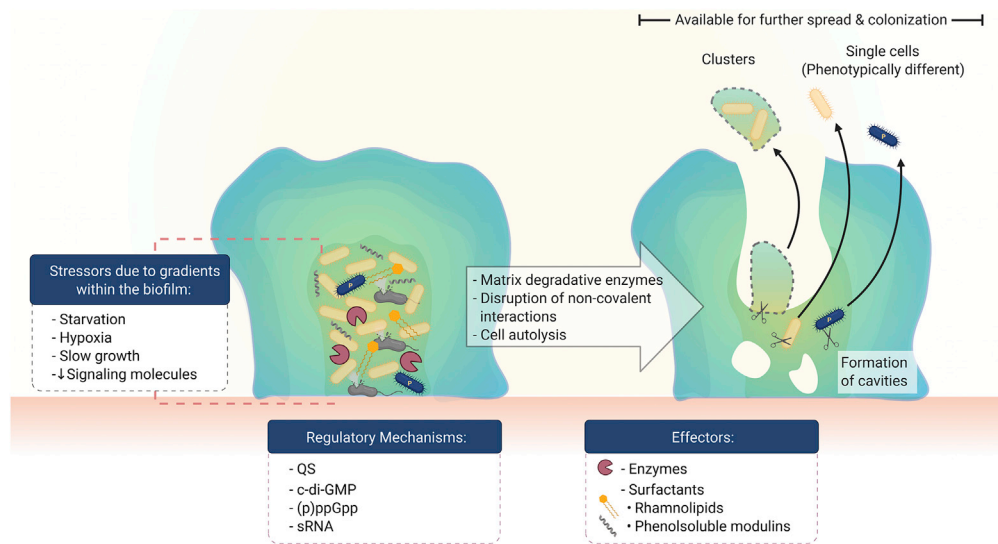


Figure 7. Biofilm dispersal

Bacterial regulatory mechanisms drive biofilm disruption for the release of individual cells and bacterial clusters into the bulk fluid where they become available for further spread and colonization.

as (p)ppGpp (Díaz-Salazar et al., 2017). Produced signals may culminate in events and self-produced bacterial substances that contribute to the remodeling process of the biofilm structure at this stage. Some of them include the production and release of enzymes to degrade the EPS matrix components (Boles and Horswill, 2008); disruption of noncovalent interactions by bacterial surfactants such as rhamnolipids (Boles et al., 2005) and phenol-soluble modulins (Wang et al., 2011); and cell death, which leads to the formation of cavities within the biofilm (Ma et al., 2009). The latter has been reported to be used by motile bacteria as points of escape from the biofilm and is often referred to as “central hollowing” or “seeding dispersal” (Ma et al., 2009; Purevdorj-Gage et al., 2005; Sauer et al., 2002). In general, individual cells or cell clusters may be released from a mature biofilm (Stoodley et al., 2001), and these become available for further spread and colonization (Figure 7).

The aforementioned mechanisms highlight the fact that biofilm dispersion is a well-regulated process affecting particular cells and regions within the biofilm. Furthermore, the dispersed cells have the advantage of having gone through changes in phenotype during biofilm development as well as further genetic modifications after the stress conditions generated by biofilm gradients. These modifications that once permitted them to express a more virulent and resistant phenotype are partially maintained; hence, dispersed cells are more virulent than their planktonic counterparts but less than those within the parent biofilm (Chua et al., 2014).

Biofilms modulate and evade immune responses from the host

As any other element recognized as foreign by the immune system, biofilm formation elicits the activation of different cells and mechanisms intended to clear it from the host. Although immune cells and mediators offer a wide variety of strategies to prevent pathogenic microbial invasion, biofilm-living microorganisms as well as biofilm molecular and structural components have the ability of modulating and evading immune attack (Figure 8). Some of the main mechanisms that contribute to this immune regulation include the following:

- (1) Activation and/or impaired activity of innate immune cells such as monocytes/macrophages (Kaya et al., 2020), neutrophils (Hong et al., 2009), and natural killer cells (Kaya et al., 2020);
- (2) Induction of an increased secretion of proinflammatory and anti-inflammatory cytokines (Kaya et al., 2020);

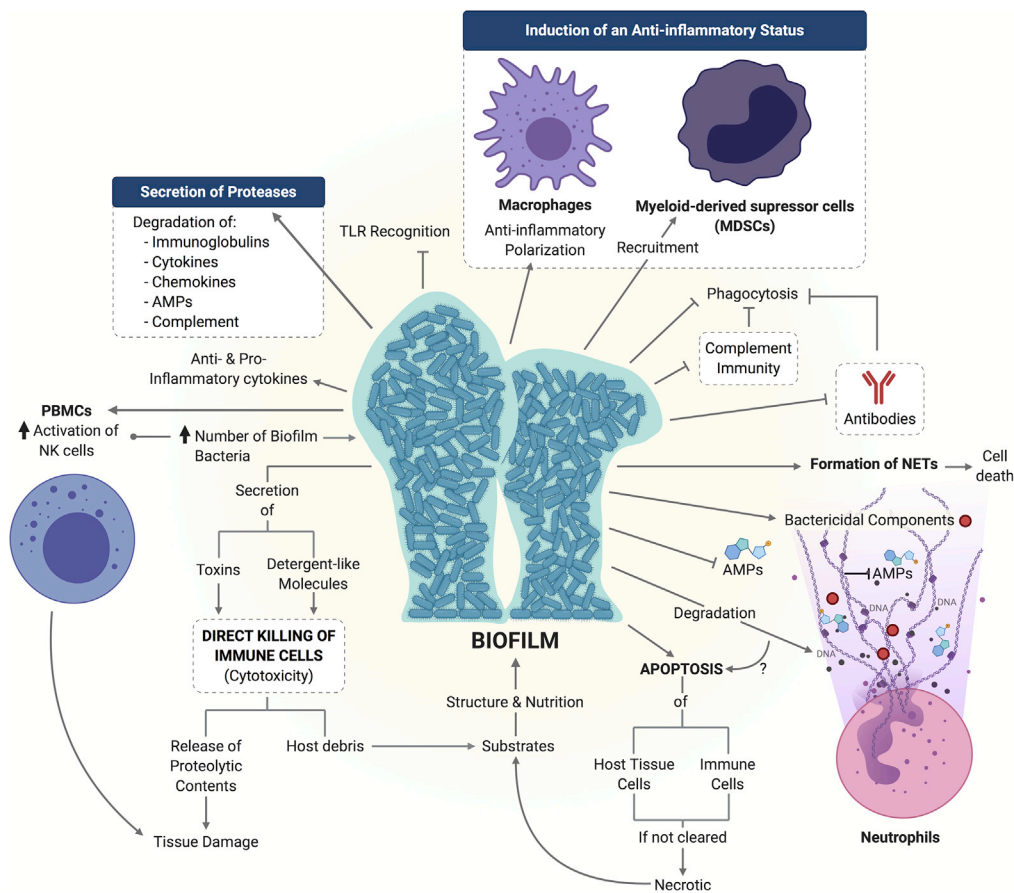


Figure 8. Biofilm mechanisms of immune evasion

Some reported strategies utilized by different biofilms to overcome and modulate host innate and adaptive immune responses are shown.

- (3) Modulation of recognition by toll-like receptors (TLRs) (Shang et al., 2019; Thurlow et al., 2011);
- (4) Shielding of epitopes recognized by immunoglobulins and impaired opsonization (Kristian et al., 2008; Langereis and Weiser, 2014);
- (5) Hindered complement activity (Kristian et al., 2008; Langereis and Weiser, 2014);
- (6) Impaired phagocytosis (Rose and Bermudez, 2014; Scherr et al., 2015; Thurlow et al., 2011);
- (7) Secretion of proteases that contribute to the degradation of cytokines (Fletcher et al., 1998; Mochizuki et al., 2014), chemokines (Mochizuki et al., 2014), complement proteins (Hong and Ghebrehwet, 1992), and antimicrobial peptides (AMPs) (Maisetta et al., 2011);
- (8) Induction of an anti-inflammatory status by recruitment of myeloid-derived suppressor cells (Heim et al., 2014) and skewing of macrophage polarization toward an M2 anti-inflammatory status (Thurlow et al., 2011); and
- (9) Direct killing of immune cells by the secretion of toxins (Scherr et al., 2015) and detergent-like molecules (Jensen et al., 2007; Wang et al., 2007), causing the release of intracellular components of immune cells, which in turn may lead to host tissue damage and further cytotoxicity (Parks et al., 2009).

Neutrophils are one of the innate immune system cells that play an active role during the initial stages of biofilm development. They are stimulated by biofilms to produce neutrophil extracellular traps (NETs) (Bhattacharya et al., 2018; Oveisi et al., 2019), one of the killing mechanisms of these immune cells. However, NETs are ineffective for biofilm clearance (Bhattacharya et al., 2018; Hong et al., 2009) as their main constituents, namely DNA, AMPs, and other microbicidal substances, are degraded by biofilm

components (Bryzek et al., 2019; Sultan et al., 2019). Moreover, formation of NETs may lead to death of neutrophils (Desai et al., 2016). If cell death occurs (Bhattacharya et al., 2018), the released products may contribute to host tissue damage (Manzenreiter et al., 2012) and/or biofilm formation (Walker et al., 2005). Furthermore, biofilms can also induce programmed cell death (apoptosis) in immune cells as well as other host tissues (Rose and Bermudez, 2014; Schwarzer et al., 2012; Singh et al., 2019b; Tateda et al., 2003). Finally, debris from dead cells may serve as nutritional and structural substrates for further biofilm development (Parks et al., 2009; Walker et al., 2005), in the same manner as dead bacterial cells within the biofilm do (López et al., 2009).

A meaningful part of the knowledge we currently have about biofilms, including their formation mechanisms, development stages, and the formulation of possible antibiotic or antibiofilm strategies, comes from biofilm models, which have been developed both *in vitro* or *in vivo*; each one with its own characteristics, uses, advantages, and drawbacks.

IN VITRO MODELS FOR BIOFILM ASSESSMENT

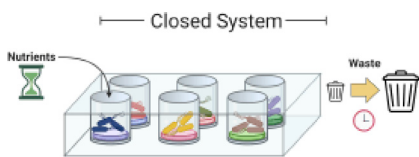
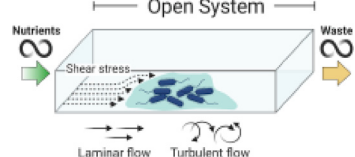
In vitro biofilm models (which do not use a living host) are used as the first step in testing new materials and methods meant to interact with biofilms. *In vitro* work is relatively simple, inexpensive, and high throughput compared with animal models, which are often used to further test promising candidates obtained from the use of *in vitro* models. The key difference between *in vivo* and *in vitro* models is the presence of the host immune system, but other important differences such as lower oxygen availability in many *in vivo* conditions are often unaddressed. *In vitro* biofilm models range from simple glass slides and Petri dishes to more elaborated systems that consider the physicochemical and mechanical properties required when trying to replicate biofilm formation in particular settings. More complex *in vitro* biofilm models may also consider cellular interactions with the host as well as the incorporation of monitoring systems for real-time assessment of biofilm formation. The variety of *in vitro* biofilm models can be classified into two main groups (Table 1) depending on the availability of nutrients over time:

- (1) Static or closed models: Nutrients and oxygen supply are limited to the initially provided conditions, or they are periodically renewed after removal of waste products (batch cultures);
- (2) Dynamic or open models: A continuous nutrient supply and waste removal over time are provided. Moreover, there is also the possibility to improve traditional *in vitro* biofilm models by translating them into *microcosm models*, either in static or dynamic setups, by including cells and/or substances that are typically found in the condition intended to be simulated. For instance, the incorporation of saliva for oral biofilm studies (Guggenheim et al., 2001) and supplementation of culture media with plasma and red blood cells to develop a chronic wound biofilm model (Sun et al., 2008) have been reported.

Currently used *in vitro* biofilm models

The currently used and well-established *in vitro* biofilm models have been extensively reviewed (Azeredo et al., 2017; Coenye and Nelis, 2010; Lebeaux et al., 2013). Biofilm formation in tubes and glass slides as models is not usually included because of their simplicity. However, it is important to acknowledge their significance and contribution during the initial biofilm experimental studies, which took place several decades ago. Particularly, the earliest reported custom-made device to analyze biofilm formation *in vitro* consisted of a lead and wood carrier suitable to hold glass slides, which was then submerged into seawater to study the initial stages of fouling (Zobell and Allen, 1935). Aside from this report, the development of more elaborate *in vitro* biofilm models really began in the 80s with the fabrication of the modified Robbins device (McCoy et al., 1981), the constant depth film fermenter (Peters and Wimpenny, 1988), the perfused biofilm fermenter (Gilbert et al., 1989), flow cell biofilm reactors (Caldwell and Lawrence, 1986; Pedersen, 1982), and the still widely used microtiter plate (Christensen et al., 1985). Several biofilm models and devices were proposed during the 90s, 2000s, and early 2010s; including the multiplaque artificial mouth (Sissons et al., 1991), drip flow reactor (Xu et al., 1998), Calgary biofilm device (CBD) (Ceri et al., 1999), colony biofilm model (Anderl et al., 2000), rotating disk reactor (Pitts et al., 2001), CDC biofilm reactor (Donlan et al., 2004), the Kadouri drip-fed biofilm assay (Merritt et al., 2005), the biofilm ring test (Chavant et al., 2007), microfluidic biochips (Lee et al., 2008; Richter et al., 2007), and the BioFlux device (Fluxion Biosciences) (Benoit et al., 2010). Although each of them offers different types of improvements for growing and studying

Table 1. General characteristics of *in vitro* static and dynamic biofilm models

	Static	Dynamic
		
Nutrient supply	Finite (may be replenished periodically)	Continuous
Flow	No	Yes, over the biofilm surface
Shear stress	Null to mild (if shaken)	Adjustable: Different hydrodynamic conditions can be adapted
Waste and planktonic cells	Accumulated	Removed as nutrients are replenished
Length of experiments	Short (days)	Long (from days to weeks)
Throughput screening	High: Different species/strains and conditions can be simultaneously evaluated	Low to medium
Technical complexity	Low	Medium to high
Complexity of setup procedures	None	Considerable, time consuming
Specialized equipment	Little (requires mostly common laboratory equipment)	Required (e.g., reactors or fermenting systems)
Cost per replicate	Low	Medium to high
Particularly useful for	Early stages of biofilm development Genetic screening	Study of mature biofilms

biofilms, all of them present inherent disadvantages and limitations (for review, see [Lebeaux et al., 2013](#)). For instance, oral biofilms are widely studied because of their clinical relevance, as they are associated with several oral diseases, such as periodontitis ([Socransky et al., 1998](#)). The Zurich model was developed in 2001 for the study of oral multispecies biofilms under controlled static conditions ([Guggenheim et al., 2001](#)). Since then, because of its simplicity and despite its limitations, this model has allowed several methodological modifications to increase the complexity of biofilm microbial composition ([Ammann et al., 2012](#)), microbial dynamics ([Thurnheer et al., 2016](#)), and to include potential cellular and molecular interactions with the host ([Belibasakis et al., 2015](#); [Belibasakis et al., 2013](#)).

Because of the fact that there is not an ideal biofilm model and since a reproducible model suitable for evaluating the efficacy of potential antimicrobials is essential, the use of standardized methods for specific biofilm devices and particular biofilm-producing bacteria has been proposed as an alternative. Specifically, the American Society for Testing and Materials (ASTM International) has implemented standardized test methods to provide the guidelines and specifications for the accurate and reproducible formation of biofilms and testing of antimicrobial substances. Four types of biofilm devices are associated to one or more ASTM standardized methods. The drip flow reactor and rotating disk reactor are used to evaluate biofilm formation in a continuous flow under low and medium shear stress, respectively, following their associated standard methods (ASTM E2647-20 and ASTM E2196-17) ([ASTM, 2017a](#); [ASTM, 2020](#)). For evaluating disinfectant capacity, the CBD (ASTM E2799-17) and the CDC biofilm reactor (ASTM E2562-17, ASTM E3161-18, ASTM E2871-19) are utilized ([ASTM, 2017c](#); [ASTM, 2019](#); [ASTM, 2017b](#); [ASTM, 2018a](#)). The colony biofilm model has also been recently adapted to develop a standard test method (ASTM E3180-18) to grow and quantify *Bacillus subtilis* biofilms ([ASTM, 2018b](#)). These standardized methods are mainly used for environmental biofilms, and there is still a need for standardized models, methods, and devices suitable for the evaluation of medical-related biofilms despite their potential associated limitations ([Coenye et al., 2018](#); [Malone et al., 2017b](#)).

The majority of scientific reports regarding the study of biofilm development and evaluation of different antimicrobial substances and methodological approaches employ biofilm models that have been used for several decades, where most of the time only slight modifications or adaptations have been proposed. The two most popular *in vitro* biofilm models (by number of citations) are the microtiter plate and CBD,

followed by flow cells. This prevalence may be explained by their simplicity and availability. Because of the great diversity of biofilm-related infections and biofilm formation methods and devices used; there is not a unique standard method for the experimental design of these studies. Instead, an open, flexible, and adaptable methodology (Figure 9) for studying biofilms allows scientists to explore and analyze them depending on the objectives being pursued.

Recently developed (from 2015 to date) *in vitro* biofilm models

Because of the high volume of biofilm models reported as “new” or “novel” in the literature, we have selected the models discussed in the following subsections by having an inclusion criteria limited to studies published from 2015 to date, which include a unique or an innovative way of biofilm formation and/or offer an improved biofilm monitoring and evaluation strategy. Also, several research articles report “new biofilm models for particular diseases and conditions;” however, in many cases, the innovative feature of the study is the way in which the disease is being reproduced, not the biofilm development on that particular condition. Hence, papers where the biofilm is formed via traditional methods were excluded.

Static *in vitro* biofilm models

The main characteristics of the recently developed static *in vitro* biofilm models (Table 2) show a tendency to (1) utilize small sized or miniaturized devices; (2) offer a platform for real-time monitoring of biofilm development; (3) evaluate biofilm properties under native conditions (undisrupted); (4) allow the *in situ* evaluation of potential antimicrobial treatments; (5) improve reproducibility by forming biofilms with customized shapes and dimensions; (6) consider the maturation state of the biofilm; and (7) include particular elements to better resemble the microenvironment of biofilm formation for certain medical conditions.

Similar to the greater antimicrobial resistance observed in biofilms as compared with planktonic bacteria, the structure and spatial arrangement of the biofilm influence this response, with a decreasing susceptibility to antimicrobials as biofilm thickness increases. For example, Ning et al. (2019) produced bioprinted bacterial biofilm constructs with thicknesses ranging from 0.25 to 4 mm using a double-crosslinked alginate bioink in a custom-made bioprinter. This technique offers the advantage of providing the user with a high degree of control over the production of constructs with predesigned shapes and dimensions. The authors printed solid and porous structures where the latter may facilitate oxygen diffusion, suggesting that these constructs may be suitable for the study of both aerobic and anaerobic bacterial biofilms depending on the design. This protocol allows the production of biofilm constructs robust enough to be manipulated for further analysis, including imaging and antimicrobial testing. The novelty of this model is that biofilm constructs can be allowed to mature for up to 28 days in culture, which is a great advantage considering that depending on the species and experimental conditions, it can take over 10 days for a biofilm to reach structural maturity (Stoodley et al., 2002). Hence, the antimicrobial efficacy of different substances can be evaluated at a particular time frame of biofilm formation for different bacterial species or strains. However, as previously mentioned, the biofilm properties are dependent on the interactions with the substrate which, in this case, would be limited to biofilms developed on soft surfaces with the additional inconvenience of other elements involved being unrepresented such as interactions with immune- and tissue-specific cells and molecules.

Bruchmann et al. (2017) also considered the importance of biofilm formation in 3D clusters to better resemble biofilm development under natural conditions. The authors addressed the need for reliable and controlled biofilm formation strategies to enable reproducibility in terms of shape and size of biofilms in order to establish proper comparisons. They took advantage of the biofilm-resistant properties of the so-called slippery lubricant-infused porous surface (SLIPS) (Epstein et al., 2012) and formed hydrophobic-hydrophilic patterns with predefined and equally sized geometries onto glass slides. As result, microcluster arrays where the biofilm can be grown into the hydrophilic areas are formed creating a set of multiple identical 3D biofilm clusters. However, after evaluating different shapes for a set of *P. aeruginosa* strains, it was found that the geometry of SLIPS as well as the size of the clusters and distance between them had an influence in biofilm formation and interactions between biofilm clusters. In order to properly apply this model for drug screening, the architectural conditions of the SLIPS for different types or strains of bacteria would require standardization. As most of the *in vitro* biofilm models for drug screening, this approach lacks the cellular and molecular interactions that have an important influence in driving biofilm formation and final properties.

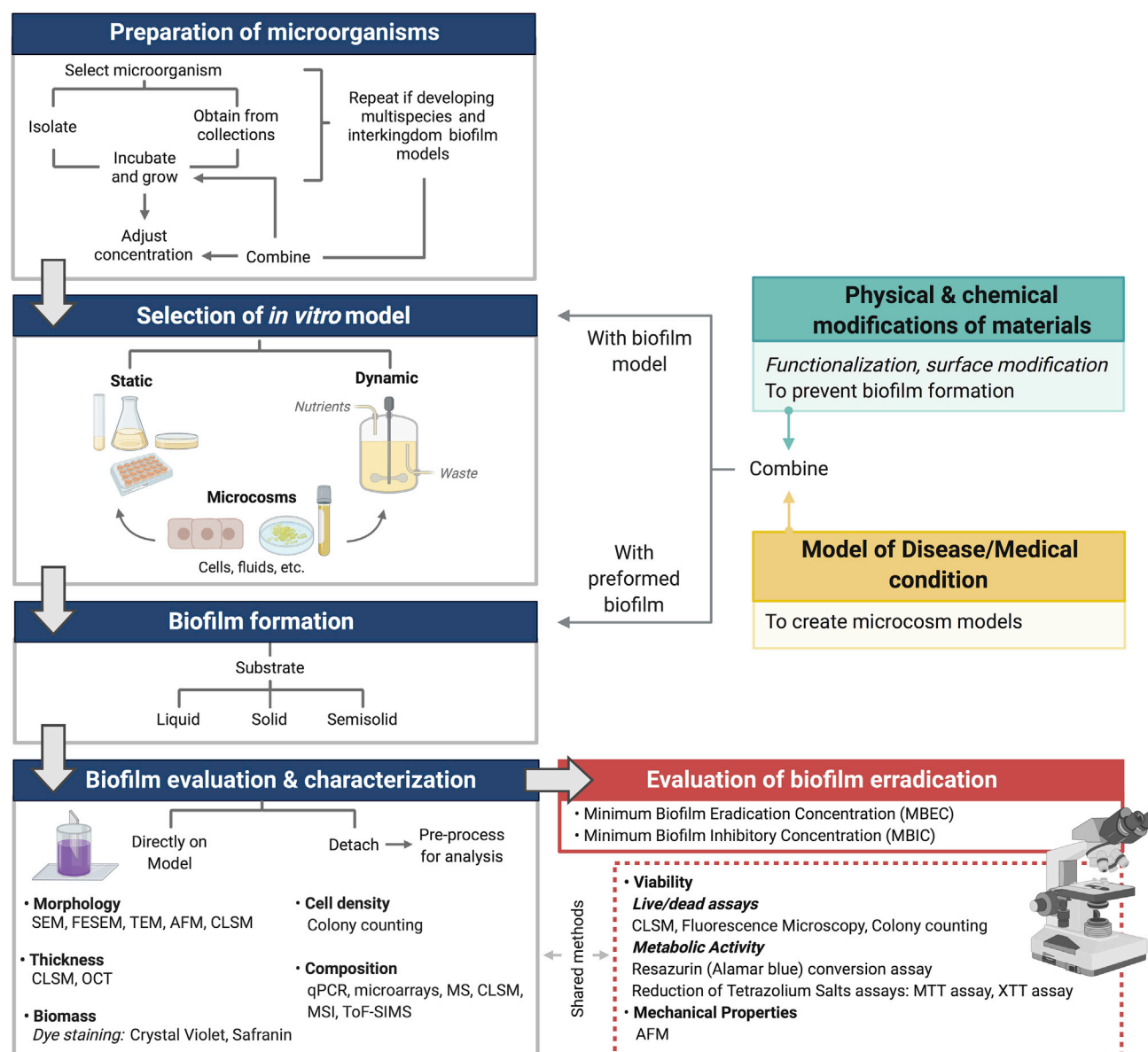
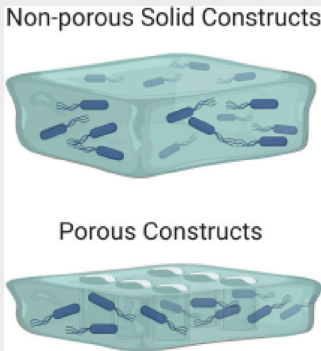
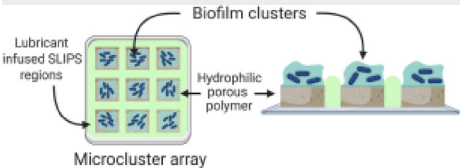


Figure 9. Schematic depiction for the overall steps involved in the current standard *in vitro* protocols of biofilm formation

Blue heading boxes show the general methodology followed in studies of biofilm development; same procedure is utilized when investigating the efficacy of biofilm eradication strategies (red box). Moreover, if prevention of biofilm formation is the goal of the study (green box) or if microcosm models are required (yellow box), functionalization/modification of materials and development of the appropriate disease models are respectively needed. After this, the materials under evaluation and the disease models may be combined with the chosen *in vitro* model for biofilm formation or with a preformed biofilm. Evaluation and characterization of the biofilm may be done directly on the model or detachment may be required for processing and analysis, depending on the chosen methods. Representative examples of the methods and techniques used for characterizing biofilms are shown; some of them are also used to evaluate the effect of antibiofilm strategies. SEM, scanning electron microscopy; FESEM, field emission SEM; TEM, transmission electron microscopy; AFM, atomic force microscopy; OCT, optical coherence tomography; qPCR, quantitative real-time PCR; MS, mass spectrometry; MSI, MS imaging; ToF-SIMS, time-of-flight secondary ion MS.

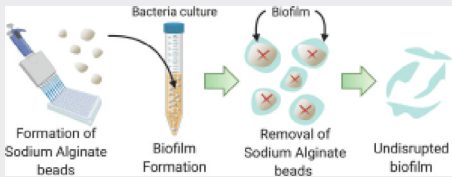
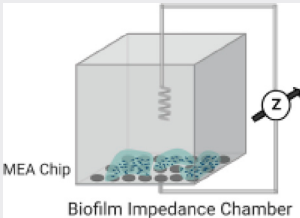
Another proposed strategy to model biofilm formation for further antimicrobial drug screening where the biofilm 3D structure is intended to be undisrupted is the dissolvable bead method (Dall et al., 2017). In this *in vitro* model, the biofilm is grown onto preformed sodium alginate beads by their incubation in culture broth. Once the biofilm has been established, the alginate beads are dissolved, and the biofilm structure remains intact. This protocol provides a rapid, simple, and cost-effective way of biofilm formation and drug screening. However, it results in a more homogeneous biofilm because of the uniform access to nutrients

Table 2. Representative examples for recent^a *in vitro* biofilm models as of 2015

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
Recent static <i>in vitro</i> biofilm models					
3D bioprinted biofilm construct (Ning et al., 2019)					
 Non-porous Solid Constructs Porous Constructs	Double crosslinked alginate-based bioink is used to print solid and porous 3D bacterial structures	Formation of mature biofilms for antimicrobial testing	<i>E. coli</i> clinical isolate (American Type Culture Collection [ATCC] 25922), methicillin-resistant <i>S. aureus</i> (MRSA, clinical isolate, ATCC 700788), methicillin-sensitive <i>S. aureus</i> (MSSA, clinical isolate, ATCC 29213) <i>P. aeruginosa</i>, PAO1 (wild-type strain, ATCC 47085) Cell density: Initial: OD_{600nm} = 1.0 Final: Visually reported (3D reconstructed CLSM z stack images)	• Customizable pre-designed biofilm thickness and dimensions • Construct stability for up to 4 weeks • Adequate cell viability • Suitable for aerobic and anaerobic biofilms • Allows biofilm formation monitoring • Suitable for antimicrobial testing (biofilm penetration and eradication) • Greater antimicrobial resistance than 2D cultures • Cost effective	• Constructs with thickness of 0.5 mm or thinner are structurally unstable • Missing interaction with cellular and molecular components • Decreased cell density as thickness increases • Restricted to biofilms grown in semisolid conditions
Patterned SLIPS ("slippery" lubricant-infused porous surface) (Bruchmann et al., 2017)					
 Microcluster array Biofilm clusters Lubricant infused SLIPS regions Hydrophilic porous polymer	Porous polymer areas with specific geometries are delimited by a lubricant that forms SLIPS regions. Biofilm formation occurs at the hydrophilic porous areas. The result is a series of 3D biofilms with uniform shapes and dimensions along the array	Biofilm screening Antimicrobial testing	<i>P. aeruginosa</i> strains PA01, PA30, and PA49 Cell density: Initial: 1×10^8 bacteria/mL Final: Not reported	• Control over the biofilm microcluster geometry • High reproducibility • Biofilm stability • Allows the study of interactions between biofilm subpopulations • Suitable for drug screening	• Biofilm formation by different bacterial strains may be influenced by the size and arrangement of SLIPS microclusters • Interactions between biofilm clusters • Missing interaction with cellular and molecular components

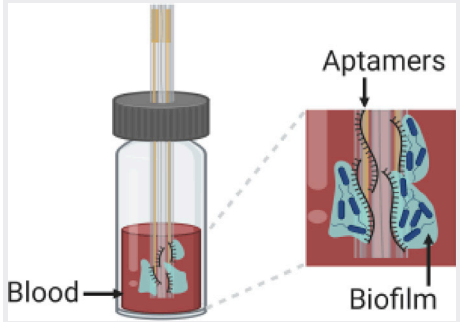
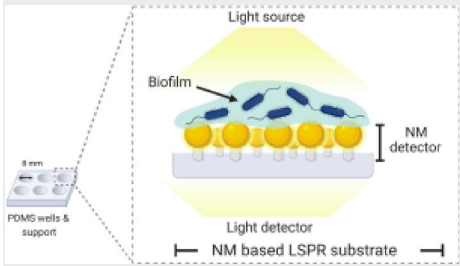
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Table 2. Continued

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
The dissolvable bead (Dall et al., 2017)  Formation of Sodium Alginate beads Bacteria culture Biofilm Formation Removal of Sodium Alginate beads Undisrupted biofilm	Sodium alginate beads are incubated in liquid bacterial culture for biofilm formation. The beads are dissolved, and biofilms are released	Antimicrobial testing	MSSA <i>S. aureus</i> (ATCC #29213), <i>S. mutans</i> (NCTC #10923), <i>E. coli</i> (ATCC #25922), clinical isolates of coagulase-negative <i>Staphylococcus</i> (CNS-J), <i>E. faecalis</i>, <i>K. pneumoniae</i>, and <i>P. aeruginosa</i> Cell density: Initial: 10^4 CFU/mL Final: Mean cell number (CFU/mL) $\text{Log}107.00 \pm 0.39$	- Localized biofilm formation onto the alginate bead surface - Uniform exposure to antimicrobials - Undisrupted biofilm - Suitable for drug screening - Greater assay responsiveness than the crystal violet assay to an antibiotic challenge - Rapid and reproducible - Cost efficient and time efficient	- Formed beads are not regular in size and shape - Potential influence of alginate beads architecture on biofilm phenotype - Homogeneous biofilm composition - Missing interaction with cellular and molecular components
Impedance-based multielectrode array (Goikoetxea et al., 2018)  MEA Chip Biofilm Impedance Chamber	Biofilm is grown onto an MEA biosensor placed into a chamber to measure impedance changes at different frequencies, which are correlated to biofilm structural changes during its development	Structural characterization of bacterial biofilms	<i>E. coli</i> TG1, isogenic ΔcsgD, ΔcsgB, and ΔbcsA mutant strains Cell density: Initial: 2.8×10^8 cells/mL Final (cells/mL): from 2.8×10^7 to 4.2×10^7	- Nondestructive and label-free characterization of biofilms - Allows distinction of biofilms with structural differences - Able to identify attachment and maturation stages of the biofilm formation cycle - Good spatial resolution - Reduced sample volume	- Unable to correlate the obtained impedance readings at certain frequencies with metabolic activity or number of cells present - Missing interaction with cellular and molecular components

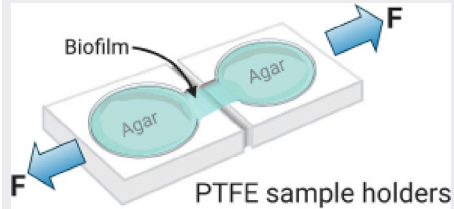
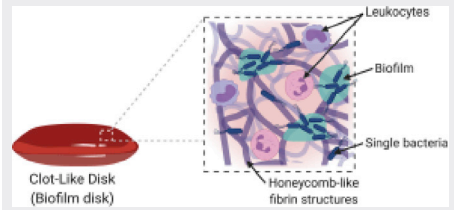
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Table 2. Continued

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
Vertical capacitance aptamer-functionalized sensor (Song et al., 2019)					
	Biosensor functionalized with bacteria-aptamers, which are vertically connected to a measurement system during biofilm formation. Biofilm electrical properties are recorded	Bacterial detection and biofilm formation monitoring in blood	<i>E. coli</i> (ATCC 25922), <i>P. aeruginosa</i> (ATCC 27853), <i>S. aureus</i> (ATCC 29213), mutant strains Δpel and $\Delta pel/\Delta psl$ of <i>P. aeruginosa</i> strain PAO1 Cell density: Initial: 10^0 , 10^1 , 10^2 , or 10^3 CFU/mL Final: Not reported	- Real-time monitoring of biofilm formation - Biofilm formation and bacterial growth can be monitored simultaneously - Suitable for antimicrobial testing - Simple, flexible, and cost effective - Detection of low bacterial concentrations	- Potential influence of the aptamer-bacteria interactions on biofilm development - Missing influence of shear forces during blood flow
Gold mushroom-like nanoplasmonic biochip (Funari et al., 2018)					
	Localized surface plasmon resonance (LSPR) biochip consisting of a detector with mushroom-like nanostructures where the biofilm is formed and monitored	Biofilm characterization and drug screening	<i>E. coli</i> (MC4100). Cell density: Initial: 2×10^7 CFU/mL Final: Not reported	- Real-time, nondestructive, label-free monitoring of biofilm formation - High sensitivity - Allows comparisons between biofilm-forming species - Suitable for antimicrobial testing - Possibility of automatization for data acquisition during several days	- Requirement of specialized equipment for device fabrication - Potential scattering in the LSPR signal if there is a high cell density per well - Sustained illumination may cause local stress to cells - Intermediate viability (65%) - Missing interaction with cellular and molecular components

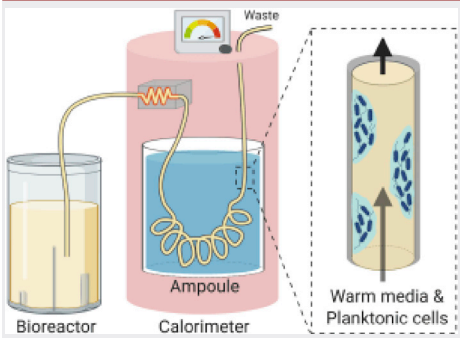
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Table 2. Continued

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
Biofilm rheometer plate (Grumbein et al., 2016) 	Biofilm is formed onto an agar surface contained along the two parts of a sample holder. A stretching force is applied to both sides	Quantification of mechanical properties (rupture forces and tensile strengths) <i>in situ</i>	<i>B. subtilis</i> B-1 Cell density: Initial: Not reported (15 μL of overnight bacterial liquid culture) Final: Not reported	- Biofilm can be measured in its naturally grown state - Allows the evaluation of antimicrobials on biofilm structural resistance - Cost effective: reusable sample holders - Biofilm deformation during the test can be followed visually	- Low force signal detection - Proper methods and filters must be applied to optimize the signal-to-noise ratio of measured data - Does not allow multiple biomechanical quantifications on the same sample - Missing interaction with cellular and molecular components
Human plasma biofilm model (hpBIOM) (Besser et al., 2020; Besser et al., 2019) 	Ellipsoid-like clot discs composed of a fibrin matrix from human plasma preserves and buffy coats plus incorporated biofilm-forming bacteria	Biofilm-infected human wounds	<i>S. aureus</i> subsp. <i>aureus</i> (DSM 799), <i>S. epidermidis</i> (DSM 20044), <i>P. aeruginosa</i> (DSM 939), <i>E. faecium</i> (DSM 2146), and <i>C. albicans</i> (DSM 1386) Cell density: Initial: 2×10^6 CFU/3 mL plasma solution Final (24 h): From 10^5 to 10^9	- Resembles a human wound milieu - Incorporates immune system components: white cells, platelets, and complement system - Personalized antimicrobial testing (donor specific)	- Results are not generalizable (effect is donor specific) - Pathogen-dependent stability - Does not include the damaged skin component of the wound: lack of skin cells

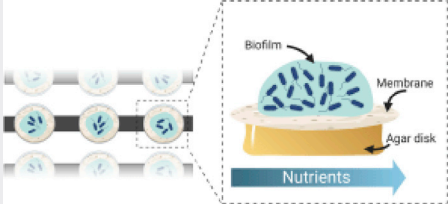
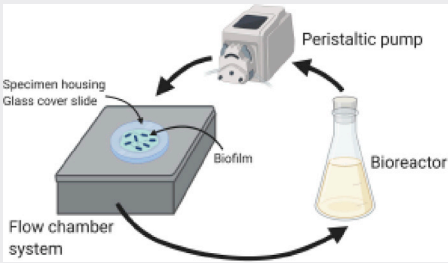
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Table 2. Continued

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
Recent dynamic <i>in vitro</i> biofilm models					
Microcalorimetry flow system (Said et al., 2015)					
 Bioreactor Calorimeter Ampoule Waste Warm media & Planktonic cells	A heat exchange unit connects an external bioreactor to the stainless-steel ampoule of a commercially available calorimeter. The heat flow, which is associated with bacterial metabolic activity, is then related to changes in the power signal of the calorimeter as an indicator of biofilm formation	Antimicrobial testing and study of biofilm formation on different medical tubing materials	<i>S. aureus</i> (NCIMB 9518) Cell density: Initial: 10^6 CFU Final: 3×10^{10} CFU/mL	• Sensitive to reduced cell density and small changes in metabolic activity • Noninvasive & nondisruptive method • Suitable for real-time monitoring of biofilm formation • Simple and reusable (removable parts permit sterilization) • Versatile: Allows monitoring of bacterial activity and evaluation of the effect of antimicrobials in a wide range of tubing materials and medical devices/implants (does not require optical clarity of the sample)	• Limited to low shear rates so as to not disturb the calorimeter • Large sized equipment • High costs for the calorimeter equipment • Complex data analysis • Limited throughput • Nonspecific: Heat flow signal involves the sum of a variety of processes taking place • It would require important adaptations if medical devices/implants are intended to be evaluated: should consider cellular and molecular interactions

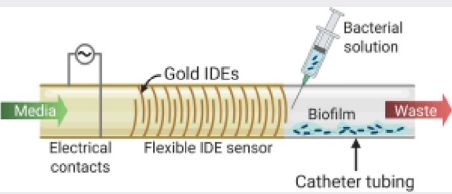
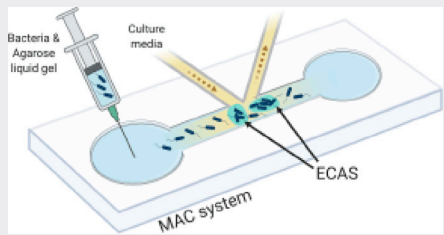
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Table 2. Continued

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
Duckworth Biofilm Device (Duckworth et al., 2018) 	3D-printed device with four channels that provide nutrients to three circular wells each. An agar disk, in direct contact with the nutritious flow, rests onto each well and supports a cellulose membrane, resembling the air-liquid interface where the biofilm is grown	Exuding chronic wound infection model for testing of antimicrobial wound dressings	<i>P. aeruginosa</i> <i>S. aureus</i> Cell density: Initial: 10^5 CFU Final: $\sim 8-10$ Log(CFU/mL)	- Mimics the air-liquid interface found in wounds - Allows temperature control and visual inspection without disrupting the biofilm - Has a lid and filter to prevent samples from crosscontamination - Good reproducibility and versatility - Small sized, inexpensive, reusable, and simple setup	- Requires fairly large amounts of media (500 mL/24 h). - Level of exudate is not regulated - Missing interaction with cellular and molecular components
Flow chamber system for medical implants (Rath et al., 2017) 	A closed flow system where the flow chamber consists of different materials and spacers to create a specimen housing where easily interchangeable implant disc samples are placed	Testing of dental implant materials	(1) <i>S. gordonii</i> (DSM, 20568) (2) <i>S. salivarius</i> (DSM, 20067) (3) <i>P. gingivalis</i> (DSM, 20709) (4) <i>S. oralis</i> (ATCC 9811) (5) <i>A. actinomycetemcomitans</i> (ATCC 2474) Cell density: (CFU/mL, $\times 10^6$) Initial: (1) 1.94; (2) 4.19; (3) 7.88; (4) 3.5; and (5) 1.25 Final: Not reported. Higher thickness: $38.85 \mu\text{m}$ for 7.88×10^6 CFU/mL	- Mimics physiological flow conditions of the oral cavity - Setup can be adjusted to aerobic or anaerobic conditions - Direct microscopic observation of the implant surface is possible - Easy removal of implant samples - Minimization of detachment effects because of removable cover and discs - Fully autoclavable and reusable system - Reproducible	- Culture conditions do not mimic the wide variety of nutrients found in the oral cavity - Optimization of conditions is necessary for the formation of multi-species biofilms - Missing interaction with cellular and molecular components

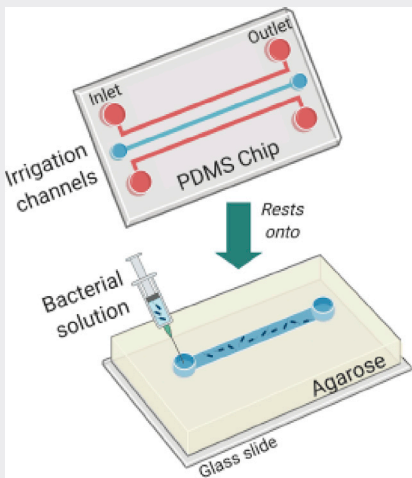
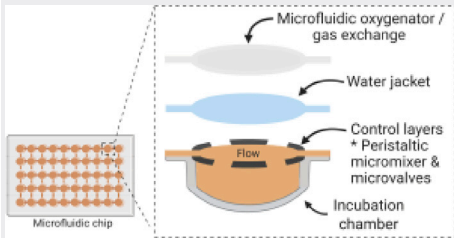
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Table 2. Continued

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
Flexible impedimetric detection platform (Huiszoon et al., 2019) 	A flexible platform of gold interdigitated electrodes (IDEs) is fitted into the lumen of a urinary catheter using a polyimide film as substrate. Bacterial growth medium is circulated through the catheter to allow biofilm formation, which is evaluated as changes in impedance	Real-time monitoring of biofilm development and evaluation of bioelectric effect as potential treatment	<i>E. coli</i> K-12 W3110 Cell density: Initial: 6×10^7 CFU/mL Final: Not reported. Impedance decrease of ~30% after 24 h of biofilm growth	- Flexible nature of the platform allows its use in clinically relevant curved surfaces and potentially in other types of geometries - Compact size - Allows for real-time nondestructive monitoring of biofilm development - Suitable for evaluation of combined therapies/treatments	- Variability of impedance measurements limits sensitivity - Technically challenging - Missing interaction with cellular and molecular components
Microfluidic agarose channel device (Jung et al., 2015) 	The system consists of a glass slide-sized microfluidic chip with multiple channels where bacterial cells are embedded in an agarose matrix, generating biofilm-like structures (ECAS). Nutritious flow is irrigated onto the surface resulting in a shear-free environment	Study of surface-free biofilms, similar to those formed within mucosal or viscous environments Testing of antimicrobial agents	<i>P. aeruginosa</i> (ATCC 27853), <i>E. coli</i> (ATCC 25922), <i>E. faecalis</i> (ATCC 29212), <i>S. aureus</i> (ATCC 29213), <i>B. subtilis</i> (ATCC 6633), <i>P. aeruginosa</i> PA14 and its mutants <i>wspF</i> and <i>pel</i> Cell density: Initial: $OD_{600nm} \approx 1.0$ Final: Not reported	- Allows for direct microscopic investigation - Better mimics the environment found on shear-free clinically relevant intra-cellular- and mucosal-associated biofilms - Simple, small-sized, and inexpensive device - Full biofilm development cycle is reached in few hours (9–12 h)	- Model not relevant to environments under shear stress - Maturation and integrity of ECAS is affected by fixing stress, viscosity, and nutrition - Missing interaction with cellular and molecular components

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Table 2. Continued

Graphic depiction	Description	Application	Microorganism(s) evaluated	Advantages	Limitations
Microfluidic wound model (Wright et al., 2015) 	A PDMS chip rests onto an agarose sheet with a central channel where a bacterial solution is added. The chip contains irrigation channels that contact and go along each side of the agarose central channel to create a concentration gradient	Study of wound bio-film structure and development Suitable for drug testing	<i>P. aeruginosa</i> (PAO1 & GFP-PAO1) <i>E. coli</i> (ATCC 700926) Cell density: Initial: 10^8 CFU Final: Not reported. Maximum biofilm surface coverage area ~55%	• Small sized • Resembles concentration gradients of wounds • Viewing channels allow high-resolution imaging techniques for real-time monitoring • Allows identification of individual species in dual-species biofilms	• Limited throughput • Missing interaction with the cellular and molecular components found in wound biofilm microenvironment
Microfluidic artificial teeth device (Lam et al., 2016) 	A microfluidic device consisting of 8×16 parallel incubation chambers, on top of which a series of microstructures with particular functions are included to allow the control of different environmental factors during biofilm growth and development	Study of biofilm growth and development under adjustable environmental conditions pertaining to dental disease Suitable for testing of potential antimicrobials	<i>Streptococci</i>, <i>F. nucleatum</i>, <i>A. naeslundii</i>, <i>P. gingivalis</i> Cell density: Initial: 10^5–10^7 cells/mL Final: Not reported. Biofilm thickness of 100 μm	• Facilitates the real-time and long-term monitoring (≥ 1 weeks) of biofilm development • Ability to independently adjust several microenvironmental conditions in scheduled times • Allows quantitative determination of biofilm thickness, cell viability, and spatial distribution • Small-sized and inexpensive device	• Requires specialized technical abilities for device fabrication and experimental setup • Missing interaction with cellular and molecular components

^aThe models listed in this table include some examples of how different multidisciplinary research groups have proposed to approach and overcome certain limitations of previous and currently used biofilm models. Because of specific goals being pursued, equipment and/or methodologies proposed are mostly particular of certain laboratories and may not be easily implementable in most research facilities. Despite this, they provide out-of-the-box strategies to study and analyze biofilms and may serve as inspiration for the development of more accurate biofilm models.

by bacterial cells, a situation that does not occur in natural biofilm formation where the bacteria in the deepest zones have limited access to nutrients and oxygen, originating a gradient of cells phenotypically different. Furthermore, as the size and shape of alginate beads is not exactly the same, it is possible that differences between biofilms in different beads arise because of the variability in the bacterial cell-alginate bead interactions. The same publication year, a different research group also reported the use of alginate beads as a platform for biofilm formation (Sønderholm et al., 2017). However, in this case, a less concentrated alginate solution was combined with a bacterial solution prior to the formation of beads. The resulting biofilms were observed as dense bacterial clusters mainly accumulated at the periphery of the beads but were also found within the beads.

The following three biofilm models have as a common objective the monitoring of biofilm development. There are many interesting reports about the employment of microsystems for the noninvasive characterization and sensing of bacterial biofilms (for review, see Subramanian et al., 2020), including microfluidic platforms and optical, mechanical, and electrochemical microsystems.

Biofilm structural and cellular components possess dielectric properties that can be exploited for their characterization by electrochemical systems (Subramanian et al., 2020). A representative model based on this class of biosensors is the biofilm impedance chamber reported by Goikoetxea et al. (2018). In this *in vitro* model, the microelectrode arrays (MEAs) served two functions: act as the substratum for biofilm formation and provide combined electrical recording and optical stimulation. Impedance was then recorded between working electrodes placed at the bottom of the MEA and an external platinum coil electrode. The MEA-based electrochemical impedance spectroscopy measurements allowed the characterization of structural differences of bacterial biofilms by detecting changes on the electrode surface at particular frequencies. According to the authors, changes in the low frequencies are related to the production of ECM and cell growth on the MEA surface, whereas those detected in the middle frequencies correspond to alterations in the supernatant that are associated to metabolites and planktonic cells. Although this model represents a fast, label-free, sensitive, and nondestructive method to characterize biofilms in terms of attachment and maturation, it requires important technical and theoretical considerations for appropriate data interpretation.

Song et al. (2019) developed an aptamer-functionalized sensor to analyze the biofilm formation in blood samples. In general, if bacteria were present in the sample, they would become attached to the sensor, affecting the electrical properties. The system was designed to allow the simultaneous measurement of capacitance, conductance, and impedance for the parallel monitoring of bacterial growth and biofilm formation. The sensor was vertically oriented in order to ensure that the alterations of electrical properties were being caused by bacteria and not by deposition of blood cells onto the sensor. An important advantage is that most of the materials and equipment required to replicate this system are part of most research laboratories. Also, apart from being a useful *in vitro* model for the evaluation of biofilm life cycle, this monitoring can also be done in the presence of antimicrobials to evaluate their efficacy. Moreover, the sensor can be removed from the system to allow further analysis of the formed biofilm. However, one implicit disadvantage is the potential influence of the aptamer-bacteria interactions on biofilm development.

An interesting optical microsystem for studying biofilm formation *in vitro* was developed by Funari et al. (2018). The authors fabricated what they called a “nanomushroom-based localized surface plasmon resonance (LSPR) substrate” for the real-time monitoring of biofilm formation and drug screening. Briefly, it consisted the formation of mushroom-like structures (nanomushrooms [NMs]) with stems of silicon dioxide and gold caps. The glass support containing this NM substrate was fused to a polydimethylsiloxane support with preformed wells. The principle of this *in vitro* model is that the charge on the NM substrate will increase as the bacterial growth and EPS production do. As a consequence, the frequency of plasmonic resonances will increase as well, causing a decrease in the LSPR wavelength of the NM caps allowing a real-time monitoring of biofilm formation (Funari et al., 2018). This optical microsystem is suitable for both biofilm characterization and antimicrobial drug screening. Although this model offers important advantages, one of the main inconveniences is that it may not be easily reproduced in different research settings because of the requirement of specialized equipment for its fabrication. Moreover, design refinements would be required in order to reduce the scattering in the LSPR signal and decrease the local stress of cells to improve cell viability.

Furthermore, apart from characterizing biofilms by monitoring changes during their formation to study life cycle, the evaluation of rheological properties is fundamental to establish multidisciplinary studies for the

development of innovative strategies to elucidate unanswered questions on biofilm fitness (Araújo et al., 2019; Hohne et al., 2009; Köstgens et al., 2001). Grumbein et al. (2016) designed and built a device suitable for the *in situ* evaluation of biofilm mechanical properties, namely rupture forces and tensile strengths. The device consisted of two polytetrafluoroethylene sample holder sections, which form a chamber that serves as a support for a solid nutrition layer (Singh et al., 2019a). Each end consists of a semicircular cavity that narrows into a straight connection area between both holder parts. Once the agar solidifies into the chamber forming a continuous layer, an incision is made on it at the contact zone of both holders. They are kept in close contact, and a certain volume of liquid bacterial culture is evenly spread over the agar layer. The device is incubated, and once the biofilm is formed over the sliced agar, this is placed in a system where a lateral stretching force is applied to the biofilm at the contact zone of the sample holders. A sensor detects the force, and this is then amplified and processed for data acquisition. Apart from allowing the measurement of native biofilm properties, the setup could be modified for the evaluation of multiple samples, treatments, or conditions at the same time. However, this device does not permit the evaluation of multiple biomechanical properties in the same sample. Also, it is restricted to the evaluation of biofilms formed on solid or semisolid substrates, excluding biofilms not physically attached to a surface. An important consideration is that a low force signal detection is obtained, which leads to the need of specialized users who can adequately select and apply the proper methods and filters to optimize the signal-to-noise ratio of measured data.

Finally, Besser et al. (2019) and (2020) developed a human plasma biofilm model (hpBIOM), which considers the influence of cellular and molecular components on nonhealing chronic wounds-associated biofilms (Besser et al., 2020; Besser et al., 2019). It consists of ellipsoid-shaped clot discs comprising human blood plasma preserves and buffy coats. For the fabrication of the hpBIOM, a bacterial solution with the pathogen of interest is prepared and combined with a plasma/buffy coat mixture. The resulting coagula-like discs with integrated pathogens are suitable for the evaluation of potential antimicrobial substances. The hpBIOM has noteworthy characteristics; first, the structural support for biofilm development is a human-based material present in the *in vivo* condition; second, it incorporates immune system components (white cells, platelets, and complement system); and last, it structurally resembles the chronic wound milieu. These qualities are finally translated into the development of a more realistic *in vitro* model where biofilm formation is influenced by its interaction with the cellular and molecular immunological components of the host. However, in order to better resemble the chronic wound microenvironment during biofilm formation, it should also consider the incorporation of “damaged” skin cells. Moreover, stability of the hpBIOM is dependent on the microbial pathogens being incorporated to the matrix. Furthermore, observed results are not generalizable, which is both an advantage and a disadvantage. It would be a drawback in the sense that immune competence would be donor specific, influencing biofilm formation and response to antimicrobial testing, requiring the elaboration of hpBIOMs for each patient. This last point emphasizes its advantage of offering personalized medicine in patients with nonhealing chronic wounds. In this case, bacteria could be isolated from the patient’s wound, cultured, and included in an hpBIOM to test a range of antimicrobials and variable doses to find the best antibiofilm treatment for that particular patient. However, further improvements are required to avoid the problem of short time stability of the hpBIOM for certain pathogens as well as the consideration of design modifications to include dermal layers to the model. A research study by Crone et al. (2015) previously reported a similar end up product for the *in vitro* modeling of chronic wound biofilm infections. This consisted a semisolid gel where bacteria were entrapped between two layers of agar-enriched media casted in a plastic holder. With the aim to better mimic the wound microenvironment, they included chopped meat powder as damaged tissue protein in a culture media supplemented with 25% defibrinated and lysed horse blood and 25% bovine plasma. Nonetheless, these modifications did not influence biofilm growth in terms of number of cells and structure of the aggregates as compared with regular culture media, but it did have an impact on the biofilm response to the treatment evaluated being more susceptible to disruption under these conditions. A peculiar reported result was the obtention of four microcolonies within the gels that were visible to the naked eye. Hence, although the authors also presented an *in vitro* biofilm model for chronic wounds, which has a similar appearance to the hpBIOM, these are actually different approaches where the hpBIOM shows greater potential.

There is a substantial amount of research studies where the development of new biofilm models is considered an important factor to increase the potential of transitioning antimicrobial therapies and products to the clinic. However, most of the time, these studies consider the inclusion of slight modifications to previously reported models (Brown et al., 2019; Garuglieri et al., 2018; Pabst et al., 2016) pulling along inherent

drawbacks of the original ones or, some other times, they focus on a particular characteristic that wants to be replicated or improved, leaving important conditions unaddressed (Pinck et al., 2017).

In general, although the new *in vitro* models presented in this section offer special advantages for the study of biofilms and evaluation of potential antimicrobials, they still face important drawbacks. These are found summarized in Table 2, where apart from the microbial species used, the different strains chosen as well as the initial and final cell density also show the high variability in the conditions reported when studying biofilms. Moreover, it is worth mentioning that the purpose of presenting the previously discussed models was not to propose them as “to use models” but to show how some of the limitations of previous models had been tackled by different research groups. Biofilm models cannot, and should not, be limited to a single formation/evaluation strategy because of the wide variety of microenvironments under which biofilms are formed. Ideally, models that better recapitulate native conditions of biofilm development would consider combining different strategies to improve biofilm formation and evaluation, depending on the objectives being pursued.

Dynamic *in vitro* biofilm models

Compared with most static models, dynamic models incorporate the effect of shear forces in different and varied ways, ranging from fluidics systems that push a mobile liquid phase through a solid phase container (e.g., the high-throughput Bioflux device; Benoit et al., 2010) or by simply placing glass beads inside the wells of a microtiter on a rotating platform (Konrat et al., 2016). While dynamic models offer the advantage of more closely replicating certain *in vivo* conditions, their disadvantages frequently come in the form of higher costs and difficulty of use. Clinically important conditions such as chronic wounds, where ~80% of wounds contain biofilms (Malone et al., 2017a), demand dynamic conditions (such as those caused by the flow of fluids from the vasculature into a wound) to be considered in the development and evaluation of potential antimicrobials.

New dynamic models. Most of the recently published *in vitro* dynamic models involve slight modifications of previously developed models instead of a completely novel approach for biofilm development and/or evaluation. Although this implies carrying already identified limitations associated to that particular device or model, the adaptation of currently available technologies is also a convenient approach if it provides significant improvements (Table 2). For instance, Said et al. (2015) incorporated a bioreactor to a commercial calorimeter (2277 TAM Thermal Activity Monitor; TA Instruments) to allow the supply of bacterial culture flow from a bioreactor to the calorimeter, passing by a heat exchange system. This simple setup allowed the real-time *in situ* monitoring of biofilm development in the lumen of medical tubing materials without disruption. Although the heat flow signal implies the net result of different processes occurring in the system (Braissant et al., 2010), the authors were able to relate it with bacterial metabolic activity as an indicator of biofilm development, as previously reported (Wentzien et al., 1994). Because of the ability of the system to support a replenishing flow, the length of experiments is not limited by nutrient exhaustion as in batch conditions, and thus, longer experiments can be performed to investigate the entire life cycle of the biofilm. However, because of the high costs of the calorimeter equipment, this setup is limited to laboratories where the instrument is already available. Moreover, this is a large sized equipment that also limits its applicability. Combining this approach with miniaturization of the calorimeter (Lerchner et al., 2008) could be an alternative to overcome these limitations. Another proposed model that is based on the principle of flow cells is the Duckworth Biofilm Device (Duckworth et al., 2018). The Duckworth Biofilm Device is a custom-made 3D printed flow system device intended to replicate the biofilms found in wound infections by delivering a flow of nutritious media at the bottom of an agar disc that is topped with a semipermeable membrane upon which a biofilm is formed. This arrangement serves to model the air-liquid interface found in infected wounds, and the flow provided by the model mimics the exudate found in some chronic wounds. However, it does not allow regulation of the exudate level and requires a considerable amount of media.

Taking into account the importance of evaluating biofilm formation on medical implants, Rath et al. (2017) proposed a dental implant infection model that consists of a reusable flow chamber system where discs of different materials are tested on their capacity to allow biofilm formation. An interesting feature is that the experimental setup included the recirculation of nutritious media and bacteria through the chamber containing the material to be evaluated, mimicking physiological flow conditions of the oral cavity. Although this chamber system showed to be reproducible for monospecies biofilms, it may require further optimization if the characteristic multispecies biofilms found in the oral cavity want to be replicated. Moreover, real-

time detection and monitoring of biofilm formation is of vital importance in clinical settings as it would allow the implementation of treatments that may prevent biofilm development if detected at early stages. A recently published study reported the potential of a flexible impedimetric detection platform to monitor biofilm development into the lumen of urinary catheters (Huiszoon et al., 2019). It consists of gold interdigitated electrodes, which are fitted into the lumen of the catheter to provide impedimetric readings that, according to the authors, can be associated to biofilm formation and growth. Moreover, apart from their utility as detectors, gold interdigitated electrodes were used to produce an electric field to evaluate their potential as a platform for treating biofilms in combination with lower dosages of antibiotics. Despite this may be a promising alternative for biofilm detection, monitoring, and treatment, it still faces some technical challenges that would need to be addressed before moving to a clinical setting.

As previously mentioned, there is a tendency to develop biofilm models in miniaturized devices. Here, we will present some recent representative examples that may find clinical application for biofilms of medical importance. For an extended revision on microsystems for biofilm development and characterization, the reader is referred to Subramanian et al. (2020).

Apart from growing onto a great diversity of surfaces, bacteria are able to colonize soft mucous tissues (Bjarnsholt et al., 2009) as well as the intracellular space (Anderson et al., 2003) where shear stress is barely present (Blake, 1973; Worlitzsch et al., 2002). In this sense, Jung et al. (2015) adapted a previously reported device (Choi et al., 2013) to develop a microfluidic agarose channel system that exposes bacterial colonies embedded in an agarose gel to a constant supply of fresh nutrients delivered at very low shear forces. The resulting bacterial colonies developed into embedded cell aggregative structures (ECASs), which were found to present several characteristics that resemble biofilms. For instance, ECAS produced EPS and became eventually burst to release planktonic cells, thus suggesting that this model replicates the full biofilm life cycle. Although gel-entrapped bacteria somehow resemble mucous environments, it was found that agarose concentration and fixing process influence the maturation and integrity of ECAS; which would require further improvement of experimental conditions. Wright et al. (2015) also utilized an agarose gel in a microfluidic wound model, which, in this case, served the function of allowing the diffusion of amino acids from the lateral injection ports of a PDMS chip placed on top of it, toward a central bacterial solution channel to resemble the concentration gradients of wounds. Notably, this arrangement provided an easy way of incorporating a chemotactic stimulus while allowing real-time monitoring of biofilm development via an observation port. Finally, a recently published Microfluidic Artificial Teeth Device offered the possibility of studying biofilm formation and development in 128 incubation chambers where different microenvironmental conditions, such as shear stress, nutrients, and oxygen levels, can be independently adjusted (Lam et al., 2016). Even though this device offered several advantages over other models, including the real-time monitoring and quantitative characterization of biofilm development, it demands specialized technical abilities for both device fabrication and operation, which limits its applicability.

It is noteworthy that all the dynamic models presented here have as a common limitation the fact that the interaction of biofilms with the cellular and molecular components of the host is not being considered or intended to be simulated. This is, generally speaking, the main issue found in most of the *in vitro* biofilm models that have been used for decades and are, unfortunately, still being developed in such a way most of the times. However, there are some published models that are exempted from this tendency. For instance, Millhouse et al. (2014) reported an *in vitro* static model where the coculture of epithelial cells with a multispecies biofilm was considered with the intention to simulate the conditions under which periodontal biofilms develop *in vivo*. Although in this study the influence of host cells was taken into account, biofilm formation was performed onto coverslips prior to the coculture setting, which ultimately limited the resemblance of this model to the development of periodontitis.

IN VIVO MODELS FOR BIOFILM ASSESSMENT

While *in vitro* biofilm models are powerful tools when it comes to reproducibly testing the effects of simple treatments and factors, they fail to account for the dynamic and complex nature of the interactions at play between the host (i.e., immune system) and bacteria that make up the biofilm as well as the potential for interaction with other bacteria. As such, the development of robust *in vivo* models is key in validating *in vitro* results and a necessary step in the testing of new treatments and devices on the path toward clinical translation.

The use of *in vivo* models to study pathogenic biofilms as well as the development of antibiofilm therapies is not new and has been a key and essential step in the development of our understanding of biofilm

formation, treatment, and prevention. The earliest example of an *in vivo* model being used to study biofilms dates back to the 1940s (Scheman et al., 1941), where rabbits were used as a model for studying osteomyelitis. Since then, there has been a significant amount of research dedicated to the development and study of *in vivo* models. Nowadays, *in vivo* models of biofilm formation related to tissue infections, device-related infections, and systemic infections can be found (Lebeaux et al., 2013). Notable tissue infections that have been investigated include models for lung infections, chronic wounds, and ear, nose, and throat infections. Common device-based infections have focused on models for orthopedic implants, subcutaneous devices, and urinary tract catheters. While mammalian *in vivo* models employing mouse, rat, rabbit, and pigs are most prevalent because of the fact that they allow for the presentation of representative human pathology through similar anatomies, healing processes, and immune response, (Barré-Sinoussi and Montagutelli, 2015), other animals as well as several nonmammalian models have been developed. Although nonmammalian models eliminate some of the practical problems associated with the cost of raising, and housing small and large animals (Ziegler et al., 2016), their inability to present complex immune responses, narrow optimal growth temperature, and short life span have limited their applicability in studying certain infections/pathogens and make them unsuitable for the study of chronic infections. That being said, there are a number of situations in which these simple nonmammalian models (i.e., *C. elegans* (Diard et al., 2007), *D. rerio* (Neely et al., 2002), and *D. melanogaster* (de Bentzmann et al., 2012)) are ideal, such as high-throughput screening studies (Letamendia et al., 2012).

In the next sections, we will highlight some of the currently employed *in vivo* biofilm models, discuss recent advancements in the development of models that target tissue- and device-related infections that were previously not addressed and touch on the methods used to characterize the biofilm both *in vivo* and postmortem/*ex vivo*.

Currently used *in vivo* biofilm models

In vivo biofilm models have enabled researchers to amass a considerable amount of information regarding biofilm-related infections. Most of the currently employed and well-established *in vivo* biofilm models have been previously reviewed (Lebeaux et al., 2013). These models have varied in levels of complexity ranging from the simple injection of an inoculate into a catheter (Hagberg et al., 1983) to more complex orthopedic surgeries (Williams et al., 2012), and although there are models for almost all tissue-related infections as well as device-related infections, there is no standard model for each type of infection as the model chosen is highly dependent on the research question one is interested in addressing and may be influenced by the immune system of the host, the size and surface area of the device or implant, and environment in which they find themselves (Lebeaux et al., 2013). Further complicating the use, selection, and development of *in vivo* models is accessibility to the biofilm for characterization. Unlike *in vitro* models where the biofilm is typically grown on an easily accessible transparent substrate, most *in vivo* biofilms can be difficult to characterize and monitor in real time under *in situ* conditions because of the fact that they are typically internal. While surface wounds, dental caries, and biofilm-related eye infections are exceptions and can be monitored using techniques such as *in vivo* confocal laser scanning microscopy (CLSM) (Schlafer and Meyer, 2017) and optical coherence tomography (Dsouza et al., 2019; Lenton et al., 2012), typically the biofilm analysis of *in vivo* models must be carried out postmortem on *ex vivo* tissue. While there are few examples of *in situ in vivo* monitoring of biofilms using advanced imaging techniques, such as microcomputed tomography (Stadelmann et al., 2015) and micropositron emission tomography (Garrido et al., 2014); these techniques are highly specialized, expensive, and require trained technicians and as such are typically difficult to access. Some of the key steps in the design and study of *in vivo* biofilms are presented and summarized in Figure 10.

It is evident in examining the *in vivo* models developed to date, that there is not much standardization (Coenye et al., 2020). While it is understandable that researchers want to modify the models to better answer their research question, at the same time by using different animals (species and/or strain), modifying the route and/or dosage of inoculant, and varying the length of the experiment it becomes quite difficult to compare results from different studies. This lack of comparability can be a problem when trying to move devices and techniques toward translation in the clinic, considering there are many who question the validity of certain *in vitro* models because of the low efficiency of translation in the clinical setting (van der Worp et al., 2010; von Herrath and Nepom, 2005).

Currently, one can find established and well-characterized *in vivo* biofilm models for tissue-related infections, such as cystic fibrosis (Semaniakou et al., 2019), chronic obstructive pulmonary disease (Pang et al., 2008), bronchitis (Yanagihara et al., 1997), tract infections (Anderson et al., 2003), kidney stones (Satoh

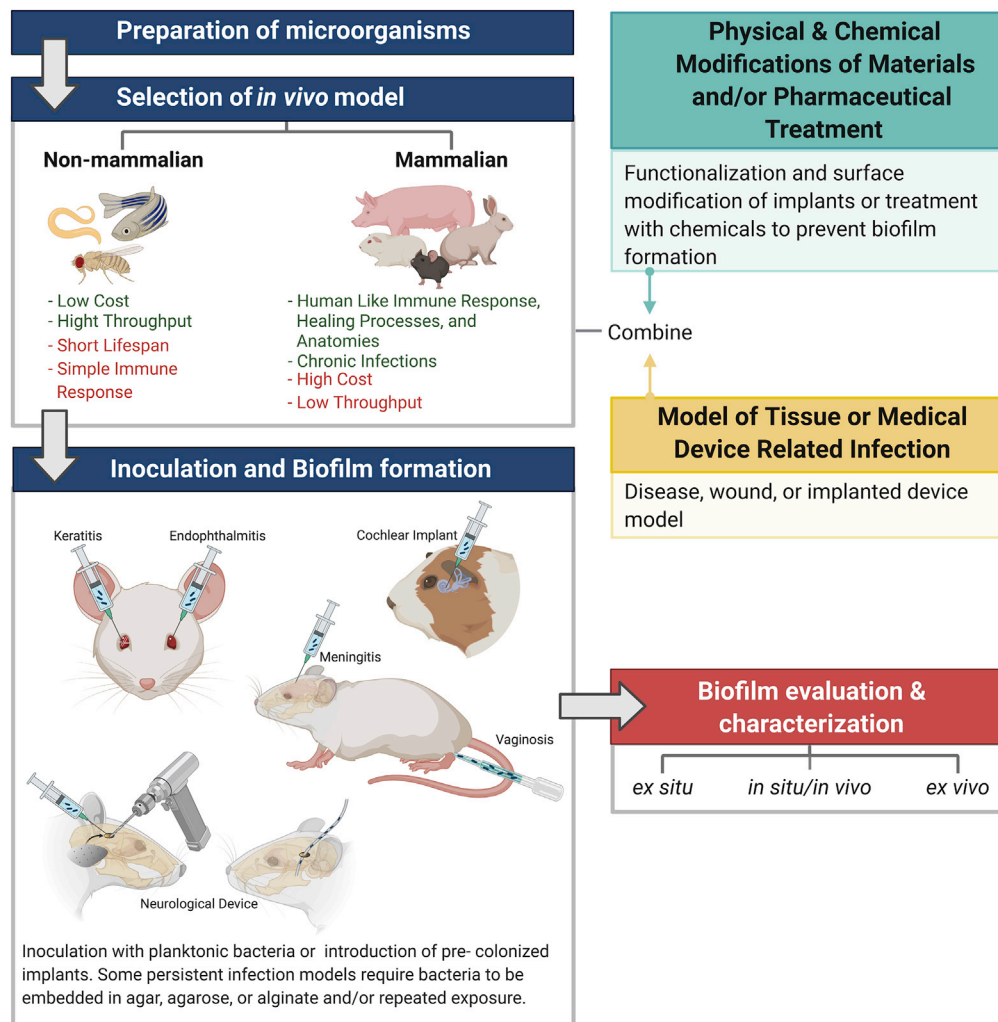


Figure 10. Schematic depiction for the overall steps involved in the design of *in vivo* protocols of biofilm formation and characterization

Blue heading boxes show the general methodology followed in studies of *in vivo* biofilm development. The red heading box highlights the methods that can be used to characterize biofilm progression. If prevention of biofilm formation is the goal of the study (green box) or if disease or implanted medical device models are required (yellow box), functionalization/modification of materials and development of the appropriate disease models are respectively needed. After this, the materials under evaluation and the disease models may be combined to generate an appropriate *in vivo* model for biofilm formation. Evaluation and characterization of the biofilm can be done *in situ* using advanced *in vivo* techniques, *ex situ* through the analysis of swabs and excised tissue samples, and *ex vivo* (i.e., histological analysis of tissue at end point of study).

et al., 1984), pyelonephritis (Nickel et al., 1987), intestinal infections (Bohnhoff et al., 1964; Nell et al., 2010), gall bladder infections (Sukupolvi et al., 1997), chronic wounds (Dai et al., 2010; Simonetti et al., 2008; Akiyama et al., 1996; Nakagami et al., 2008; Gurjala et al., 2011; Davis et al., 2008; Mastropaolo et al., 2005), infective endocarditis (Dai et al., 2010; Durack et al., 1973; Veloso et al., 2011), chronic otitis media (Chaney et al., 2011; Dohar et al., 2005; Ehrlich et al., 2002; Eriksson et al., 2006; Swords et al., 2004), chronic rhinosinusitis (Abreu et al., 2012; Ha et al., 2007; Johansson et al., 1988), dental caries (Bainbridge et al., 2010; Catalán et al., 2011; Fitzgerald and Keyes, 1960), periodontitis (Bainbridge et al., 2010; Hasturk et al., 2007; Settem et al., 2012), and osteomyelitis (Scheman et al., 1941; Brady et al., 2006; Funao et al., 2012; Poepl et al., 2011). While the development of many of these models followed closely with the introduction of new techniques for biofilm characterization and imaging, the introduction of bioluminescent bacterial strains and bioluminescence imaging had a great impact on the development of chronic wound

models (Avcı et al., 2018). Through the isolation and transfection of genes coding for light-emitting proteins in biofilm forming bacterial strains, *in situ* biofilm conditions can now be visualized and monitored. One of the more commonly employed biofilm-forming bioluminescent bacteria that has been employed is *S. aureus*, specifically strains Xen29, Xen36, Xen40, and ALC2906 (Pribaz et al., 2012).

Established models pertaining to device-related infections include vascular catheters (Fernández-Hidalgo et al., 2010; Chauhan et al., 2012; Rupp et al., 1999), urinary catheters (Davis et al., 1995; Olson et al., 1989; Guiton et al., 2010; Kurosaka et al., 2001; Fung et al., 2003; Allison et al., 2011; Cirioni et al., 2011; Haraoka et al., 1995), orthopedic implants (Mayberry-Carson et al., 1984; Sanzén and Linder, 1995; Del Pozo et al., 2009; Evans et al., 1993; Poelstra et al., 2000; Lucke et al., 2003; Prabhakara et al., 2011; Fitzgerald, 1983; Petty et al., 1985; Philipov et al., 1995; Williams et al., 2012), prosthetic joints (Schurman et al., 1978; Blomgren and Lindgren, 1981; Southwood et al., 1985; Belmatoug et al., 1996; Bernthal et al., 2010), endotracheal tubes (Berra et al., 2004; Fernández-Barat et al., 2012; Olson et al., 2002b), vascular grafts (Tollefson et al., 1987; Aboshady et al., 2012), tissue fillers (Arad et al., 2013; Jacombs et al., 2012), contact lenses (Sun et al., 2010), dental implants (Freire et al., 2011; Rimondini et al., 1997), as well as several other subcutaneous models (Ensing et al., 2005; Hansen et al., 2009; Illingworth et al., 1998; Nakamoto et al., 1994; Anguita-Alonso et al., 2007; Engelsman et al., 2010; Rediske et al., 2000).

Recently developed *in vivo* biofilm models

Many tissue-related infections and commonly used medical devices have well-established animal models that allow the study of biofilm-related disease progression and testing of new drugs, treatments, materials, and coatings. However, over the last decade, there has been a push to develop new models that address the shortcomings of available models or fill the holes where no model currently exists. In Table 3, we highlight several new *in vivo* biofilm models, which allow for the study of tissue- and device-related infections. Since *in vivo* biofilm models imply a more complex scenario because of all the potential interactions that may arise, and considering that differences in the species, sex, and strain of the animal chosen, as well as differences in age and weight influence the outcome of the model developed, we considered important to include these data as reference for comparison between the models presented. Moreover, the methods employed for analyzing these biofilm models and the main outcomes achieved with them are briefly summarized in the same table.

While several *in vivo* models to study biofilm adhesion and formation on contact lenses have been reported (Sun et al., 2010), until recently there was no accepted model in which to study the association of noncontact lens-related corneal biofilms with bacterial keratitis. In 2015, Saraswathi and Beuerman (2015) developed a mouse model in which they could study the role of *P. aeruginosa* biofilms in the progression of corneal keratitis. Using 7-8-week-old C57BL/6 mice, they were able to follow the progression of *P. aeruginosa* derived corneal biofilms from planktonic state to microcolony formation in a corneal injury model. The injury model was created by introducing four superficial abrasions of 1–2 mm into the corneal epithelium to which an aliquot of *P. aeruginosa* was added. By following the progression of the infection using slit lamp microscopy over a period of 7 days and imaging the biofilm formed on the enucleated eyes with a combination of CLSM, scanning electron microscopy, and transmission electron microscopy at days 1, 2, 3, 5, and 7, they were able to illustrate biofilm formation on the corneal surface in a standard infection, which suggests that mature biofilms are a common component of keratitis and could account for therapeutic difficulties when resistance to treatment is observed (Saraswathi and Beuerman, 2015). This study was followed by one from Ponce-Angulo et al. (2020) in which they examined biofilm formation in a mixed keratitis immunosuppressed murine model. The coinfection of *S. aureus* and *F. falciforme* was introduced through inoculation of a micropocket incision that was made in the limboscleral border of the cornea. Using optical and fluorescence microscopy techniques along with transmission electron microscopy of excised tissue, they were able to confirm and characterize the growth of a mixed bacterial and fungal biofilm (Ponce-Angulo et al., 2020).

Another biofilm-related tissue infection that afflicts the eye is endophthalmitis. In 2014, Sadaka et al. (2014) developed a mouse model in which biofilm-related anterior chamber infections characteristic of endophthalmitis could be studied. Interested in showing a link between the branched-chain amino acid responsive transcription regulator CodY and endophthalmitis, the study looked to investigate the impact of CodY deletion on *S. aureus* virulence within a murine anterior chamber model. Briefly, the anterior chamber of the right eye was inoculated with *S. aureus* using a 35G needle on a NanoFil syringe just anterior to the limbus without

Table 3. Recently developed *in vivo* biofilm models

Description	Application	Microorganism(s)	Biofilm analysis	Outcome(s)
Recent <i>in vivo</i> biofilm models for tissue-related infection				
Endophthalmitis (Sadaka et al., 2014)				
The anterior chamber of the right eye is inoculated with <i>S. aureus</i> using a 35-G needle on a NanoFil syringe just anterior to the limbus without touching the iris. The left eye is used as an untreated control. Animals: ♀C57BL/6J mice	Investigate the impact of CodY deletion on <i>S. aureus</i> virulence in a murine anterior chamber infection model	<i>S. aureus</i> (MS7/SA564/CDM7) Initial cell density: 1 μ L of OD _{600nm} = 0.4–0.8	- Bacterial growth assessed after 24 h by extraction and homogenization of the entire eye followed by quantitative plating - Light microscopy of histological samples	- Developed a model of anterior chamber infection, characteristic of endophthalmitis - Revealed a link between branched-chain amino acid responsive transcription regulator CodY and endophthalmitis repression - Addition of branched-chain amino acids to postoperative eyedrops could reduce progression of endophthalmitis
Keratitis (Saraswathi and Beuerman, 2015)				
Four superficial abrasions of 1–2 mm are introduced into the corneal epithelium to which an aliquot of <i>P. aeruginosa</i> is then delivered Animals: 7–8-week-old C57BL/6 mice	Follow the progression of corneal biofilms from planktonic to microcolony formation in a corneal injury model	<i>P. aeruginosa</i> (ATCC 9027) Initial cell density: 5 μ L of 1×10^8 CFU/mL	- Slit lamp - Light microscopy - CLSM - SEM - TEM	- Developed a model for studying biofilm infections of the corneal surface induced by <i>P. aeruginosa</i> - Suggests that mature biofilms are a common component of keratitis - Biofilm formation explains occasional resistance toward therapeutic treatments
Keratitis (Ponce-Angulo et al., 2020)				
A micropocket incision in the limboscleral border of the cornea is made to which a bacterial inoculate is injected Animals: 6–8-week-old ♀BALB/c mice	Characterize and analyze biofilm formation in mixed keratitis induced by coinfection	<i>S. aureus</i> (IOM2617228) <i>F. falciiforme</i> (IOM325286) Initial cell density (CFU/mL): <i>S. aureus</i> 3 μ L of 1×10^5 <i>F. falciiforme</i> 8 μ L of 1×10^5	- Histopathological analysis via optical and fluorescence microscopy using Gomori-Grocott and periodic acid-Schiff staining - TEM	Developed a model for studying mixed biofilm infections of the corneal surface
Vaginosis (Nash et al., 2016)				
Intravaginal infections are induced through bacterial inoculation Animals: 8–10-week-old ♀C57BL/6 mice, age matched C3H/HeN mice (KK.Cg-A ^{Y/J}), 9–11-week-old KK.Cg-A ^{Y/J} mice	Test whether <i>C. glabrata</i> like <i>C. albicans</i> results in inflammatory immunopathogenic vaginitis in a diabetic mouse model	<i>C. glabrata</i> (BG2) <i>C. albicans</i> (DAY185) Initial cell density: <i>C. glabrata</i> 2 $\times 10^6$ –1 $\times 10^7$ CFU <i>C. albicans</i> 5 $\times 10^4$ –5 $\times 10^6$ CFU	- Colonization was assessed by quantitative culture of vaginal swabs - Confocal microscopy of excised and stained vaginal tissue	- Developed a model for studying vaginal infections caused by <i>C. glabrata</i> - Determined that <i>C. glabrata</i> does not elicit the same immunopathology as <i>C. albicans</i> during vaginal colonization <i>C. glabrata</i> does not enhance <i>C. albicans</i> pathogenesis

(Continued on next page)

Table 3. Continued

Description	Application	Microorganism(s)	Biofilm analysis	Outcome(s)
Vaginosis (Hymes et al., 2013)				
Intravaginal infections are induced through inoculation with <i>G. vaginalis</i> along with DNase or gelatin as control Animals: 8-week-old ♀C57BL/6J mice	Demonstrate that eDNA secreted from <i>G. vaginalis</i> biofilms is critical for the structural integrity of newly forming and established biofilms	<i>G. vaginalis</i> (ARG37) Initial cell density: 5×10^6 CFU	Colonization was assessed by quantitative culture of vaginal swabs	- Developed a model for studying vaginal infections caused by <i>G. vaginalis</i> - Illustrated that intravaginal treatment with DNase inhibits <i>de novo</i> biofilm formation while also liberating <i>G. vaginalis</i> from existing biofilms without killing, a process that decreases biofilm density and could potentially enhance the effect of antimicrobials
Meningitis (Grumbein et al., 2016; Zhang et al., 2018)				
Injection of a bacterial inoculate through the intracranial subarachnoidal route of infection. Injection site is located 3.5 mm rostral from the bregma Animals: 6-week-old ♀ BALB/c mice	Investigate the roles of <i>S. suis</i> biofilms in meningitis	<i>S. suis</i> (P1/7) Initial cell density: 3×10^7 CFU	- Colonization was assessed by quantitative culture - Light microscopy of histopathological samples	- Developed a model to study the mechanism of <i>S. suis</i> meningitis and the pathogenesis of other meningitis causing bacteria as well as the efficacy of new drugs against bacterial meningitis - Showed that disruption of the blood-brain or blood-cerebrospinal fluid barrier by <i>S. suis</i> are important steps in the development of meningitis
Chronic abscess infections (Pletzer et al., 2017)				
Bacteria are injected onto the right side of the dorsum underneath the thin skeletal muscle Animals: 7-week-old ♀ (25 ± 2 g) & ♂ (35 ± 5 g) CD-1 mice, 15-week-old ♀ (35 ± 5 g) CD-1 mice, 7-week-old ♀ & ♂ (17 ± 2 g) C57BL/6 mice	Test the efficiency of treatments and drugs against chronic subcutaneous infections caused by Gram-negative bacteria	(1) <i>P. aeruginosa</i> (LESB58, PA14) (2) <i>A. baumannii</i> (AB5075) (3) <i>K. pneumoniae</i> (KPLN49) (4) <i>E. coli</i> (MG1655) (5) <i>E. cloacae</i> (218R1) Initial cell density (CFU): (1) 50 μ L of 10^7 ; 50 μ L of 10^6 ; (2), (3), & (4) 50 μ L of 10^8 ; (5) <i>E. cloacae</i> 50 μ L of 10^7	- Progression of the infection was monitored by measuring the size of the abscess lesion - Skin abscesses were excised and homogenized to determine bacterial counts by serial dilution - Histology, qPCR, and IVIS	- Developed a murine model that can be used as a rapid and easy <i>in vivo</i> secondary screening assay for testing novel compounds, enabling toxicity studies, and determining their efficacy against a variety of Gram-negative bacteria - Testing of organs showed that infections did not disseminate

(Continued on next page)

Table 3. Continued

Description	Application	Microorganism(s)	Biofilm analysis	Outcome(s)
<i>Recent in vivo biofilm models for device-related infection</i>				
Cochlear implant (Cevizci et al., 2015)				
Bacteria are instilled into the middle ear Guinea pigs through the tympanic membrane. Small pieces of cochlear implant are then implanted under the skin in the retroauricular area after being soaked in a pneumococcal solution	Assess the effect of a QS inhibitor against otitis media and biofilm formation on cochlear implants	<i>S. pneumoniae</i> (19F) Initial cell density: 500 μ L of 1×10^5 CFU	SEM	- Developed a model for cochlear implant infection - Illustrated a novel QS inhibitor that could prevent biofilm formation on cochlear implants
Animals: 500–600 g ♀ & ♂ Dunkin Hartley Guinea pigs				
Neurological device (Glage et al., 2017)				
Two holes are drilled in the cranium. The holes are inoculated with bacteria and then fitted with titanium screws	Test whether intraoperative infection of intracranial screws would lead to biofilm formation and inflammatory tissue reaction	<i>S. aureus</i> (36/07, ZTL) Initial cell density: 5 μ L of 1×10^7 CFU	Bacterial growth and biofilm formation on the screws was analyzed by CLSM after staining with propidium iodide and Syto 9	- Developed a model for cranial implant infection - Allows for the study of pathophysiologic mechanisms leading to implant failure, test novel therapeutic interventions, antibacterial coatings, and novel materials
Animals: 210–260 g ♀ Sprague-Dawley rats				
Neurological device (Snowden et al., 2012)				
Catheters are colonized with bacteria and then placed into the lateral brain ventricle. After catheter placement, the burr hole is sealed with bone wax, and the incision sealed with surgical tissue glue	Characterize the nature of the inflammatory response during biofilm growth within central nervous system catheter	<i>S. aureus</i> (MSSA) Initial cell density: 2×10^4 CFU/mL	- SEM of catheters after removal - Quantitative bacterial plating of catheters and surrounding homogenized tissue	- Developed a model of central nervous system catheter infection - Provides tool for screening patients at high risk of infection and testing adjunctive therapies to current antibiotic treatment regimens
Animals: 8–9-week-old ♂ C57BL/6 mice				

touching the iris while using the left eye as an untreated control. In order to assess the growth of the bacteria at 24 h after inoculation, some of the eyes were enucleated and homogenized to allow for quantitative plating, whereas others were fixed for histological analysis via light microscopy. In addition to illustrating *S. aureus* biofilm formation in the anterior chamber, the study revealed a link between CodY regulation and endophthalmitis repression while also showing that the addition of branched-chain amino acids to eye drops could possibly reduce the progression of bacterial-related endophthalmitis (Sadaka et al., 2014).

Like many of the other *in vivo* models, we have touched on the models that have been recently developed are rather simple in design. Vaginitis is another tissue-related infection for which there was up until recently no animal model in which to study biofilms. In 2013, Hymes et al. (2013) developed a murine vaginitis model to demonstrate that eDNA secreted from *G. vaginalis* biofilms is critical for the structural integrity of newly forming and established biofilms. Vaginal biofilms were induced through intravaginal infections through inoculation with *G. vaginalis*. The progression of the infection was assessed by quantitative culture of vaginal swabs. During the initial inoculation, the *G. vaginalis* was either added in combination with DNase or a gelatin control. The results from this study showed that intravaginal treatment with DNase inhibits *de novo* biofilm formation while also liberating *G. vaginalis* from existing biofilms without killing, a process that decreased biofilm density and possibly enhance the effect of antimicrobials (Hymes et al., 2013). While this finding is interesting, there was actually no direct analysis of biofilm formation in this model, something that was improved on by Nash et al. (2016), who were looking to develop a similar diabetic murine model in which to study vaginal infections caused by *C. glabrata*, *C. albicans*, and whether the presence of *C. glabrata* enhances the pathogenesis of *C. albicans*.

Again, intravaginal infections were induced through inoculation with either *C. glabrata* or cocultures with *C. albicans*. Colonization was assessed by quantitative culture of vaginal swabs and visualization of biofilm infection, and inflammatory immunopathogenic response was accomplished through CLSM. Overall, they were able to determine that *C. glabrata* neither does elicit the same immunopathology as *C. albicans* during vaginal colonization nor does its presence enhance *C. albicans* pathogenesis (Nash et al., 2016).

The first animal model of biofilm-mediated meningitis was reported by (Zhang et al., 2018) to investigate the role of *S. suis*-derived biofilms in meningitis. The infection was introduced via injection of *S. suis* inoculum through the intracranial subarachnoid route of infection, which is located 3.5 mm rostral from the bregma. Colonization was assessed by quantitative culture and light microscopy of histopathological samples after sacrifice. This model can be not only used to study the mechanism of bacterial meningitis or the efficacy of new drugs but also showed that disruption of the blood-brain or blood-cerebrospinal fluid barrier by *S. suis* are important steps in the development of meningitis (Zhang et al., 2018).

Recently, Pletzer et al. (2017) developed a simple murine abscess model for studying subcutaneous chronic Gram-negative infections. Unlike previous chronic infection models, this model is not technically challenging and can be easily tracked without sacrificing the animals. Subcutaneous injection of the Gram-negative bacteria results in the formation of a visible abscess whose progression can be monitored visually and sized using calipers. Furthermore, inoculation with luminescent bacteria allows for visualization of disease progression using noninvasive *in vivo* imaging system. Extraction of the abscess followed by homogenization and plating allows for quantitative analysis of colony-forming units. Furthermore, dissemination of the infection can be determined by postmortem analysis of the surrounding organs.

In addition to these tissue-related infection models, a few new biofilm models pertaining to device-related infections have been recently established. These include a model for biofilm formation on cochlear implants (Cevizci et al., 2015) and a couple of neurological devices such as central nervous system catheters (Snowden et al., 2012) and surgical screws (Glage et al., 2017). In 2015, Cevizci et al. (2015) developed a guinea pig model in which to study biofilm-mediated infection of implanted cochlear devices and to test a QS inhibitor. The model was generated by instilling *S. pneumoniae* into the middle ear of the guinea pigs through the tympanic membrane, while small pieces of cochlear implant were implanted under the skin in the retroauricular area after being soaked in a pneumococcal solution. Using scanning electron microscopy analysis, they were able to visualize biofilm formation on the explanted device pieces and confirm that their novel QS inhibitor could prevent biofilm formation on the implants (Craig et al., 2004). As for the neurological devices, Glage et al. (2017) developed a rat model to test whether intraoperative infection of intracranial screws with *S. aureus* would lead to biofilm formation and inflammatory reaction, whereas Snowden et al. (2012) developed a mouse model to characterize the nature of the inflammatory response during biofilm growth within a central nervous system catheter.

The more we learn about the underlying complexity of biofilm formation in living organisms, the more evident is the need to further advance in developing *in vitro* models that more closely mimic the *in vivo* setting where biofilms form and mature. By now, we expect the reader to agree with us that the “one-fit-all” approach is certainly unrealistic and not practical. Further, when one is presented with the choice of selecting or developing an *in vitro* biofilm; the first step should be assuming that no model will ever be able to fully recapitulate how bacteria will be challenged *in vivo*. Next, deciding on what critical factor(s) must be incorporated in an *in vitro* biofilm model (Figure 11) are equally important as acknowledging the intrinsic limitation of that specific model. Thus, for example, not including the immune response and cellular interactions for most of the current *in vitro* biofilm models have impacted on how we understand biofilm formation and bacterial colonization in living organisms.

Although recent progress in the field has considered incorporating the 3D feature component of biofilms (Ning et al., 2019), most studies have focused on using flat surfaces as main target. This rather oversimplistic conception is a consequence of not considering the multifactorial, and interdependent, mechanisms involved in biofilm formation (“fundamental concepts on biofilm development” section). Thus, a large proportion of currently available *in vitro* models focus on surfaces (including mechanical deformation and geometry), and availability of nutrients (*in vitro* models for biofilm assessment section; Table 2). When looking at the inherent polymicrobial nature of biofilms formed in living organisms; most of the currently available *in vitro* models are limited to a single bacteria strain (Table 2). Modeling biofilm formation in a single-

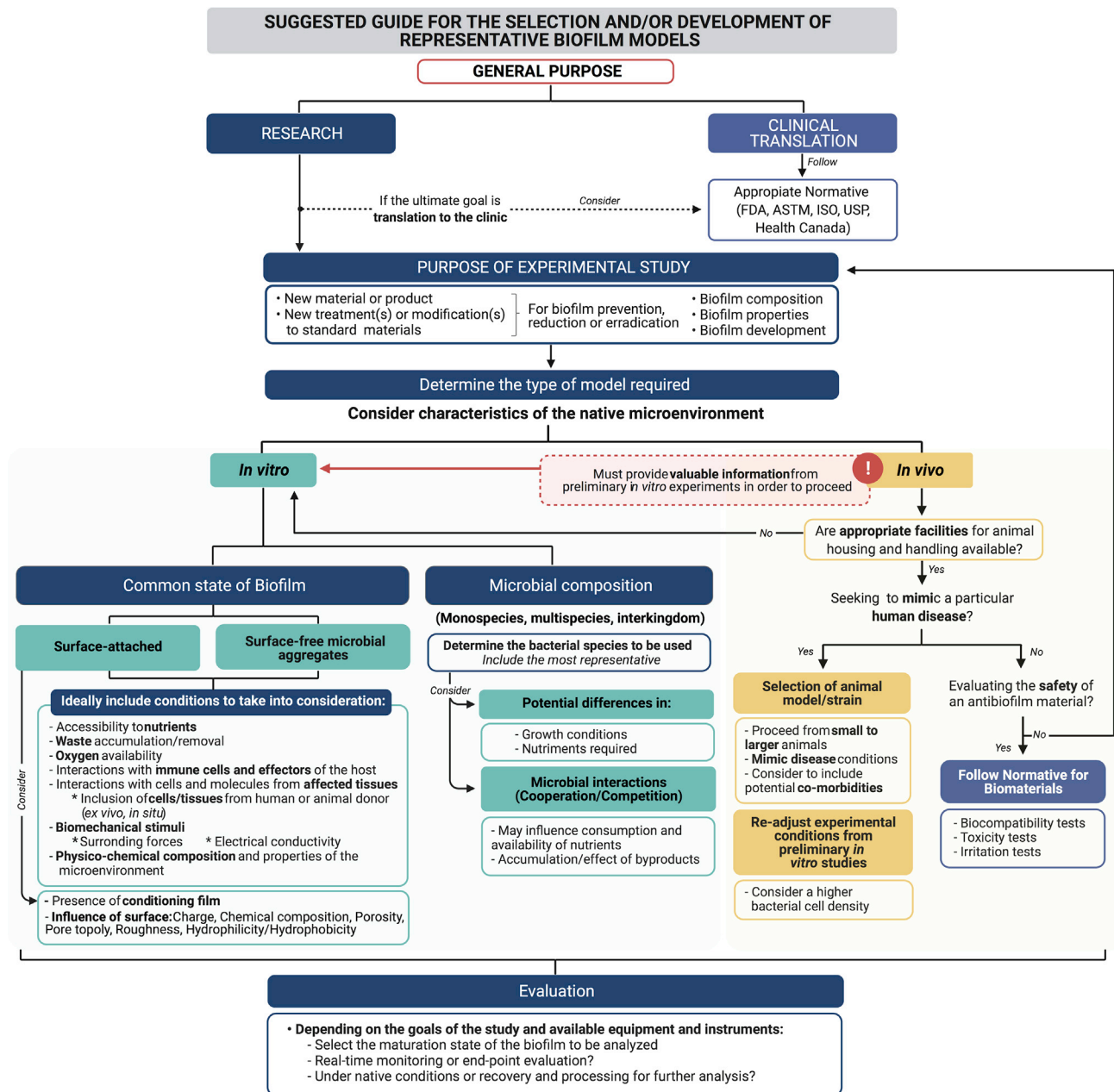


Figure 11. Suggested decision-making system for selecting a biofilm model

species fashion has also intrinsic limitations, particularly when testing materials/therapies designed for preventing, eradicating, and even sensing biofilms. Such limitations, however, go beyond of what can be recapitulated in the laboratory, as the polymicrobial flora of biofilms, despite of having common components, is truly a fingerprint of each individual, including microorganisms that cannot be cultured in the laboratory. Further limitations for *in vitro* models have more to do with variability between the same model used in different laboratories and include using (1) bacteria of the same species but different strains, (2) different media composition and nutritional content, (3) growth time of the biofilm, and (4) chosen method for evaluating biofilm formation and maturity (including real-time monitoring), to name some factors.

In vivo models (*in vivo* models for biofilm assessment section) have also their own limitations. For starters, the immune system in animals, particularly in small rodents, is not fully comparable to that from humans. Apart

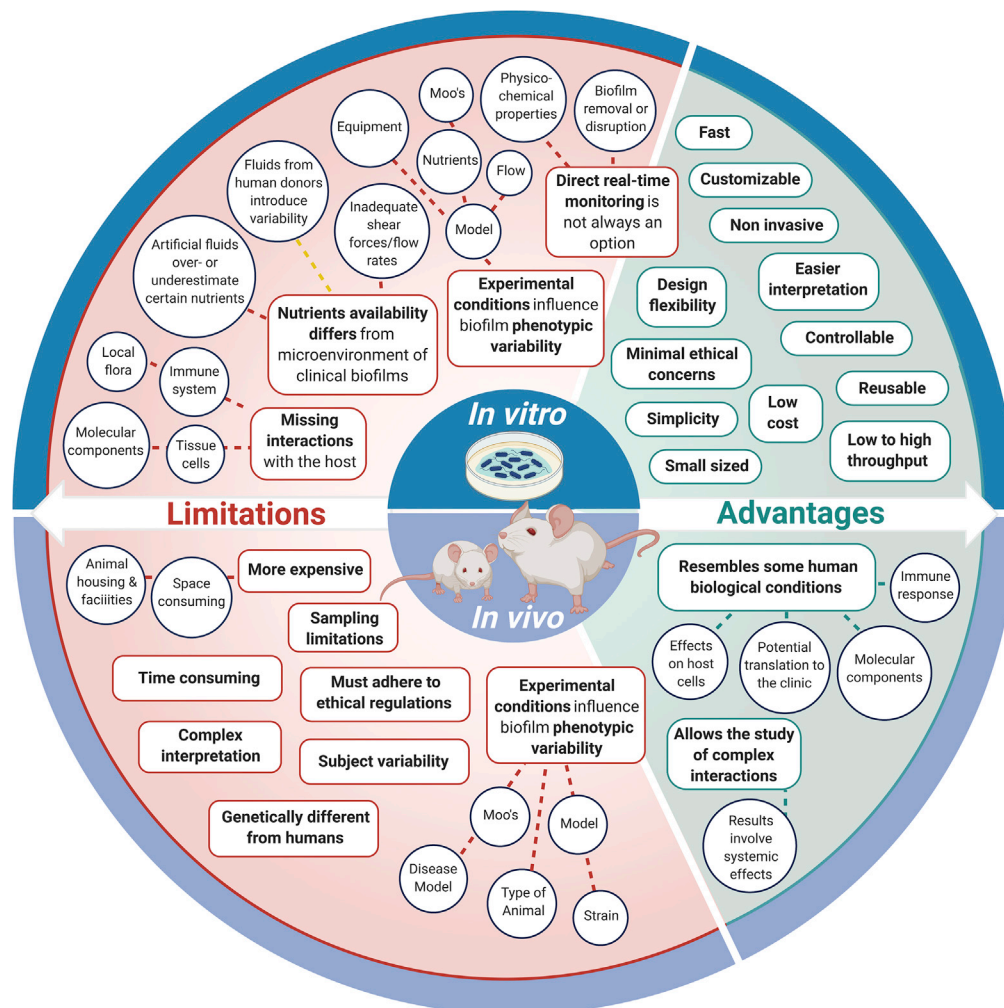


Figure 12. Schematic representation for the limitations and advantages of *in vitro* and *in vivo* biofilm models

from being expensive, assessing biofilm *in vivo* needs of special setup for preventing potential infection of other colonies within the facilities. Further, most studies are short term and carried out in small sample sizes. To test the susceptibility of biomedical devices to bacterial infection using nonclinical bacterial isolates, most tests rely on using relatively large bacterial densities (Table 3). Moreover, most of the *in vivo* models, including those regulatorily approved, use healthy animals that are even further apart from what is seen in patients who need to have implants or interventional procedures. A representative example of this was recently reported by part of our team when assessing bacterial biofilm prevention (*P. aeruginosa*) in skin repair. In that study, diabetic mice showed impaired wound healing and were more prone to bacterial infection, when compared with their nondiabetic pairs (Lazurko et al., 2020). Another important factor to consider as a drawback for *in vivo* models of biofilms rely on the variability of methodologies for evaluating and quantifying biofilm formation (Table 3). As a final note, one must also acknowledge the ethical boundaries for developing *in vivo* models that more closely recapitulate human diseases. Thus, for example, in the case of diabetic foot ulcers, despite the relevance of incorporating the pressure factor in the lower limb wounds; the currently available animal models for that disease aim for assessing biofilms that do not compromise the animal mobility. In Figure 12, we have schematically summarized some of what we consider limitations and advantages of *in vitro* and *in vivo* biofilm models. This list is by no means exhaustive but aims to highlight some of the factors revised in the present review.

NEXT GENERATION OF INTEGRATIVE BIOFILM MODELS

The last 50 years of research in the biofilm and infectious diseases field have brought us closer to developing more functional models of bacterial infection in living organisms. Despite the limitations present

in both *in vitro* and *in vivo* models of biofilms, the advantages of the models we have currently available would be a microbiologist's dream in the 1950s. Biofilms can be modeled *in vitro* at a relatively low cost, with a somewhat decent array of customizable alternatives, including high-throughput capabilities and other options (Table 2; Figure 12). Despite the current limitations, *in vivo* biofilm models allow for more mechanistically accurate assessment of their development and progress within a complex organism. In Figures 9 and 10, we included a road map on how *in vitro* and *in vivo* biofilm studies are normally carried out. Moreover, we proposed a schematic in Figure 11 to offer a simplified guide for scientists on where to start and what is important to consider when there is the need to choose or develop more representative biofilm models for a given condition or an experimental setting.

Ex vivo models could be a good compromise for providing a more biologically relevant *in vitro* bacterial colonization. The cornea is considered an immune-privileged organ, meaning triggering an immune response to the corneal structure is relatively tricky. Very recently, rabbit and porcine corneas have been proposed as an alternative for developing "in vitro" models that allow the evaluation of potential antimicrobials in fungal and bacterial keratitis (Okurowska et al., 2020; Pinnock et al., 2017). However, when working in perfused tissues, such as the heart, modelling endocarditis needs to encompass the physiological temperature, volume, flow, and shear conditions (Lauten et al., 2020) with the innate immune response. The rapid progress in microfluidics and organ-on-a-chip, in what is called "body-on-a-chip" (Dehne and Marx, 2020), could open a new door for seemingly integrating endogenous immune response and high-throughput capabilities in the study of biofilms. Such technologies could be, for example, coupled with the fabrication of 3D bioprinted structures to more closely mimic the complex structure present in living organisms.

Further refinement of biofilm models will need to integrate advanced computing tools to pinpoint critical features in biological systems that need to be more finely replicated for *in vitro* models. Humanized animal models could be the next frontier for developing more precise *in vivo* models for biofilms, but the ethical issues must be carefully considered. A more viable avenue that could be explored for *in vivo* studies are animal models with comorbidities, such as diabetes, cardiovascular diseases, or obesity, that could more closely mimic underlying medical conditions that human patients would present.

It is difficult to predict what the next generation of advanced models in biofilms would look like. Nevertheless, the recent advances in biofilm models discussed in this review illustrate how crossdisciplinarity is contributing to accelerating the development of more physiologically accurate biofilm models.

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The article was written through contributions from all authors. All authors have given approval to the final version of the article. Figures in this work were created using the BioRender.com platform.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

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