CHAPTER

21

In silico and in vivo methods for designing antibiofilm agents against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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1. Introduction

Biofilm is a term used to describe microbes that are accumulated on a surface and have a polymer-encased community. After the microbial cells adhere to the surfaces, biofilm development is initiated. Suspended counterparts are differentiated from the biofilm associated cells by the up/down gene regulation, extracellular polymeric matrix generation, and reduction in the growth rate. Some basic requirements needed to be fulfilled for the biofilm formation are transcription of a variety of genes different from the planktonic cell and the intracellular signaling (Donlan, 2002; Kokare et al., 2009). Embodiment of surfaces for the attachment of microorganisms results in the substantial enhancement of bacterial activity and growth (Bottle effect) (Kokare et al., 2009).

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Microbial Biofilms
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Biofilms are liable for a plethora of diseases with high levels of difficulty in effective treatment because of their resistance to host defense systems and antibiotics (Wilson, 2001). In case of oral infection, the oral biofilm contains a complex concoction of microorganisms, which constitutes a community with a suitable composition required to remain stable (termed a climax community) because of both antagonistic and advantageous variety of interactions such as coaggregation, bacteriocin production, competition for nutrients, and food webs between the constituent species (Marsh & Bradshaw, 1997). Apart from oral biofilms, biofilm-associated human diseases usually occur when the host defense systems display some sort of impairment like in the case of cystic fibrosis patients with pulmonary infections or due to the existence of some medical device implant such as joint prostheses, catheters, etc. (Costerton et al., 1999).

Due to the problems caused by the biofilms, prevention or proper eradication of biofilms is necessary, which leads to the formulation of antibiofilm strategies. Complete knowledge of the biofilm formation process is necessary for the designing of antibiofilm activity. So to fight against the biofilms, two strategies have been proposed after studying the biofilm formation process—first strategy comprises the eradication of biofilm cells and the second strategy is more of a prophylactic strategy, which aims toward the prevention of bacterial cell adhesion to the surface. Also at the same time, to limit the nosocomial infections, some preventive hygiene protocols such as cleaning the hands, rooms, or apparatus in a hospital environment have been formulated and followed (Jones et al., 1995; Thebault et al., 2013).

After the formulation and execution of antibiofilm strategy, it is studied and compared among various biofilm-forming microorganisms to understand the effectiveness of the eradicative or preventive strategy used.

2. Biofilms

A biofilm can be defined as a systematically participating, properly coordinating association of microorganisms. Biofilm formation is a cyclic step by step process comprising—conditioning layer formation, bacterial adhesion followed by bacterial growth, expansion of biofilm, and at last active dispersal (Fig. 21.1). Biofilms can be seen prevailing on almost all types of surfaces such as food products, materials for medical implantation, tissue, various soil particles, glass, wooden, metallic and plastic surfaces, etc. (Kokare et al., 2009). A bridge between the conditioning film and the bacteria is formed, which is mediated by the locomotive organelle of bacteria such as pili, flagella, and fimbriae to help in the production of extracellular polymeric substance (EPS) by the accretion of multilayered cells. Microbial biofilms naturally have a high level of organization with some special characteristics that they can develop a 3D structure or a single layer and may exist in communities of multiple or single species (Form, 2002; Kumar & Anand, 1998) (Table 21.1).

3. Biofilms and human diseases

The formation of biofilms not only makes bacteria less susceptible to antibacterial agents but also kills the host's immune effector mechanism, creating a new niche that provides a protective mechanism that allows pathogens to survive and enable them in hostile environments,

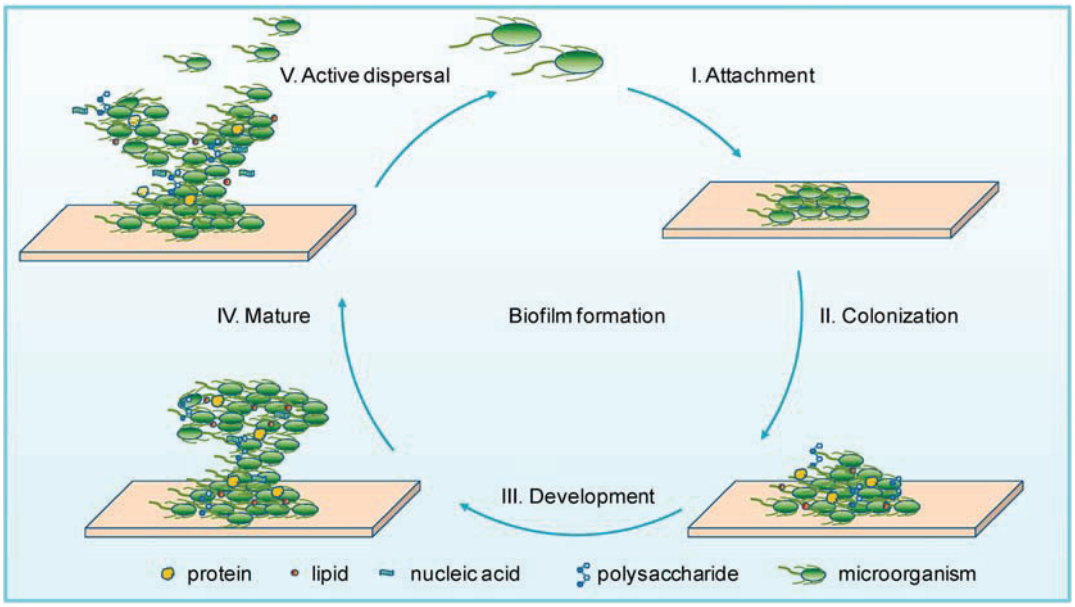

FIGURE 21.1 Cyclic step by step process of microbial biofilm formation consisting of five specific stages: I. Attachment: Reversible adhesion of microbial cells on the surface through weak forces (such as Van der Waals). II. Colonization: Irreversible attachment of microbial cells to the surface via locomotive organelle such as pilli, flagella, and fimbriae. III. Development: Production of EPS by the accumulation of multilayered cells. IV. Mature: Formation of a 3D stable community. V. Active dispersal: Return of microorganisms to the planktonic state after their dissemination from the accumulated biofilm (Yin et al., 2019).

TABLE 21.1 Biofilm-forming organisms and their respective site (Wilson, 2001).

Sr. No.	Microorganism	Biofilm formation site
1.	*Staphylococcus aureus*	Implantable medical devices
2.	*Staphylococcus epidermidis* and other coagulase-negative staphylococci	Implantable medical devices
3.	*Pseudomonas aeruginosa*	Lungs of cystic fibrosis patients
4.	*Escherichia coli* and other enterobacteria	Urinary catheters
5.	*E. coli*	Intestinal tract
6.	*Streptococcus* spp.	Teeth
7.	*Actinomyces* spp.	Teeth
8.	*Lactobacillus* spp.	Vagina, teeth

and allows the formation of colonies. Biofilm diseases include device-mediated infections, medical device malfunctions, and even chronic nonforeign body infections. Many infections in developed countries include myelitis, endocarditis, urinary tract infections, chronic

prostatic inflammation, sinusitis, periodontitis, middle ear infections, and chronic lung infections in patients with cystic fibrosis included in illness. It is caused by biofilm-related microorganisms (Hall-Stoodley et al., 2004). Nasal infections are specifically associated with indwelling devices (Del Pozo & Patel, 2007). This poses a major public health problem, as the burden on this medical facility is enormous. Due to the inherent antibiotic resistance of sessile bacteria, biofilm diseases are difficult to treat and generally result in the failure of antibacterial treatment. Biofilm-related diseases contribute to increased mortality and patient morbidity, representing a significant financial burden, and device-mediated biofilm infections cost more than $ 1 billion annually in hospitals in the United States (Darouiche, 2004).

3.1 Device-related biofilm disease

The use of embedded medical devices has become essential in modern medicine. Apart from the excellent benefits rendered by these foreign body implants, infectious complications are frequently observed (Fux et al., 2003). Vascular prosthesis, electrodialyzer, joint prosthesis, cardiac pacemaker and heart valve prosthesis, intravenous catheter, intratracheal tube, allogeneic dysplastic orthopedic device, urinary stent, peritoneal dialysis catheter, CSF shunt, breast implant, voice prosthesis, abdominal drains, intrauterine devices, bile duct stents, tissue fillers, contact lenses, and renal shunt tubes are just a few cases that have proven important to patients. However, these devices are also associated with an increased risk of biofilm-mediated infection. These infections can be fatal and are often caused by bacterial species that are ubiquitous in soil, air, water, or human skin. Bacterial colonization on these devices can occur within 24 h and can be enhanced by host-produced conditioning films such as platelets, tissue proteins, and plasma (RaadBodey, 1992). Medical devices not only serve the main purpose of mimicking the normal functioning of vital organs, but also serve as a competitive ground for the host cells and microorganisms (Gristina et al., 1988). Device-related nosocomial infections are initiated by colonization of the material surface of medical equipment, but the origin of microbial infections is either endemic in vivo or extrinsic in vitro, followed by biofilm formation (Rutala & Weber, 1997).

3.2 Chronic biofilm diseases not related to devices

Cystic fibrosis, chronic obstructive pulmonary disease, tuberculosis, chronic wound infection, chronic sinusitis, osteomyelitis, tooth rot, bile duct infection, bacterial prostatic inflammation, endocarditis, periodontitis, and otitis media are a part of a biofilm disease that is not associated with implantable devices (Luis Del Pozo, 2017). It has been found that biofilm microflora present in soft tissues can be regularly exposed to antibiotics below minimal inhibitory concentrations. It affects the physiology of bacteria, causes phenotypic and genotypic changes in biofilms, and accelerates the spread of antibiotic-resistant strains from biofilms (Andersson & Hughes, 2014).

3.3 Malfunction of biofilm-related devices

The final stage of the inflammatory and wound healing response after transplantation of medical devices is the foreign body reaction between macrophages and foreign body giant

cells. During the first 2–4 weeks after implantation of a medical device, the surface properties of the biomaterial play an important role in regulating the foreign body reaction (Glage et al., 2017a). Biofilm microorganisms that grow in a foreign body reaction environment are known to cause deterioration of biomaterials and lead to failure of clinical equipment (Del Pozo et al., 2009). Implant dysfunction presents with minor clinical symptoms such as slight soft tissue contraction and pain, as well as dysfunction due to negative inflammatory markers. The clinical manifestations of biofilm-related device malfunctions can be devastating. These include breast augmentation, capsule contraction, bile duct obstruction, endovascular catheter dysfunction, CSF shunt dysfunction, chemical deterioration and physical damage to pacemaker leads, crystalline attachment to urinary stents, and artificial joint dysfunction. It is included. All of this requires device removal, increases mortality and morbidity, and can result in additional hospital costs for the patient. Understanding these complex interactions between microorganisms and biofilms will lead to the emergence of new biomaterials and tissue engineering structures that can guide biological reactions.

4. Antibiofilm strategies

Two strategies are best known to fight against biofilms: the first one is to eliminate biofilm cells, and the second one is to prevent the formation of biofilm by blocking bacterial attachment. Simultaneously, novel hygiene protocols have come up to reduce nosocomial infections. These preventive hygiene protocols constitute cleaning of apparatus, hands, or rooms in the hospital arena. Jones et al. observed that the number of sessile staphylococci bacteria reduced by 50% when apparatus were cleaned on yearly basis as compared with uncleaned materials (Jones et al., 1995). Since some materials induce microbial contamination whereas others exhibit intrinsic antimicrobial properties, it has been proposed to use the "right materials" for the manufacturing of hospital equipment or fittings such as toilet seats, tap handles, ward entrance push plates, and door knobs. For instance, due to the antimicrobial activity of copper, a hospital in Ireland replaced door knobs in stainless steel by some in copper (Thebault and Lequeux).

4.1 Biofilm eradication

Elimination of biofilms requires high concentration of antibiotics or disinfectants, leading to severe environmental damages and emergence of multiresistance. Hence, some new strategies have been developed. One reliable strategy is the usage of quorum sensing (QS) inhibitors. The process by which bacteria achieve cell–cell communication is QS, which is mediated by small molecules secreted by microorganisms called self-inducing substances. By the inhibition of QS, the communication between the microorganisms gets disturbed. Gene modification is another strategy as seen in a study conducted by Jayaraman et al., where *lasI*, a structural gene for N-(3-oxododecanoyl) L-homoserine lactone (an autoinducer) synthetase in *Pseudomonas aeruginosa* was deleted (Jayaraman & Wood, 2008). This showed defects in biofilm formation due to loss of exopolysaccharide production. Another strategy is the usage of competitive molecules that interfere with autoinducer receptors (Lazar, in press). Nonclassical biocidal compounds can also be used. The mechanical strategy of

removal of biofilm by simple friction can also be considered; however, it can be painful for the patient (Thebault and Lequeux). Enzymatic strategies could also be considered for the biofilm destruction, e.g., dispersin B (Fekete et al., 2011).

4.2 Biofilm formation prevention

This strategy constitutes the elaboration of antiadhesive and/or antimicrobial surfaces to prevent biofilm formation. It either aims to avoid bacterial adhesion or to kill cells that come in contact with the surface. To elaborate antiadhesive materials, the roughness is changed by physical modification (Mei et al., 2011) on surface energy (Churchley et al., 2008) or by immobilization of antiadhesive compounds such as polyethylene glycol (PEG) or polysaccharides (Dong et al., 2011). For the elaboration of biocidal surfaces, incorporation or covalent binding of antibiotics, silver, and quaternary ammonium is often used for immobilization. Another promising tool is the use of antimicrobial peptides (AMPs). This is because AMPs exhibit a wide range of antibacterial activity at very low concentrations and do not promote bacterial resistance. For instance, Humblot et al. immobilized gramicidin A on gold magainin I by self-assembled monolayer (SAM) methods. A reaction between the ester function of magainin and the amine function of gramicidin with the peptides has generated the antimicrobial surfaces. These surfaces showed antibacterial activity against gram-positive and gram-negative bacteria for about 6 months (Thebault and Lequeux).

5. Antibiofilm studies

Antibiofilm research suggests a good strategy to counter antibiotic research, as biofilms show increased resistance to most conventional antimicrobial agents prescribed by doctors. In recent years, the focus has been on the discovery of new bioactive compounds with desirable antibacterial properties. Recently, literature reports have pointed to the use of potential compounds that disrupt the biofilm-forming ability of microorganisms. Researchers have identified natural antibiotics due to the low efficacy of various treatments and the in vivo toxicity of available antibiotics (Cattò & Cappitelli, 2019). These approaches interfere with certain important steps associated with biofilm formation: (1) easy removal of microorganisms is possible prior to biofilm formation through a surface sensing process, keeping breakthrough cells in a floating form; (2) destruction of biofilm structure by attacking the matrix, or destruction of biofilm physical integrity by disrupting cell-cell communication; (3) helps spread biofilm by forcing a plankton state. Several natural and synthetic compounds, as well as matrix-targeting enzymes, are immobilized on the polymer surface based on the mechanism of action of prebiocide-free antibiofilms. This provides a promising bioinspired, environmentally friendly antibiofilm material that can replace or integrate with the currently mainstream biocide-based approaches (Cattò & Cappitelli, 2019).

For example, biocompatible poly(lactic-co-glycolic acid) coatings containing natural clove oil or eugenol exhibited efficient biofilm inhibition and less toxicity on solid surfaces against Enterohemorrhagic Escherichia coli O157:H7 (EHEC) (Cattò & Cappitelli, 2019). Natural compounds such as zosteric acid and salicylic acid were covalently grafted onto the surface of low density polyethylene, reducing *E. coli* adhesion and ultimately reducing biofilm formation by

73%. Phytochemicals are known to inhibit QS mechanisms by blocking QS inducers such as AHL and autoinducers (Ciric et al., 2019). Antiadhesion properties of *Psidium guajava* L. ethanol and acetone extracts have been studied (Razak & Rahim, 2003). Biosurfactants prevent the formation of biofilms by reducing the hydrophobicity of the cell surface, interfering with cell adhesion capacity through inhibition of the electron transport chain, membrane disruption, and controlling the energy demand of cells (Satpute et al., 2016). Analysis of the effects of the biosurfactants *Pediococcus acidilactici* and *Lactobacillus plantarum* on the QS signaling molecule revealed downregulation of biofilm-related genes such as autoinducer-2 and cidA, respectively (Yan et al., 2019). Antimicrobial Peptides (AMPs) esculentin-1a, esculentin-1a(1-21)NH_2 corresponding to the first 20 residues of esculentin-1a, as well as its diastereomer, Esc(1-21)-1c inhibits *P. aeruginosa* biofilm formation through its membrane-destroying activity (Casciaro et al., 2019). Efforts have been made to create specifically targeted multidomain AMPs containing species-specific peptides linked to broad-spectrum antibacterial killer peptide domains (Sztukowska et al., 2019). Researchers have set some of the limitations of natural antibiofilm drugs, including unexplored toxicity, degradation by host proteases, long amino acid sequences that boost production costs, and limited knowledge of the structure–function relationships of these compounds. Identified. Attempts to overcome the implementation (Fjell et al., 2012) include deletion and/or substitution of amino acid residues, cyclization, retro-inverso-peptide designs using D-enantiomer amino acids and hybrid construction, sequence truncation, or computational methods (Fuente-Núñez et al., 2016). The next step in this area is to confirm the activity of antibiofilm compounds in animal models of biofilm infection. Although some success has been achieved so far, much work must be done to establish these molecules as viable alternatives to antibiofilm agents and traditional antibiotics.

5.1 In silico

5.1.1 Molecular targets in Pseudomonas aeruginosa

An opportunistic pathogen *P. aeruginosa* is capable of performing group behaviors, including biofilm formation and swarm motility. HHQ (2-heptyl-4-hydroxyquinoline) and PQS (*Pseudomonas* quinolone signal) perform a compelling functions during biofilm provenance as well as in the virulence factor production and its regulation in *P. aeruginosa* (Storz et al., 2012). Transcriptional regulator PqsR is activated by PQS and HHQ, which enhances its own biosynthetic operon pqsABCDE (Wade et al., 2005). Production of signal molecules rapidly increases because of this autoinduction, and their diffusion into the environment leads to a more controlled and coordinated population behavior. In addition, HHQ and PQS show involvement in expressing various genes encoding for virulence factors such as rhamnolipids (a class of biosurfactants with sugar moiety being rhamnose), LasB (elastase B), HCN (hydrogen cyanide), LecA (lectin A), and pyocyanin (Déziel et al., 2005; Van Delden & Iglewski, 1998). Furthermore, PQS has some specific capability in regulating biofilms, even though its mechanism of action is not yet clearly understood (Diggle et al., 2003; Yang et al., 2009).

To identify the potential drug targets of *P. aeruginosa* (PAO1), Rajkumari et al. (2019) docked natural QS signaling molecules and 5-HMF to the transcriptional regulators of RhIR, LasR, and QS circuit. In this study, they used the crystal structure of *P. aeruginosa* regulatory protein LasR bound to its autoinducer solved at 1.80 Å (PDB ID: 2UV0). For RhIR, as

the experimentally determined structure was unavailable, they have utilized their previously published homology modeled structure (Cordeiro et al., 2020a; Rajkumari et al., 2018, 2019) for docking studies. Using the AutoDock Vina program (https://vina.scripps.edu/), they docked the autoinducers C4-HSL, 3-oxo-C12HSL, and 5-HMF onto the binding site of RhIR and LasR proteins. LigPlot+ was used for analyzing hydrophobic and hydrogen bond interactions in between proteins and ligands. Based on docking scores and binding interactions, the best protein−ligand complex was chosen and further subjected to Molecular Dynamics Simulations in GROMACS 5.0.4 with Gromos force field. To further estimate the binding free energy and stability of the docked complexes, MM/PBSA method was used. Gibbs free energy was calculated, and the Visual Molecular Dynamics (VMD) tool was used to analyze MD trajectories (Rajkumari et al., 2019).

This in silico study clearly shows that the natural autoinducer molecules C4-HSL and 3-oxo-C12HSL exhibit a good binding affinity with the cognate receptors RhIR and LasR with the docking energies of −5.4 kcal/mol and −8.8 kcal/mol, respectively. In the catalytic site of RhIR, there is a formation of an H-bond between RhIR and Ser135 (2.99 Å). Similarly, in the catalytic site of LasR, four hydrogen bonds were formed by 3-oxo-C12HSL Asp73(2.6 Å), Ser129(2.5 Å), and Tyr56(2.8 Å, 3.1 Å). The RMSD (root mean square deviation) between natural ligands and biomolecular complexes was within 2 Å, indicating the docking accuracy and precise binding pocket. Molecular dynamics simulation output shows the stability of the LasR-HMF and LasR-3-oxo-C12HSL complexes. The binding free energy estimation from the molecular dynamics simulation trajectory shows that 5-HMF binds stronger with LasR than RhIR (Rajkumari et al., 2019).

5.1.2 *Molecular targets in* Staphylococcus aureus

Biofilm-related resistance of *Staphylococcus aureus* to persistent infections and conventional antibacterial drugs has elevated. Hence a demand is rapidly rising for discovery and creation of various advanced therapeutic alternatives as there is low availability of new antibacterial drugs.

To determine the antibacterial activity of myrtenol against *S. aureus*, Cordeiro et al. (2020a, 2020b) measured the MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration), and MBEC (minimum biofilm eradication concentration). In addition to this, they evaluated the effect of myrtenol in combination with existing FDA-approved antibacterial drugs to assess their synergistic activity against *S. aureus* biofilm formation. They also performed molecular docking and pharmacokinetic predictions to identify possible drug targets and drug likeness (Thomsen & Christensen, 2006).

They performed molecular docking to determine the possible interactions between myrtenol and PBP2 (penicillin-binding protein 2), a transpeptidase enzyme that plays a crucial role in the cell wall biosynthesis. Molecular redocking was performed to validate the efficiency of the docking algorithm in reproducing the crystal pose. Binding energy displayed by myrtenol with PBP2 was 52.3 kcal/mol, and on the data on the active site interactions of the enzyme, hydrogen bonding interactions were performed by the hydroxyl group of myrtenol with residues of Thr600 and Ser403, and with Lys406 van der Waals, hydrophobic interactions were observed. These set of interactions play a vital role as they are executed by the β-lactam drugs after they are get effectively anchored at the active site of PBP2 (Lim & Strynadka, 2002; https://vina.scripps.edu/).

The complete elucidation of the antimicrobial mechanism of action of myrtenol is not yet done. Here the results indicate that for myrtenol to act against *S. aureus*, PBP2 is a possible target. Thus, the substance would show interference in the bacterial cell wall synthesis, therefore leading to cell death (Lim & Strynadka, 2002; https://vina.scripps.edu/).

5.1.3 Molecular docking methods

To discover potential lead molecules and understand their possible drug targets against *P. aeruginosa* and *Staphylococcus*, several researchers used virtual screening and molecular docking as the primary in silico approach. Many different tools, algorithms, and software were utilized for this.

5.1.3.1 AutoDock Vina

AutoDock Vina is an open-source program developed by Scripps Research Institute for molecular docking purposes (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/). It substantially elevates the moderate accuracy of predictions related to binding mode in comparison to AutoDock4. This software was successfully used for evaluating ligand molecules binding onto sites of LasR and RhIR proteins to predict the best possible docked conformations. The best-docked poses were analyzed based on their docking scores (Rajkumari et al., 2019).

5.1.3.2 LigPlot+

LigPlot+ is the successor to the LIGPLOT program. It is an open-source software used to study protein–ligand complex interactions. This algorithm automatically generates a 2D ligand–protein interaction diagram for the given protein–ligand docked complex. It mainly displays hydrogen bonds and their length and nonligand residues in hydrophobic contact(s). Hydrophilic contacts are characterized by spiked arc radiating toward contacted ligand atoms, whereas dashed lines in between involved atoms indicate hydrogen bonds. Spikes radiating back represent the contacted atoms (https://www.gromacs.org/About_Gromacs) (Fig. 21.2).

5.1.3.3 GROMACS

GROMACS is a package full of versatility and is mainly used to perform molecular dynamics simulations of proteins, nucleic acids, and lipids using Newtonian laws and equations of motion. It helps monitor the physical movements of atoms and molecules at specific time intervals. It also helps to study the behavior of systems such as protein–ligand complexes under a particular temperature, pressure, and pH (An Andy et al., 2021).

5.1.4 Recent advances

The current status of antibiofilm drug discovery significantly focuses on the process involved in regulating biofilm formation and inhibiting it. Hence an improved understanding of biofilm formation is essential for discovering various new potential drug targets during the development of antibiofilm agents. On the other hand, a detailed mechanism of action was not well studied for certain existing antibiofilm agents. Therefore, realizing the impact of these agents on biofilms can pave the way for discovering various new targets and pathways to be modulated by new agents.

The target genes, processes, and pathways necessary for biofilm formation can be well explored by omic approaches, including transcriptomics, genomics, and proteomics.

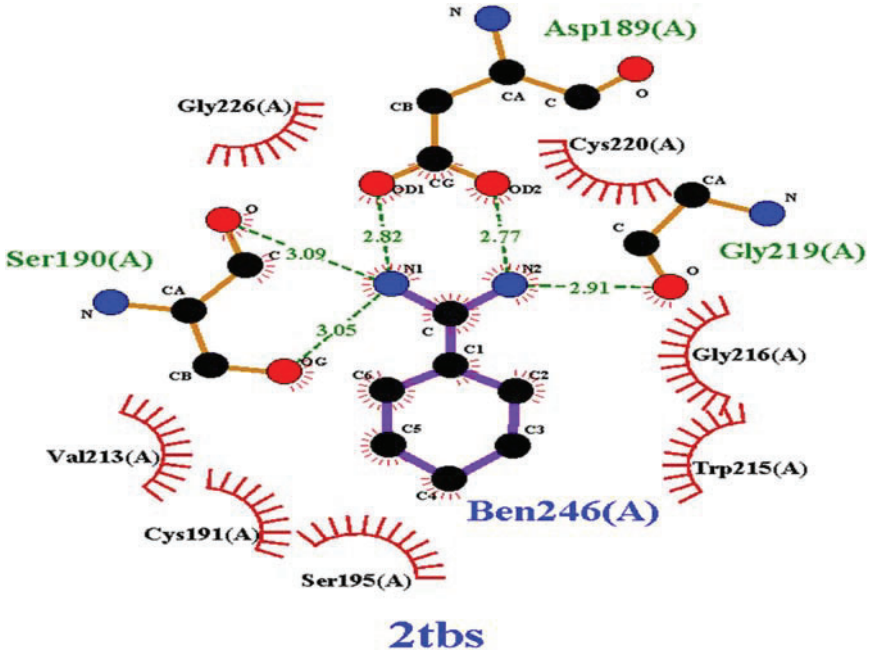

FIGURE 21.2 Ligplot of Benzamide in 2TBS crystral structure. *Courtesy: https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/gif/ligplot_2tbs.gif.*

Generally, these approaches focus on the abundance of different biological molecules such as metabolites, nucleic acids, and proteins in various conditions. Through the comparing molecular changes between diverse conditions in bacteria such as planktonic growth versus biofilm, ± an antibiofilm agent, mutant versus wild-type strains, it can be hypothesized that the changes observed indicate the treatment/condition and possibly affirm the details regarding potential causes behind the resistance and their mechanisms (Noble, 2006).

5.1.4.1 Machine learning

Machine learning is considered to be a more flexible approach as instead of focusing on the target to infer antibiofilm activity, it pays much attention to the properties of molecules. Machine learning is a set of systematic and powerful statistical methods used to make predictions in diverse scenarios, including the prediction of new antimicrobial agents specially targeted to biofilm infections. Usually, an algorithm is created using large significant data sets to learn a relationship between the characteristics expressing the data and the prediction task at hand. With respect to small molecule antibiofilm agents, these characteristics (also known as molecular descriptors) include lipophilicity, steric size, and 3D structure yet there are hundreds of physical–chemical parameters that can be used (Fig. 21.3). The aim of a perfect machine learning model is being unspecified to unseen examples; hence the precision of predictions is generally assessed on examples, which are not used for training (a validation set). Many algorithms have been developed to extricate complex linear and nonlinear

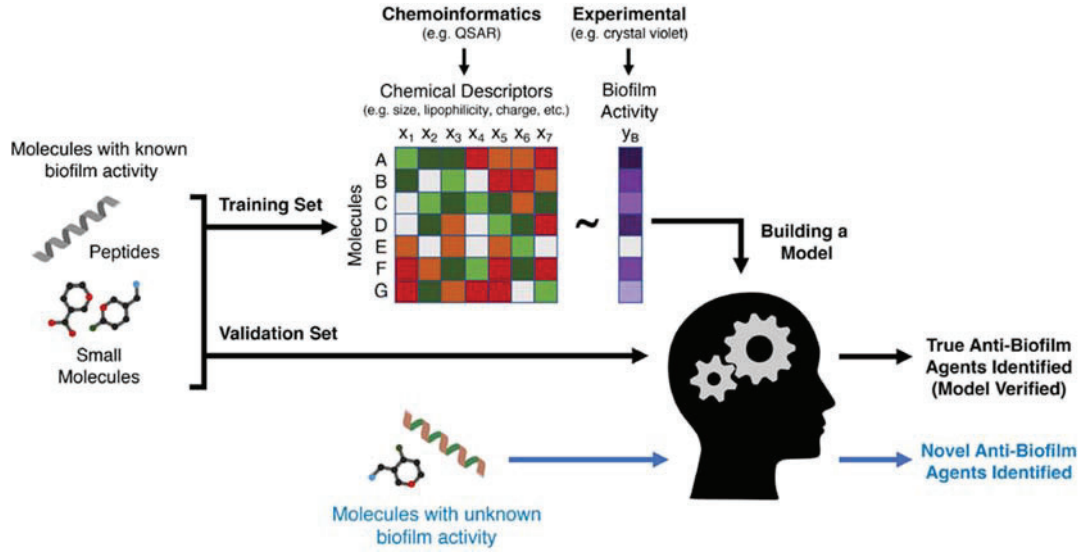

FIGURE 21.3 A schematic diagram of how machine learning can be used to discover new antibiofilm agents.

relationships and modify them into predictive models, including logistic regression, random forest, support vector machines (SVMs), and neural networks (Di Luca et al., 2015; LeCun et al., 2015). The Biofilm-active AMPs database (BaAMPs) was developed to provide access to researchers to a source of peptides to train machine learning models with antibiofilm activity (Gupta et al., 2016). In a recent study, BaAMPs was used to choose 178 antibiofilm peptides for training an SVM model; however, the non−antibiofilm set consisted of randomly produced peptides from all SwissProt database sequences (Sharma et al., 2016).

Likewise, BaAMPs database was used to select 80 antibiofilm peptides to train an SVM model, whereas only QS peptides with no antimicrobial effects were under nonbiofilm set (Haney et al., 2018b). Even though the model precisely predicted known antibiofilm peptides, its potential to predict unknown was not verified. However, it is essential to consider that such validations assume a reproducible standardized assay for evaluation, with in vitro MIC in contrast to biofilm inhibitory concentrations (BICs) and minimal biofilm inhibitory concentrations (MBICs) (Artini et al., 2018). Machine learning models that predicted the antibiofilm activity of naturally occurring essential oils were effectively implemented (Artini et al., 2018; Patsilinakos et al., 2019). The essential oils for two strains of *S. aureus* and two strains of *S. epidermidis* were assayed and utilized the results to train separate models for each strain. The value of training strain-specific machine learning models was highlighted by the fact that there is huge variation of antibiofilm activity of each essential oil (Lo et al., 2018).

The other approaches to represent molecules numerically for the application in machine learning are through fingerprinting and chemical graphs, which capture the atomic structure and connectivity of the molecule (Srivastava et al., 2020). These representations are commonly valid for small molecules instead of peptides with complex secondary structures.

In a study, a hybrid random forest model based on QSAR-type descriptors and chemical fingerprints to identify prospective antibiofilm molecules was trained (Stokes et al., 2020). A 10,208-unit chemical fingerprint was isolated, which was further combined with QSAR descriptors to produce a hybrid classifier. Neural networks are a category of machine learning models that impersonate the operations of neurons in the brain. Explicitly, they permit the models to learn the features through hidden layers and then utilize them to perform the prediction task. A directed message passing a deep neural network to learn a class of chemical fingerprints based on the graph structure was generated (Stokes et al., 2020; Yang et al., 2019). Even though it was not aimed to discover an antibiofilm agent, they predicted and validated the capability of antibiotic halicin for use against *E. coli* infections. This study also showcased the utilization of neural networks and feature learning, which can be applied to identify new antibiofilm agents.

5.2 In vivo

5.2.1 In vivo model biofilm studies

With regard to infection and pathogenesis, there is a continuous interaction between the host and the microorganism and between the microorganism itself. These interactions are complex and dynamic and can affect the fate of infection. Several approaches have been proposed to study this complexity of bacterial biofilms in vivo. Knowledge of physiology and biofilm formation gained through in vitro experiments is an important part of early research on biofilms. However, the urgent need to investigate biofilm-related infections and develop treatment strategies has led to a translational approach that allows the discovery of pathogenicity and pathogenicity determinants and the identification of new therapies. The in vitro biofilm model is a powerful tool in terms of easy treatment and reproducible testing of the effects of factors, but it is the interaction between the host's immune system and the bacteria that make up the biofilm. Therefore, in vitro and ex vivo studies are complemented by in vivo experiments to investigate the immune response to bacterial biofilm infections. Developing a robust in vivo model is the key to supporting in vitro results and is an important step in testing of new therapies and devices on the road to clinical implementation.

The earliest example of an in vivo model for analyzing biofilms was a rabbit for studying osteomyelitis (Scheman et al., 1941). Since then, much research effort has been devoted to the development and exploration of in vivo models. In vivo models of biofilm formation associated with tissue infections, device-mediated infections, and systemic infections are currently being found (Labeaux et al., 2013). Pulmonary infections, chronic wounds, ear, nose, and throat infections are notable tissue infections studied using an in vivo model. These models are also used in orthopedic implants, subcutaneous injection devices, and device-induced infections associated with the urethral catheter. Mammalian and nonmammalian models have been developed for this purpose. The nonmammalian model eliminates certain practical problems such as low cost associated with the breeding and maintenance of small and large animals (Ziegler et al., 2016) but fails to show a complex immune response and optimal growth temperature. It is narrow and has a short lifespan, making it inappropriate for the investigation of relevant chronic infections. On the other hand, an in vivo mammalian model consisting of rats, mice, rabbits, and pigs is used as a representative presenter of human

pathology through similar anatomy, healing process, and immune response (Barre-Sinoussi & Montagutelli, 2015). However, there are situations where nonmammalian models are ideal such as high-throughput screening study (Letamendia et al., 2012).

5.2.2 *Animal models for* Pseudomonas aeruginosa *infections*

P. aeruginosa is an important opportunistic pathogen that can cause acute respiratory infections in immunocompromised and cystic fibrosis patients. These bacteria are encapsulated in a biofilm structure that can withstand a variety of environmental conditions such as immune response and antibacterial therapy. Therefore, *P. aeruginosa* is ubiquitous not only in the environment but also in the in-hospital environment.

5.2.2.1 Invertebrate models

Invertebrate models are inexpensive and relatively easy to set up and maintain. Although the invertebrate model lacks an adaptive immune response, it can be considered a simplified model of innate immunity and is highly responsive to infectious pathogens. The three major invertebrate models of *P. aeruginosa* are described in the following (Fig. 21.4).

5.2.2.2 Nematodes

Caenorhabditis elegans are nematodes that live in the soil. Easy adaptation to laboratory conditions, simple body organization, and a male–female way of life make it one of the broadest subjects of study for cell biologists and geneticists. Since nematodes mainly eat bacteria, they may ingest pathogens such as *P. aeruginosa*. A secondary metabolite called piocyanin is the main virulence factor for *P. aeruginosa*. It has been widely studied in C. elegans. Elevated levels of pyocyanin released into the environment have been shown to be positively correlated with clinical lung deterioration (Mowat et al., 2011). The use of *C. elegans* to study the effects of pyocyanin on the host was reported by Tan et al. (1999).

Infected *C. elegans* respond to pyocyanin with mitophagy, a lysosomal degradation of damaged mitochondria (Kirienko et al., 2015). This mechanism allows the organism to survive and resist infection. Perhaps bacterial killing does not correlate directly with autophagy but plays a role in the destruction of necrotic cells from infected intestines caused by bacterial infection. Autophagy is induced by bacteria in the gut, resulting in upregulation of the EGF

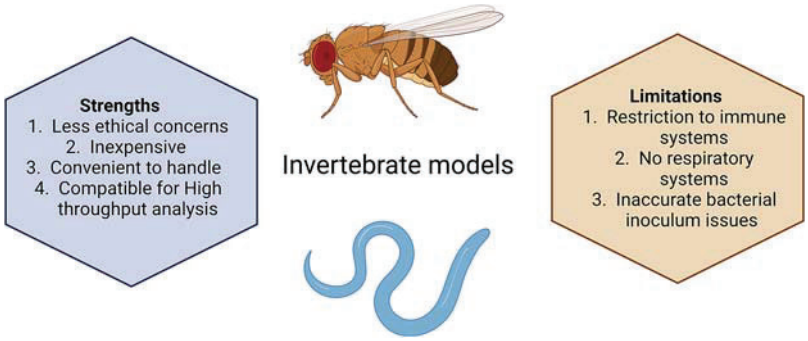

FIGURE 21.4 Overview of strengths and limitations using invertebrate animal models for the study of *Pseudomonas aeruginosa* infections (Lorenz et al., 2016).

homologous LIN3. This activates the MAP kinase-ERK cascade via the EFGR homolog LET2. The intestine is easily destroyed by bacteria, and without this mechanism, it can lead to a systemic infection. It emphasizes the value of considering elegance as a model of infection. *C. elegans* can be used to test the potential for newly induced antibiotic-resistant *P. aeruginosa* (Cabot et al., 2016). Therefore, *C. elegans* can be seen as a bridge between in vitro systems such as cell culture and the most complex in vivo vertebrate models. This helps in the evaluation of new compounds and leads to the establishment of translation potential. Metabolic experiments at *C. elegans* also show the potential of this model system.

While the *C. elegans* model has proven to be a powerful tool for infection research, it also has some drawbacks. First, worms feed on *P. aeruginosa*, but because the number of bacteria that can infect a worm is limited, it cannot affect the absorption of bacterial pathogens. For example, experiments using a high-density transposon mutant library for efficient genome-wide screening of infection-related genes can cause serious bottleneck problems. Also *C. elegans* lack important immune cells and appear unable to signal through the TLR/NF-κB pathway, a cellular signaling pathway that is highly valued in both the plant and animal kingdoms (Irazoqui et al., 2010).

5.2.2.3 Insects

Insects such as *Galleria mellonella* and *Drosophila melanogaster* have certain defense mechanisms that nematodes do not have. This is advantageous for use as an infection model for invertebrates. Pathogen phagocytosis, nodule formation, and encapsulation are characteristic cellular responses of insects to bacterial infections. In addition, the humoral response of insects forms the induction of coagulation, melanization, and antibacterial peptides. Insect blood cells, such as mammalian neutrophils and macrophages, can function as professional phagocytes that can ingest and kill bacteria (Kounatidis & Ligoxygakis, 2012).

Greater wax moths and wax moths belong to the order Lepidoptera. It is also known as a comb moth because female wax moths lay eggs in their nest boxes, eggs hatch, and larvae eat pollen and wax on combs. They have proven to be notable model organisms and are increasingly used in bacterial infection studies. They can be cultivated at 37°C, a natural mammalian host temperature. Caterpillars are available at feed stores and do not require special breeding or care in the laboratory. Caterpillar melanization can be used as a parameter to indicate a fatal infection (Koch et al., 2014). The potential of the *Galleria* larvae model to test *P. aeruginosa* virulence factor, which also affects mammals, has been previously evaluated (Jander et al., 2000). Thirty-two variants of pathogenicity-related genes were compared between *Galleria* and mouse models and showed a positive correlation between them. This emphasizes the value of wax moth larvae as a simple and practical in vivo model that is as close to mammals as the invertebrate model. The *Galleria* in vivo system has recently gained popularity by prechecking the effectiveness of antibiotic ingredients. For example, a potential combination of double or triple antibiotic therapy against multidrug-resistant strain revealed a new therapeutic strategy (Krezdorn et al., 2014). Strategies to counteract virulence factors such as pyoverdine (Ross-Gillespie et al., 2014) and QS effectors (Thomann et al., 2016) were investigated in the *Galleria* model.

D. melanogaster is a favorite subject of genetics and developmental research. Also known as *Drosophila*, its potential use as a model organism has not been fully investigated and is used in the study of bacterial infections. There are two main models of infection: (1) a feeding model

in which a fly ingests a pathogen from the intestine and the etiology can be studied for several days, and (2) a septic injury model in which a fly is stung. They carry bacteria with needles. The power of *Drosophila* as a model for *P. aeruginosa* infection is demonstrated by coinfection experiments using QS variants (Schuster et al., 2013; Lutter et al., 2012). Isolates showing such mutations were compared to wild-type (WT) *P. aeruginosa*. Variants of the nicking system behaved almost like Wt, but in the feeding system, all mutants are nontoxic and require the presence of both QS systems to infect flies. This shows the diversity of the *Drosophila* in vivo system (Lutter et al., 2012).

The *D. melanogaster* model facilitates comparison of multiple genes involved in antioxidant reactions. Recently, FldP, a long-chain flavodoxin from *P. aeruginosa*, was discovered and characterized. It is presented as a virulence factor because it provides bacterial survival to the fly's infectious system and is required for most fly mortality (Moyano et al., 2014). In summary, all these examples demonstrate the importance of fruit fly and wax moth larvae as models of infection. However, the vertebrate model system is essential for more detailed characterization of molecular interactions between the host and bacteria (Fig. 21.4).

5.2.2.4 Vertebrate models

In the arms race between the host and the pathogen, some pathogenic microorganisms can overcome the barriers of the innate immune system. In primitive animals, host death is caused by the defeat of the innate immune system, whereas in higher animals, the adaptive immune system efficiently handles infection under these conditions.

5.2.2.5 Zebrafish

Adult zebrafish Daniel Orerio has both an innate and adaptive immune system. However, depending on the stage of fish development, there is a temporary disruption between the maturation of the two immune systems. Innate immune system or adaptive system "adult" is involved only in the immune response elicited by *Pseudomonas*, depending on the stage of development in which the infection occurred. Zebrafish have gills instead of lungs and are not directly associated with CF-related diseases, but their traceability and genetic characteristics are of importance. This shows that Rerio is a valuable in vivo model system. Zebrafish-fertilized eggs are transparent and fast-growing, so they can be used in combination with appropriate fluorescent markers to monitor the progression of infection in real time. It works by lockers. He emphasized that the bacteria can be injected intravenously in the case of systemic infections and into specific sites such as the posterior ventricle in the case of local infections (Rocker et al., 2015). Bacterial microcolonization, which represents the early stages of biofilm development, was observed in *P. aeruginosa* introduced into the posterior ventricle of the larva to avoid systemic spread and rapid death of the larva. The diversity of zebrafish as an infection model has been demonstrated by Peter et al. SOD2 detected by *P. aeruginosa* zebrafish infection has been shown to be upregulated in hematopoietic and myeloid hematopoietic organs (Peterman et al., 2015).

Detoxification of ROS in these cells has been shown to be important by removing SOD2 and infecting larvae at an early stage of development where only macrophages exist as professional immune cells. These larvae were susceptible to systemic infections and increased mortality. At the same time, zebrafish can be used to investigate the defense mechanisms present in pathogens against ROS. For example, the *P. aeruginosa* transcription factor PA2206 is activated by

ROS stress and also triggers 58 genes that successfully infect zebrafish (Reen et al., 2013). In addition, zebrafish can be used to characterize new concepts and alternative treatment strategies for *P. aeruginosa* infections. Due to the increasing complexity of vertebrate models, zebrafish raise ethical issues related to animal welfare. Apart from that, the temperature of the zebrafish shell is 28°C, which is different from the temperature of the human body.

5.2.2.6 Mammalian models

Mammalian models are specifically used to study a variety of pathogenic symptoms of *P. aeruginosa* infections, including biofilm-mediated infections. Various types of mammals have been used as model systems for studying infectious diseases from chinchillas to pigs and nonhuman primates.

5.2.2.7 Murine models (mouse, rat)

A small rodent model, primarily a mouse, was used to obtain most information about *P. aeruginosa* infection. Bacteria can be administered intranasally, intratracheally, or through the oropharyngeal route of administration. This is a factor that influences final lung colonization (Fisher et al., 2014), infection level, bacterial strain, and outcome of infection. It has little effect on the genetic background of the affected host. Systematic examination of various mouse strains revealed a significant difference between the strains tested and the immune response they exhibited (De Simone et al., 2014). Molecular interactions between pathogens and hosts can be studied in detail by mapping and characterizing these genetic differences.

The role of bacterial factors has been widely characterized in vitro and confirmed using the acute mouse infection model. For example, hemolytic phospholipase C (PlcH), which is involved in the degradation of phosphatidylcholine, a component of pulmonary surfactant, has been mentioned in a recent study (Jackson et al., 2013). It is emphasized that metabolites such as choline regulate the ANR-dependent promoter induced under hypoxic conditions. By the way, PlcH and its regulator GbdR were involved in biofilm formation of lung epithelial cells and colonization of the mouse respiratory tract in acute lung infections. Recently, a new two-component system BfmS/BfmR was discovered that controls quorum detection (Cao et al., 2014). The response regulator BfmR is negatively regulated by the sensory kinase BfmS. Removal of BfmS causes upregulation of BfmR and then rhlR, leading to biofilm maturation, which, along with reduced cytotoxicity to mouse lung epithelial cell lines, is a model of acute mouse infection. It showed a decrease in fitness.

Mammalian models are specifically used to study a variety of pathogenic symptoms of *P. aeruginosa* infections, including biofilm-mediated infections. Various types of mammals have been used as model systems for studying infectious diseases from chinchillas to pigs and nonhuman primates.

The effects of host factors on the establishment and progression of infection can be studied in mouse models. For example, in mice lacking lung-specific vascular endothelial growth factor (VEGF), severe infections and increased inflammatory responses were observed (Breen et al., 2013). The development of antibacterial strategies by discovering new bacterial etiological pathways and metabolic adaptations can be tested in an in vivo model system. Migiyama and others convincingly demonstrated AHL-lactase AiiMP in a mouse model of acute pneumonia. Expression of *P. aeruginosa* by bacterial strains facilitates the investigation of the in vivo efficacy of QS inhibitors (Migiyama et al., 2013). Benefits that mice offer as a test

system include economic factors such as acquisition, care, housing, or genetic variation or alteration. Despite all these aspects, the small size can complicate certain experimental procedures. For example, the rat lung function test is cheaper than the mouse due to its large body size (Fig. 21.5).

5.2.2.8 Combination of different animal models

Collaborative research on various animal models has proven useful. This highlights the possibility of combining different in vivo infection models for an integrated view of the potential pathogenicity of *P. aeruginosa*. The nonvertebrate model is used to screen for new antibacterial compounds that provide information on efficacy and toxicity. You can then advance promising candidates to more complex mammalian models. A comparative study was conducted by Dubern et al. to understand the pathogenicity of C. elegans, and P. aeruginosa transposon mutants in various animal models such as melanogistor, mouse models. In some mutants, the pathogenicity profiles between the models were consistent, while in other mutants, variability was observed (Dubern et al., 2015). This highlights the possibility of combining different in vivo infection models for an integrated view of the potential pathogenicity of *P. aeruginosa*.

5.2.3 *Animal models for* Staphylococcus aureus *infections*

S. aureus is an important human pathogen that causes a variety of clinical infections. It is the leading cause of bacterialemia and infective endocarditis, skin and soft tissue infections, and chest lung and device infections. It is a symbiotic bacterium and a human pathogen. It is known that 30% of the population is colonized by *S. aureus*.

5.2.4 *Advantages and limitations of animal models*

The use of animals for research has been a long-standing practice and is the subject of ongoing debate in the scientific community. Due to the striking similarities between humans

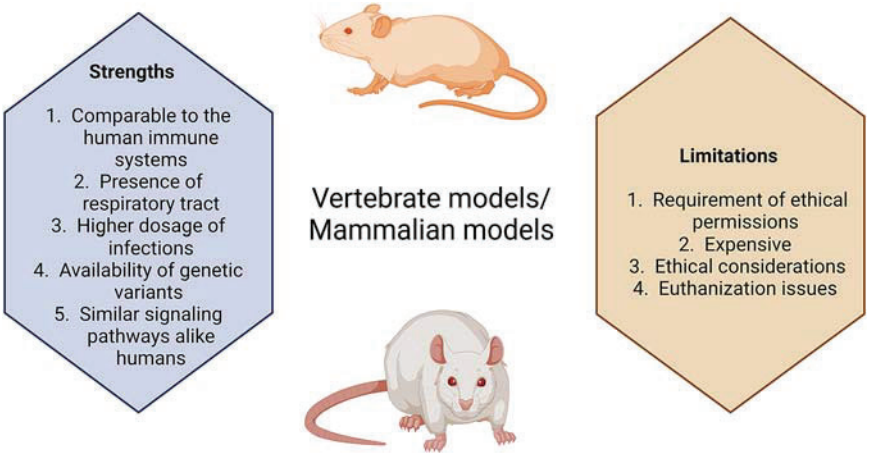

FIGURE 21.5 Overview of strengths and limitations of vertebrate and mammalian in vivo systems in *Pseudomonas aeruginosa* infection research (Lorenz et al., 2016).

and animals, especially mammals, both anatomically and physiologically, researchers have introduced new treatments for animals before testing them in humans. However, not all results obtained in animals can be extrapolated directly to humans. The right to use animals for human benefit, for example, in research, is still controversial as it can harm animals.

In vivo models have been used to answer a variety of scientific questions, from preliminary science to the development and evaluation of new vaccines or therapies. Animal species are also based on being susceptible to human illnesses such as type 1 diabetes, allergies, cancer, high blood pressure, myopathy, and epilepsy, in addition to infectious diseases. Due to the similar mechanisms involved in these diseases, 90% of veterinary drugs are very similar to those used in human treatment. Trials conducted solely on in vivo models are the reason for the great progress in medical research. Banting and McLoed won the 1921 Nobel Prize for establishing a treatment for canine type 1 diabetes. Cell therapies that use stem cells for tissue regeneration have been developed and tested in animals. After being tested in animals, even various surgical techniques have been developed and improved.

Complex organisms, such as humans and other mammals, have organs that have performed different physiological functions. Relationships between organs depend on hormones, circulatory factors, cell networks, and signal transduction between cells in all compartments. To explain and understand the mechanism, it is necessary to investigate at all levels such as molecules, cells, and organs. All three organizational levels can be examined in the in vitro model. However, the whole organism needs to explore the physiological functions and systemic interactions between organs. Hypotheses and models from in vitro study need to be tested and evaluated throughout the organism. If not, it remains speculative. Despite discussions by the Animal Rights Commission, there is still a long way to go to completely replace animal models in vivo.

Although some models of biofilm-related infections are lacking, the use of various in vivo models provides extensive information on most biofilm-related infections over the past two decades. Multiple models per infection to answer questions about initial adhesion, various surface assessments, and prophylactic approaches are shown in the multiple models used in the study of CVC-related infections. An in vivo model is being developed. Depending on the host's immune system, device size or surface area, and environment, each model provides answers to specific questions. Therefore, there is no "gold standard" model specific to each infectious disease.

It should also be noted that the results obtained in animals are not always confirmed in human studies. First, despite the great similarity, there are differences between the given human model and the animal model. For example, 95% of genes between mouse and human are homologous, but there is a clear difference. These genetic differences are later converted into physiological differences. A deep understanding and knowledge of these differences is required before disproving the value of animal models. It can also provide an opportunity to discover new mechanisms and develop innovative strategies. The second reason is genetic and physiological mutations within and between closely related species. A single animal model cannot mimic a particular human disease, which is also polymorphic between patients, but the strain–species difference is novel and explores the difference between disease onset and host response. It offers a great opportunity to develop step-by-step treatments.

The in vivo model has certain limitations. First, the immune system of animals, especially small rodents, is not comparable with the human immune system. Special equipment is

required to evaluate biofilms in vivo to prevent the possibility of infection from other nearby colonies. The use of in vivo models is also considered expensive. In general, these studies are short-term and are conducted with a small sample size. In addition, regulatory in vivo models represent healthy animals that differ significantly from those observed in patients requiring implants or intervention procedures. For example, while the prevention of bacterial biofilms (*P. aeruginosa*) in skin repair is being studied, diabetic mice show impaired wound healing and have recently been reported to be more susceptible to bacterial infections than nondiabetic pairs (Lazurko et al., 2020). Another important parameter that can be considered a disadvantage of biofilm in vivo models is based on various methods for validating and quantifying biofilm formation.

Finally, we must also recognize the ethical limitations of developing an in vivo model that accurately represents human disease. The European Directive has created a regulatory framework for all animal testing. First, if a non−animal-based experimental approach is available, there is no need to use animal models. Second, we need to minimize the number of animals we use. Third, all necessary steps must be taken to reduce the harm done to animals throughout the research process. All projects, including the use of animals, are evaluated against these standard principles known as the Three Rs (Replacement, Reduction and Refinement).

Therefore, research into relevant, carefully designed, well-characterized, and controlled animal models is an essential step in basic discoveries, testing of biological-level hypotheses, and evaluation of human data. Animal welfare is taken into account as more reliable and informative in vivo models need to be improved. These two goals need to be balanced to produce high-quality science (Barre-Sinoussi & Montagutelli, 2015).

5.2.5 Alternate models

Since experiments on animal models are considered to be immoral and time-consuming and most of the time do not resemble human physiology, the researchers are now coming up with new models and animal-free methods, which are more relatively more relevant to human health to study diseases and to test products. These alternatives to in vivo models include advanced tests such as in vitro tests using human cells and tissues and sophisticated computer-modeling techniques popularly known as in silico models. These methods are quick, and research is not hampered by species differences.

5.2.5.1 In vitro models

A simplified in vitro model is important for answering questions about biofilm structure and physiology. Low cost, ease of setup, and high-throughput screening trends are the advantages offered by in vitro models. Based on the restoration of growth medium and the nutrients, these models are classified into static or dynamic type. Static models are generally used to substantiate the formation of biofilm and biomass accumulation (Barre-Sinoussi & Montagutelli, 2015).

Since the medium is not changed during the culture period, the nutritional supply of the static model is limited. These models are simple, cheap, and reproducible. In addition, they are often used to study the growth dynamics of biofilm and antibiofilm compounds (Salli & Ouwehand, 2015). The dynamic model creates an environment for biofilm growth that mimics the natural environment. This is made possible by a constant supply of nutrient

throughout the assay. These models are used for long-term analysis of biofilm growth dynamics as the medium is continually updated and metabolites are discarded. The use of in vivo models has many advantages, such as nutrients for biofilm growth, which can lead to its incomplete maturation, and the lack of an immune response is another limitation of these models (Salli & Ouwehand, 2015; Tolker-Nielsen & Sternberg, 2014).

Currently, there are several commercially available in vitro models for testing biofilms. Microtiter plate-based system with 12-, 24-, or 96-well plates are the commonly used one for studying biofilms (Tolker-Nielsen & Sternberg, 2014). The cultured biofilm grows on the bottom and sides of the microtiter plate, or on specific surfaces placed in the wells of the plate. This system acts as a static model because the medium does not flow in or out (Roberts et al., 2015). The main drawback of these models is that they oversimplify the in vivo environment without in vivo simulation conditions during the assay procedure. The environment in the well changes over time, as nutrients gradually decrease and signal molecules accumulate. Due to the advantages of these plate-based methods, they are regularly used in research. This plate-based system is cost-effective because it uses only a small amount of reagents. It can be used to run many tests at the same time, demonstrating its usefulness in screening tests for further research (Labeaux et al., 2013). Therefore, these models can be used to explore new antimicrobials, disinfectants, and other compounds such as QS modulators with antibiofilm activity against plant extracts and biofilms (Heersink et al., 2003). The method of quantifying biofilms by the microtiter plate-based method is variable and relies on indirect measurements. Crystal violet staining, an XTT reduction assay used to check biofilm viability, is another major drawback of this method (Niu & Gilbert, 2004).

In vitro microplate-based methods can be supported on multiple devices such as biofilm ring tests and Calgary plates (Peeters et al., 2008; Sidrim et al., 2019). The biofilming test is performed on a microtiter plate (Goeres et al., 2009), and an assessment of biofilm growth is performed by quantifying the immobilization of paramagnetic beads by adherent cells and biofilm polymer matrix. This device can be used to study the dynamics of biofilm formation by a variety of bacteria (Goeres et al., 2009). It is also used to determine the effect of matrix components on biofilm growth (Rudney et al., 2012), to detect QS molecules (Chavant et al., 2007), and to compare biofilm-forming abilities of different bacterial species (Badel et al., 2008). Calgary biofilm devices are recognized as a new tool for rapid and reproducible antibacterial biofilm susceptibility testing. This is a plate-based model with a 96-well plate and a fixed lid (plastic invasion) inoculated with bacterial inoculum into wells containing a biofilm growth medium. This forms a biofilm on the surface of the vortex. This method makes it easy to transfer the biofilm from one plate to another, and the availability of fresh medium makes the study model relatively dynamic. It also reduces interference with biofilm growth in media containing metabolites and regulatory molecules (Huang et al., 2009). Due to differences in adhesive surfaces, nutritional support, and molecular interactions, the in vitro model cannot completely replace the in vivo model. However, this limitation can be overcome to some extent by associating these in vitro models with specific medical devices.

5.2.5.2 Microcosm-based biofilm models

It has been recommended to handle microcosms to represent the consumption of nutrient-rich sources or areas similar to location of infection to create a host-like microenvironment, thereby creating environmental conditions similar to infection (Sulaeman et al., 2010). The main goal of these models is to bring samples with minute modifications comprising clinical

and fluid-based samples from various anatomical microenvironments such as reproductive and gastrointestinal tract as well as oral cavity to laboratory. Attempts to create an in vitro condition. Microcosms are broadly differentiated into three types—natural, mixed, and artificial microcosms. Natural microcosm is composed of natural liquids such as saliva and plaque, as well as the biochemical properties of microorganisms and the microenvironment (Goeres et al., 2009). The artificial microcosm contains a medium simulating necessary organic conditions (such as Lubbock's chronic wound model) and is essentially fully synthesized (Junka et al., 2016). In contrast, mixed microcosms contain samples from anatomical sites but are cultured in artificial medium (Sun et al., 2008). Oral microbial biofilms are easy to collect and exhibit a wide variety of microbial species, including those that cannot be cultivated in vitro, so microcosms are primarily developed through them. For primary development of microcosms, oral biofilms are used as they can be easily collected and also display a plethora of microbial species, comprising species that are unable to cultivate by in vitro methods. Moreover, dealing with the microcosm is difficult because it requires skilled human resources, special equipment, and experimental methods to ensure that the microenvironment of that particular anatomical site remains in a state similar to that of the microenvironment (Verardi et al., 2016).

Studies are underway to create reproducible oral microcosm models and to validate existing models because of certain inadequacy of specific methods and diversification within bacterial species (Sun et al., 2008). For example, Fernandez et al. (2017) (Sissons et al., 2007) created a reproducible subgingival biofilm model. They extracted subgingival plaque samples at four depths and used glass slides on the substrate for biofilm growth in patients with periodontitis. Biofilms were cultured under anaerobic conditions and analyzed every 2 weeks. This model is considered simple and effective, producing complicated yet powerful biofilms of various compositions.

Also, the producing and evaluating models of microcosm biofilm are important because research on in vitro biofilm models usually involves a single species that is far from reality. For example, the oral microbial flora contains hundreds of microorganisms that do not form a single biofilm (Fernandez y Mostajo et al., 2017). Microcosms are essentially more representative as they bring a complex microbial environment to the laboratory setting. For example, plaque and saliva infusion of human teeth extracted from the same individual can be performed to monitor tooth biofilm formation (Goeres et al., 2009), which can be used with antibiofilm compounds and more accurate evaluation of compounds with anticariogenic activity (Braga et al., 2018).

To evaluate the oral microcosm experiments and its reproducibility, various studies were conducted. For example, Rudney et al. (2012) to generate a dynamic state biofilm used oral microcosm (CDC biofilm reactor) including human saliva and plaque (Peeters et al., 2008) to assess whether saliva and dental specimen inoculations from the same donor have a similar composition when harvested on different days compared to the composition of inoculations between different donors. It was observed that in the first condition, the biofilm did not possess varied composition but in the second case where inoculum was collected from different individuals presented a different composition. Since, all the samples produced a thick biofilm which implied that they are reproducible (Peeters et al., 2008).

But there are only a few standard models for microcosm of anatomical sites other than the oral cavity. By observing the physicochemical, microbiological, and nutritional aspects of this

environment, an improved understanding and studying of microorganisms and their anatomical insights on interaction with the environment can lead to the various concepts and uses proposed. Some researchers to discover an alternative for genesis of microcosms that can mimic the required environmental conditions for microorganisms are using the provided information on the anatomical sites regarding the biochemical and microbial composition (Woodworth et al., 2008). To study chronic sinusitis, an in vitro model of microcosm of *P. aeruginosa* was prepared by culturing cells of rat respiratory epithelium. Through this study, it was concluded that this model was dependable for assessing interactions among bacterial biofilms and rat respiratory epithelium (Woodworth et al., 2008). In another study, cells of human respiratory epithelium were taken for monitoring the biofilm formation *Aspergillus fumigatus* in patients undergoing EGD (esophagogastroduodenoscopy). *A. fumigatus* was found to have a complicated yet powerful biofilm formed on cells of epithelium. This helps in the further investigation of fungus and its pathogenic characteristics in chronic sinusitis (Singhal et al., 2011).

Cordeiro et al. (2020a, 2020b) using vaginal discharge from women suffering from candidiasis, proposed a microcosm-based model of biofilm to evaluate the infection (Singhal et al., 2011). The secretions were incubated for about 72 h in a medium, i.e., vaginal discharge simulator medium, which resulted in the production of a biofilm with the largest value of cellular density; also a biomass with resistance to fluconazole was obtained after incubating for 48 h. Metagenomic analysis of these biofilms showed that they were more sensitive to fluconazole when they carried high levels of *Lactobacillus* spp. compared with biofilms lacking this genus of bacteria. This model helped study strategies for treating and controlling vulvar vaginal candidiasis. A study was conducted by adding collagen and artificial skin for stimulating an environment similar to that of a protracting injury (Junka et al., 2016). To create a chronic wound environment, Sun et al. developed a Lubbock's chronic wound model using a variety of bacterial species such as *S. aureus*, *Enterococcus faecalis*, and *P. aeruginosa* for identifying the main components in protracting injuries. Therefore, a culture medium containing bovine plasma, meat hydrolyzate, and horse erythrocytes is used to produce plasma, erythrocytes, and necrotic tissue. Due to its inclusion of the main components of chronic wounds, this model is suitable because it allows the study of multiple biofilms along with anaerobic biofilms and the evaluation of antimicrobial agents against microbial biofilms. It also helps improve treatment strategies for these infections (Junka et al., 2016).

Using cell culture as a substrate, these models, which resemble body fluids, allow the interaction between bacteria and adhesive surfaces and the assessment of performance during the formation of microbial biofilm (provided peculiar nutritional support). It is important to be aware of what you do. However, it is not possible to analyze the interaction between symbiotic organisms or the effect of physicochemical properties of the anatomical environment on biofilm growth.

5.2.5.3 Ex vivo models

Investigation and evaluation of infectious processes associated with biofilm formation is possible through the development of many methods (Cordeiro et al., 2020b). To fill this gap, the ex vivo model was created as in vitro models cannot be considered reliable as it lacks valuable biological data on host (Eslami et al., 2019). For creating host-like environmental conditions, ex vivo models are used. Therefore, with certain modifications, an in vitro method can be used for stimulating an environment with in vivo conditions (Gabrilska & Rumbaugh, 2015) and used by the tissue itself as a substrate to generate a really naturally occurring sticky

community in biofilm-related infections of common bacteria and fungi such as infective endocarditis, caries, periodontitis, osteomyelitis. The ex vivo model is used for biofilm research because it is likely to be (Cordeiro et al., 2020b; Eslami et al., 2019). Actually, teeth from various animals and humans were widely implemented for studying various dental ex vivo biofilm models on related diseases such as cavities (Vila et al., 2015), endodontic biofilms, and pulpitis (109). With the exception of pulp, teeth are primarily composed of calcified tissue, which makes them less prone to deterioration (Huang et al., 2019) and easier to sterilize, making them useful for ex vivo biofilm research. The reliability of the results obtained is improved by assigning them to the microcosm of the oral cavity. By creating an environment similar to the in vivo model, the ex vivo model allows large-scale experimental assays to be performed under a variety of conditions and at a relatively lower cost than the in vivo model (Eslami et al., 2019). It can be said as follows: The usefulness of the ex vivo model has provided diverse opportunities for studying multiple bacterial−host interactions. These studies can be performed for proper in vivo guidance and optimization of various assays while animal lives are saved (Carrasco et al., 2017). Therefore, the main advantages of using these ex vivo models are for significantly reducing the number of animals used in the study, addressing ethical concerns related to animal welfare, and reducing the costs associated with raising laboratory animals. These models should also consider using slaughterhouse by-products for maximizing the animal usage that humans will consume. However, from ex vivo point of view, these models have a major shortcoming, and that is the absence of interaction between biofilm structure and the host immune system, the potential lack of normal microbial flora at the studied anatomical site, and the in vivo model which is lack of fluid flow (Yang et al., 2013).

6. Recent advances

Many tissue-borne infections and commonly used medical devices have established animal models that allow biofilms to study disease progression and test new drugs, treatments, materials, and more. There was a push to create a search for a new model that exists to fill the gap where there is no model. A mouse model was developed by Saraswathi and Beuerman in 2015 to study *P. aeruginosa* biofilms in the process of corneal keratitis. They were able to track the progression of *P. aeruginosa*. A corneal biofilm derived from microcolonization of *P. aeruginosa* suspension in a corneal injury model using 7- to 8-week-old C57BL/6 mice. They can visualize and treat biofilm formation on the corneal surface from standard infections when mature biofilms are a common component of keratitis and treatment resistance is observed by Masu (Mao et al., 2018). This will be followed by a study by Ponce-Angulo et al. in 2020, we studied biofilm formation in an immunopreserved mouse model of mixed keratitis. A mixed bacterial and fungal biofilm was identified and characterized by coinfection with *S. aureus* and falciforme (Chuang-Smith et al., 2010) by inoculating a micropocket incision at the edge of the sclera of the cornea. Recently, Prezer and his group have developed a simple mouse abscess model for studying subcutaneous chronic gram-negative bacterial infections. Unlike other chronic infection models, this model is technically feasible and can be pursued without sacrificing animals. The formation of a visible abscess is caused by a subcutaneous injection of gram-negative bacteria, the progression of which is visually monitored and measured with a caliper. In addition, the course of the disease is visualized with a

noninvasive in vivo imaging system by inoculation with luminescent bacteria. Subsequent homogenization and extraction of abscesses by plating enables quantitative analysis of colony-forming units. In addition, postmortem analysis of surrounding organs can be performed to determine the spread of the infection (Plezer et al., 2017). Zhang reported in 2018 on the first animal model of biofilm-related meningitis that investigated the role of *S. aureus*. A biofilm native to Switzerland that causes meningitis has been identified. After infection, quantitative culture and light microscopy of postsacrificial histopathological specimens confirmed the formation of bacterial colonies. This model was used to study the mechanism of bacterial meningococci and the efficacy of new drugs. Furthermore, the destruction of the blood–brain barrier and blood–cerebrospinal fluid barrier by the above streptococci has been shown to be an important step in the development of meningitis (Zhang et al., 2018).

In addition to these tissue-borne infections, several new biofilm models related to device-borne infections have recently been developed. These include new models of biofilm formation in cochlear implants, surgical screws, and central nervous system catheters. The guinea pig model was published by Cevizci et al. in 2015 by studying biofilm-borne infections from transplanted cochlear devices and testing QS inhibitors (Cevizci et al., 2015). This model was developed by injecting a cochlear implant through the eardrum into the pneumonia middle ear and implanting the cochlear implant under the skin behind the pinna. Visualization of biofilm formation on explanted device components using scanning electron microscopy demonstrates that these novel QS inhibitors can prevent biofilm formation on implants. For neurological devices, a rat model was developed to test whether intraoperative infection of the intracranial screw with *S. aureus* causes biofilm formation (Glage et al., 2017b).

6.1 Integrative biofilm models

In live organisms, the formation of bacterial infection models that are functional has been made possible by extensive research in the field of biofilms and infectious diseases over the past 50 years. A wide range of tailorable alternatives comprising high-throughput abilities can be performed for biofilm in vitro modeling at comparatively little cost. A mechanically accurate assessment of the development and progression of biofilms in sophisticated organisms is possible only through In vivo models, despite current limitations in their use.

Biologically relevant in vitro bacterial colonies can also be obtained from ex vivo models. In case of a corneal structure, immune response elicitation is relatively difficult; thus, cornea can be stated as an organ with immunity privileges. While assessing the potential antimicrobial agents in bacterial and fungal keratitis instead of the in vitro model, some alternatives were used such as corneas of porcine and rabbit (Pinnock et al., 2017; Snowden et al., 2012). However, when modeling endocarditis, physiological temperature, shear conditions, volume, and flow should be considered when dealing with perfused tissue such as the heart (Goeres et al., 2009). Rapid advances in microfluidics and organ-on-a-chips (commonly known as body-on-a-chips) bring the capacities for high-throughput analysis and integrated endogenously triggered immune response closer together during biofilm research. It may be fully possible in the future. Tip (Lauten et al., 2020). Such techniques, for example, in combination with the creation of 3D bioprinting structures, have the ability to mimic the complex structures of living organisms.

6.2 Challenges and future perspectives

The next frontier for generating a more accurate in vivo model of biofilm is the humanized animal model, but ethical issues need to be carefully considered. These humanized animal models should have comorbidities such as diabetes, cardiovascular diseases, or obesity, which can firmly mimic the exact conditions similar to that of human patients. Also it is hard to anticipate the upcoming generations of advanced biofilm models (Dehne et al., 2020).

References

An Andy, Y., Choi, K. Y. G., Baghela Arjun, S., & Hancock Robert, E. W. (2021). An overview of biological and computational methods for designing mechanism-informed anti-biofilm agents. *Frontiers in Microbiology, 12*. https://doi.org/10.3389/fmicb.2021.640787. https://www.frontiersin.org/article/10.3389/fmicb.2021.640787

Andersson, D. I., & Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nature Reviews Microbiology, 12*(7), 465–478. https://doi.org/10.1038/nrmicro3270. PubMed PMID: 24861036.

Artini, M., Patsilinakos, A., Papa, R., Božoviæ, M., Sabatino, M., Garzoli, S., et al. (2018). Antimicrobial and antibiofilm activity and machine learning classification analysis of essential oils from different Mediterranean plants against *Pseudomonas aeruginosa*. *Molecules Basel Switz, 23*, 482. https://doi.org/10.3390/molecules23020482

Badel, S., Laroche, C., Gardarin, C., Bernardi, T., & Michaud, P. (2008). New method showing the influence of matrix components in Leuconostoc mesenteroides biofilm formation. *Applied Biochemistry and Biotechnology, 151*, 364–370. https://doi.org/10.1007/s12010-008-8199-y

Barre- Sinoussi, F., & Montagutelli, X. (2015). Animal models are essential to biological research; issues and perspectives. *Future Science OA, 1*(4), Fso63.

Braga, A. S., Pires, J. G., & Magalh∼aes, A. C. (2018). Effect of a mouthrinse containing Malva sylvestris on the viability and activity of microcosm biofilm and on enamel demineralization compared to known antimicrobial mouthrinses. *Biofouling, 34*, 252–261. https://doi.org/10.1080/08927014.2018.1428957

Breen, E. C., Malloy, J. L., Tang, K., Xia, F., Fu, Z., Hancock, R. E. W., Overhage, J., Wagner, P. D., & Spragg, R. G. (2013). Impaired pulmonary defense against *Pseudomonas aeruginosa* in VEGF gene inactivated mouse lung. *Journal of Cellular Physiology, 228*, 371–379.

Cabot, G., Zamorano, L., Moyà, B., Juan, C., Navas, A., Blázquez, J., & Oliver, A. (2016). Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation supply rates. *Antimicrobial Agents and Chemotherapy, 60*, 1767–1778.

Cao, Q., Wang, Y., Chen, F., Xia, Y., Lou, J., Zhang, X., Yang, N., Sun, X., Zhang, Q., Zhuo, C., et al. (2014). A novel signal transduction pathway that modulates rhl quorum sensing and bacterial virulence in *Pseudomonas aeruginosa*. *PLoS Pathogens, 10*, e1004340.

Carrasco, P. A., Brizuela, C. I., Rodriguez, I. A., Munoz, S., Godoy ∼, M. E., & Inostroza, C. (2017). Histological transformations of the dental pulp as a possible indicator of post mortem interval: A pilot study. *Forensic Science International, 279*, 251–257. https://doi.org/10.1016/j.forsciint.2017.09.001

Casciaro, B., Cappiello, F., Loffredo, M. R., Ghirg, F., & Mangoni, M. L. (2019). The potential of frog skin peptides for anti-infective therapies: The case of esculentin-1a (1-21) NH2. *Current Medicinal Chemistry, 27*, 1405–1419. https://doi.org/10.2174/0929867326666190722095408

Cattò, C., & Cappitelli, F. (2019). Testing anti-biofilm polymeric surfaces: Where to start? *International Journal of Molecular Sciences, 20*(15), 3794. https://doi.org/10.3390/ijms20153794

Cevizci, R., Duzlu, M., Dundar, Y., Noyanalpan, N., Sultan, N., Tutar, H., & Bayazit, Y. A. (2015). Preliminary results of a novel quorum sensing inhibitor against pneumococcal infection and biofilm formation with special interest to otitis media and cochlear implantation. *European Archives of Oto-Rhino-Laryngology, 272*, 1389–1393.

Chavant, P., Gaillard-Martinie, B., Talon, R., Hebraud, M., & Bernardi, T. (2007). A new device for rapid evaluation of biofilm formation potential by bacteria. *Journal of Microbiological Methods, 68*, 605–612. https://doi.org/10.1016/j.mimet.2006.11.010

Chuang-Smith, O. N., Wells, C. L., Henry-Stanley, M. J., & Dunny, G. M. (2010). Acceleration of *Enterococcus faecalis* biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. *PloS One, 5*, e15798. https://doi.org/10.1371/journal.pone.0015798

Churchley, D., Rees, G. D., Barbu, E., Nevell, T. G., & Tsibouklis, J. (2008). Fluoropolymers as low-surface energy tooth coatings for oral care. *International Journal of Pharmaceutics, 352*, 44–49.

Ciric, A. D., Petrovic, J. D., Glamoclija, J. M., Smiljkovic, M. S., Nikolic, M. M., Stojkovic, D. S., et al. (2019). Natural products as biofilm formation antagonists and regulators of quorum sensing functions: A comprehensive review update and future trends. *South African Journal of Botany, 120*, 65–80. https://doi.org/10.1016/j.sajb.2018.09.010

Cordeiro, R. D. A., de Andrade, A. R. C., Portela, F. V. M., Pereira, L. M. G., Moura, S. G. B., Sampaio, M. D., Pereira, E. M. A., de Melo Guedes, G. M., Bandeira, S. P., de Lima-Neto, R. G., et al. (2020b). Proposal for a microcosm biofilm model for the study of vulvovaginal candidiasis. *Biofouling, 36*, 610–611. https://doi.org/10.1080/08927014.2020.1785435

Cordeiro, L., Figueiredo, P., Souza, H., Sousa, A., Andrade-Júnior, F., Barbosa-Filho, J., & Lima, E. (2020a). Antibacterial and antibiofilm activity of myrtenol against *Staphylococcus aureus*. *Pharmaceuticals, 13*(6), 133.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science, 284*(5418), 1318–1322.

Darouiche, R. O. (2004). Treatment of infections associated with surgical implants. *The New England Journal of Medicine, 350*(14), 1422–1429. https://doi.org/10.1056/NEJMra035415. PubMed PMID: 15070792.

De Simone, M., Spagnuolo, L., Lorè, N. I., Rossi, G., Cigana, C., De Fino, I., Iraqi, F. A., & Bragonzi, A. (2014). Host genetic background influences the response to the opportunistic *Pseudomonas aeruginosa* infection altering cell-mediated immunity and bacterial replication. *PLoS One, 9*, e106873.

Dehne, E. M., & Marx, U. (2020). Chapter 13- Human body-on-a-chip systems. In J. Hoeng, D. Bovard, & M. C. Peitsch (Eds.), *Organ-on-a-chip* (pp. 429–439). Academic Press.

Del Pozo, J. L., & Patel, R. (2007). The challenge of treating biofilm-associated bacterial infections. *Clinical Pharmacology & Therapeutics, 82*(2), 204–209. https://doi.org/10.1038/sj.clpt.6100247. PubMed PMID: 17538551.

Del Pozo, J. L., Tran, N. V., Petty, P. M., Johnson, C. H., Walsh, M. F., Bite, U., et al. (2009). Pilot study of association of bacteria on breast implants with capsular contracture. *Journal of Clinical Microbiology, 47*(5), 1333–1337. PubMed PMID: 19261794.

Déziel, E., Gopalan, S., Tampakaki, A. P., Lépine, F., Padfield, K. E., Saucier, M., … Rahme, L. G. (2005). The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: Multiple quorum sensing-regulated genes are modulated without affecting lasRI, rhlRI or the production of N-acyl-L-homoserine lactones. *Molecular Microbiology, 55*(4), 998–1014.

Di Luca, M., Maccari, G., Maisetta, G., & Batoni, G. (2015). BaAMPs: The database of biofilm-active antimicrobial peptides. *Biofouling, 31*, 193–199. https://doi.org/10.1080/08927014.2015.1021340

Diggle, S. P., Winzer, K., Chhabra, S. R., Worrall, K. E., Cámara, M., & Williams, P. (2003). The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Molecular Microbiology, 50*(1), 29–43.

Dong, B., Manolache, S., Wong, A. C. L., & Denes, F. S. (2011). Antifouling ability of polyethylene glycol of different molecular weights grafted onto polyester surfaces by cold plasma. *Polymer Bulletin, 66*, 517–528.

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases, 8*(9), 881.

Dubern, J., Cigana, C., De Simone, M., Lazenby, J., Juhas, M., Schwager, S., Bianconi, I., Döring, G., Eberl, L., Williams, P., et al. (2015). Integrated whole genome screening for *Pseudomonas aeruginosa* virulence genes using multiple disease models reveals that pathogenicity is host specific. *Environmental Microbiology, 17*, 4379–4397.

Eslami, L. M., Vatanpour, M., Aminzadeh, N., Mehrvarzfar, P., & Taheri, S. (2019). The comparison of intracanal medicaments, diode laser and photodynamic therapy on removing the biofilm of *Enterococcus faecalis* and Candida albicans in the root canal system (ex-vivo study). *Photodiagnosis and Photodynamic Therapy, 26*, 157–161. https://doi.org/10.1016/j.pdpdt.2019.01.033

Fekete, A., Borbas, A., Gyémant, G., Kandra, L., Fazekas, E., Ramasubbu, N., & Antus, S. (2011). Synthesis of β-(1-6)-linked N-acetyl-D-glucosamine oligosaccharide substrates and their hydrolysis by Dispersin B. *Carbohydrate Research, 346*, 1445–1453.

Fernandez y Mostajo, M., Exterkate, R. A. M., Buijs, M. J., Beertsen, W., Van Der Weijden, G. A., Zaura, E., & Crielaard, W. (2017). A reproducible microcosm biofilm model of subgingival microbial communities. *J Periodont Res, 52*, 1021–1031. https://doi.org/10.1111/jre.12473

Fisher, M. L., Sun, W., & Curtiss, R. (2014). The route less taken: Pulmonary models of enteric gram-negative infection. *Pathog Dis, 70*, 99–109.

Fjell, C. D., Hiss, J. A., Hancock, R. E. W., & Schneider, G. (2012). Designing antimicrobial peptides: Form follows function. *Nat. Rev. Drug Discov., 11*, 37–51.

Form, H. D. T. (2002). *Biofilms: Forming a defense strategy for the food plant. Order*.

de la Fuente-Núñez, C., Cardoso, M. H., de Souza Cândido, E., Franco, O. L., & Hancock, R. E. W. (2016). Synthetic antibiofilm peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes, 1858*(Issue 5), 1061–1069. https://doi.org/10.1016/j.bbamem.2015.12.015. ISSN 0005-2736.

Fux, C. A., Stoodley, P., Hall-Stoodley, L., & Costerton, J. W. (2003). Bacterial biofilms: A diagnostic and therapeutic challenge. *Expert Rev Anti Infect Ther, 1*(4), 667–683. PubMed PMID: 15482163.

Gabrilska, R. A., & Rumbaugh, K. P. (2015). Biofilm models of polymicrobial infection. *Future Microbiol, 10*, 1997–2015. https://doi.org/10.2217/fmb.15.109

Glage, S., Paret, S., Winkel, A., Stiesch, M., Bleich, A., Krauss, J. K., et al. (2017). A new model for biofilm formation and inflammatory tissue reaction: Intraoperative infection of a cranial implant with *Staphylococcus aureus* in rats. *Acta Neurochir (Wien)*. https://doi.org/10.1007/s00701-017-3244-7. PubMed PMID: 28647798.

Glage, S., Paret, S., Winkel, A., Stiesch, M., Bleich, A., Krauss, J. K., & Schwabe, K. (2017). A new model for biofilm formation and inflammatory tissue reaction: Intraoperative infection of a cranial implant with *Staphylococcus aureus* in rats. *Acta Neurochir, 159*, 1747–1756.

Goeres, D. M., Hamilton, M. A., Beck, N. A., Buckingham-Meyer, K., Hilyard, J. D., Loetterle, L. R., Lorenz, L. A., Walker, D. K., & Stewart, P. S. (2009). A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor. *Nat Protoc, 4*, 783–788. https://doi.org/10.1038/nprot.2009.59

Gristina, A. G., Naylor, P., & Myrvik, Q. (1988). Infections from biomaterials and implants: A race for the surface. *Med Prog Technol, 14*(3–4), 205–224. PubMed PMID: 2978593.**First report of the so called race for the surface.

Gupta, S., Sharma, A. K., Jaiswal, S. K., & Sharma, V. K. (2016). Prediction of biofilm inhibiting peptides: An in silico approach. *Front. Microbiol., 7*, 949. https://doi.org/10.3389/fmicb.2016.00949

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: From the natural environment to infectious diseases. *Nat Rev Microbiol, 2*(2), 95–108. https://doi.org/10.1038/nrmicro821. PubMed PMID: 15040259.

Haney, E. F., Trimble, M. J., Cheng, J. T., Vallé, Q., & Hancock, R. E. W. (2018b). Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides. *Biomolecules, 8*, 29. https://doi.org/10.3390/biom8020029

Heersink, J., & Goeres, D. (2003). Reactor design considerations. In M. Hamilton, J. Heersink, J. Buckingham-Meyer, & D. Goeres (Eds.), *The biofilm laboratory: Step-by-step protocols for experimental design, analysis, and data interpretation* (pp. 13–15). Bozeman: Cytergy Publishing.

Huang, Z., Meric, G., Liu, Z., Ma, R., Tang, Z., & Lejeune, P. (2009). LuxS-based quorum-sensing signaling affects biofilm formation in Streptococcus mutans. *J Mol Microbiol Biotechnol, 17*, 12–19. https://doi.org/10.1159/000159193

Huang, Y., Thompson, T., Wang, Y., Yu, Q., Zhu, L., Xu, X., Wen, Z. T., & Townsend, J. A. (2019). Analysis of cariogenic potential of alternative milk beverages by in vitro Streptococcus mutans biofilm model and ex vivo caries model. *Archives Oral Biol, 105*, 52–58. https://doi.org/10.1016/j.archoralbio. 2019.05.033

Irazoqui, J. E., Urbach, J. M., & Ausubel, F. M. (2010). Evolution of host innate defense: Insights from *Caenorhabditis elegans* and primitive invertebrates. *Nat Rev Immunol, 10*, 47–58.

Jackson, A. A., Gross, M. J., Daniels, E. F., Hampton, T. H., Hammond, J. H., Vallet-Gely, I., Dove, S. L., Stanton, B. A., & Hogan, D. A. (2013). Anr and its activation by PlcH activity in *Pseudomonas aeruginosa* host colonization and virulence. *J Bacteriol, 195*, 3093–3104.

Jander, G., Rahme, L. G., & Ausubel, F. M. (2000). Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J Bacteriol, 182*, 3843–3845.

Jayaraman, A., & Wood, T. H. (2008). Bacterial quorum sensing signals: Signals, circuits, and implications for biofilm and disease. *Annu Rev Biomed Eng, 10*, 145–167.

Jones, J. S., Hoerle, D., & Riekse, R. (1995). Stethoscopes: A potential vector of infection? *Annals of Emergency Medicine, 26*(3), 296–299.

Junka, A. F., Szymczyk, P., Secewicz, A., Pawlak, A., Smutnicka, D., Ziołkowski, G., Bartoszewicz, M., & Chlebus, E. (2016). The chemical digestion of Ti6Al7Nb scaffolds produced by Selective Laser Melting significantly reduces the ability of *Pseudomonas aeruginosa* to form biofilm. *Acta Bioeng Biomech, 18*, 115–120.

Kirienko, N. V., Ausubel, F. M., & Ruvkun, G. (2015). Mitophagy confers resistance to siderophore-mediated killing by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA, 112*, 1821–1826.

Koch, G., Nadal-Jimenez, P., Cool, R. H., & Quax, W. J. (2014). Assessing *Pseudomonas* virulence with nonmammalian host: *Galleria mellonella*. *Methods Mol Biol, 1149*, 681–688.

Kokare, C. R., Chakraborty, S., Khopade, A. N., & Mahadik, K. R. (2009). *Biofilm: Importance and applications*.

Kounatidis, I., & Ligoxygakis, P. (2012). *Drosophila* as a model system to unravel the layers of innate immunity to infection. *Open Biol, 2*, 120075.

Krezdorn, J., Adams, S., & Coote, P. J. (2014). A *Galleria mellonella* infection model reveals double and triple antibiotic combination therapies with enhanced efficacy versus a multidrug-resistant strain of *Pseudomonas aeruginosa*. *J Med Microbiol, 63*, 945–955.

Kumar, C. G., & Anand, S. K. (1998). Significance of microbial biofilms in food industry: A review. *International Journal of Food Microbiology, 42*(1–2), 9–27.

Labeaux, D., Chauhan, A., Rendueles, O., & Beloin, C. (2013). From in vitro to *In vivo* models of bacterial biofilm related infections. *Pathogens (Basel), 2*, 288–356. https://doi.org/10.3390/pathogens2020288

Lauten, A., Martinovic, M., Kursawe, L., Kikhney, J., Affeld, K., Kertzscher, U., Falk, V., & Moter, A. (2020). Bacterial biofilms in infective endocarditis: As in vitro model to investigate emerging technologies of antimicrobial cardiovascular device coatings. *Clin. Res. Cardiol., 110*, 323–331.

Lazar, V. (2011). Quorum sensing in biofilms – how to destroy the bacterial citadels or their cohesion/power? *Anaerobe, 17*, 280–285.

Lazurko, K., Khatoon, Z., Goel, K., Sedlakova, V., Eren Cimenci, C., Ahumada, M., Zhang, L., Mah, T. F., Franco, W., Suuronen, E. J., et al. (2020). Multifunctional nano and collagen based therapeutic materials for skin repair. *ACS Biomat. Sci. Eng., 6*, 1124–1134.

LeCun, Y., Bengio, Y., & Hinton, G. (2015). Deep learning. *Nature, 521*, 436–444. https://doi.org/10.1038/nature14539

Letamendia, A., Quevedo, C., Ibarbia, I., Virto, J. M., Holgado, O., Diez, M., Izpisua Belmonte, J. C., & Callol Massot, C. (2012). Development and validation of an automated high throughput system for zebrafish *In vivo* screenings. *PLoS One, 7*, e36690.

Lim, D., & Strynadka, N. C. (2002). Structural basis for the β lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. *Nature Structural Biology, 9*(11), 870–876.

Lo, Y.-C., Rensi, S. E., Torng, W., & Altman, R. B. (2018). Machine learning in chemoinformatics and drug discovery. *Drug Discov. Today, 23*, 1538–1546. https://doi.org/10.1016/j.drudis.2018.05.010

Lorenz, A., Pawar, V., Häussler, S., & Weiss, S. (2016). Insights into host–pathogen interactions from state-of-the-art animal models of respiratory *Pseudomonas aeruginosa* infections. *FEBS Lett, 590*, 3941–3959. https://doi.org/10.1002/1873-3468.12454

Luis Del Pozo, J. (2017). Biofilm-related disease. *Expert Review of Antiinfective Therapy*. https://doi.org/10.1080/14787210.2018.1417036

Lutter, E. I., Purighalla, S., Duong, J., & Storey, D. G. (2012). Lethality and cooperation of *Pseudomonas aeruginosa* quorum-sensing mutants in *Drosophila melanogaster* infection models. *Microbiology, 158*, 2125–2132.

Mao, Y., Singh-Varma, A., Hoffman, T., Dhall, S., Danilkovitch, A., & Kohn, J. (2018). The effect of cryopreserved human placental tissues on biofilm formation of wound-associated pathogens. *J Funct Biomater, 9*, 3. https://doi.org/10.3390/jfb9010003

Marsh, P. D., & Bradshaw, D. J. (1997). Physiological approaches to the control of oral biofilms. *Advances in Dental Research, 11*(1), 176–185.

Mei, L., Busscher, H. J., Van der Mei, H. C., & Ren, Y. (2011). Influence of surface roughness on streptococcal adhesion forces to composite resins. *Dental Materials, 27*, 770–778.

Migiyama, Y., Kaneko, Y., Yanagihara, K., Morohoshi, T., Morinaga, Y., Nakamura, S., Miyazaki, T., Hasegawa, H., Izumikawa, K., Kakeya, H., et al. (2013). Efficacy of AiiM, an N-acylhomoserine lactonase, against *Pseudomonas aeruginosa* in a mouse model of acute pneumonia. *Antimicrob Agents Chemother, 57*, 3653–3658.

Mowat, E., Paterson, S., Fothergill, J. L., Wright, E. A., Ledson, M. J., Walshaw, M. J., Brockhurst, M. A., & Winstanley, C. (2011). *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. *Am J Respir Crit Care Med, 183*, 1674–1679.

Moyano, A. J., Tobares, R. A., Rizzi, Y. S., Krapp, A. R., Mondotte, J. A., Bocco, J. L., Saleh, M.-C., Carrillo, N., & Smania, A. M. (2014). A long-chain flavodoxin protects *Pseudomonas aeruginosa* from oxidative stress and host bacterial clearance. *PLoS Genet, 10*, e1004163.

Niu, C., & Gilbert, E. S. (2004). Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. *Applied and Environmental Microbiology, 70*, 6951–6956. https://doi.org/10.1128/AEM.70.12.6951-6956.2004

Noble, W. S. (2006). What is a support vector machine? *Nature Biotechnology, 24*, 1565–1567. https://doi.org/10.1038/nbt1206-1565

Patsilinakos, A., Artini, M., Papa, R., Sabatino, M., Božoviæ, M., Garzoli, S., et al. (2019). Machine learning analyses on data including essential oil chemical composition and in vitro experimental antibiofilm activities against Staphylococcus species. *Molecules Basel Switz., 24*, 890. https://doi.org/10.3390/molecules24050890

Peeters, E., Nelis, H. J., & Coenye, T. (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of Microbiological Methods, 72*, 157–165. https://doi.org/10.1016/j.mimet.2007.11.010

Peterman, E. M., Sullivan, C., Goody, M. F., Rodriguez-Nunez, I., Yoder, J. A., & Kim, C. H. (2015). Neutralization of mitochondrial superoxide by superoxide dismutase 2 promotes bacterial clearance and regulates phagocyte numbers in zebrafish. *Infection and Immunity, 83*, 430–440.

Pinnock, A., Shivshetty, N., Roy, S., Rimmer, S., Douglas, I., Macneil, S., & Grag, P. (2017). Ex vivo rabbit and human corneas as models for bacterial and fungal keratitis. *Graefe's Archive for Clinical and Experimental Ophthalmology, 255*, 333–342.

Plezer, D., Mansour, S. C., Wuerth, K., Rahanjam, N., & Hancock, R. E. W. (2017). New mouse model for chronic infections by gram negative bacteria by enabling the study of anti- infective efficacy and host microbe interactions. *mBio, 8*, e00140–17.

Ponce-Angulo, D. G., Bautista-Hernández, L. A., Calvillo-Medina, R. P., Castro-Tecorral, F. I., Aparicio-Ozores, G., López-Villegas, E. O., Ribas-Aparicio, R. M., & Bautista-de Lucio, V. M. (2020). Microscopic characterization of biofilm in mixed keratitis in a novel murine model. *Microbial Pathogenesis, 140*, 103953.

Raad, I. I., & Bodey, G. P. (1992). Infectious complications of indwelling vascular catheters. *Clinical Infectious Diseases, 15*(2), 197–208. PubMed PMID: 1520756.

Rajkumari, J., Borkotoky, S., Murali, A., Suchiang, K., Mohanty, S. K, & Busi, S. (2018). Cinnamic acid attenuates quorum sensing associated virulence factors and biofilm formation in Pseudomonas aeruginosa PAO1. *Biotechnology Letters, 40*(7). https://doi.org/10.1007/s10529-018-2557-9. In this issue.

Rajkumari, J., Borkotoky, S., Reddy, D., Mohanty, S. K., Kumavath, R., Murali, A., … Busi, S. (2019). Anti-quorum sensing and anti-biofilm activity of 5-hydroxymethylfurfural against *Pseudomonas aeruginosa* PAO1: Insights from in vitro, *In vivo* and in silico studies. *Microbiological Research, 226*, 19–26.

Razak, F. A., & Rahim, Z. H. (2003). The anti-adherence effect of *Piper betle* and *Psidium guajava* extracts on the adhesion of early settlers in dental plaque to saliva-coated glass surfaces. *Journal of Oral Science, 45*, 201–206. https://doi.org/10.2334/josnusd.45.201

Reen, F. J., Haynes, J. M., Mooij, M. J., & O'Gara, F. (2013). A non-classical LysR-type transcriptional regulator PA2206 is required for an effective oxidative stress response in *Pseudomonas aeruginosa. PLoS One, 8*, e54479.

Roberts, A. E., Kragh, K. N., Bjarnsholt, T., & Diggle, S. P. (2015). The limitations of in vitro experimentation in understanding biofilms and chronic infection. *Journal of Molecular Biology, 427*, 3646–3661. https://doi.org/10.1016/j.jmb.2015.09.002

Rocker, A. J., Weiss, A. R. E., Lam, J. S., Van Raay, T. J., & Khursigara, C. M. (2015). Visualizing and quantifying *Pseudomonas aeruginosa* infection in the hindbrain ventricle of zebrafish using confocal laser scanning microscopy. *Journal of Microbiological Methods, 117*, 85–94.

Ross-Gillespie, A., Weigert, M., Brown, S. P., & Kümmerli, R. (2014). Gallium-mediated siderophore quenching as an evolutionarily robust antibacterial treatment. *Evolution, Medicine, and Public Health, 2014*, 18–29.

Rudney, J. D., Chen, R., Lenton, P., Li, J., Li, Y., Jones, R. S., Reilly, C., Fok, A. S., & Aparicio, C. (2012). A reproducible oral microcosm biofilm model for testing dental materials. *Journal of Applied Microbiology, 113*, 1540–1553. https://doi.org/10.1111/j.1365-2672.2012.05439.x

Rutala, W. A., & Weber, D. J. (1997). Water as a reservoir of nosocomial pathogens. *Infection Control & Hospital Epidemiology, 18*(9), 609–616. PubMed PMID: 9309431.

Salli, K. M., & Ouwehand, A. C. (2015). The use of in vitro model systems to study dental biofilms associated with caries: A short review. *Journal of Oral Microbiology, 7*, 26149. https://doi.org/10.3402/jom. V7.26149

Saraswathi, P., & Beuerman, R. W. (2015). Corneal biofilms: from planktonic to microcolony formation in an experimental keratitis infection with Pseudomonas aeruginosa. *The ocular surface, 13*(4), 331–345.

Satpute, S. K., Banpurkar, A. G., Banat, I. M., Sangshetti, J. N., Patil, R. H., & Gade, W. N. (2016). Multiple roles of biosurfactants in biofilms. *Current Pharmaceutical Design, 22*, 1429–1448. https://doi.org/10.2174/1381612822666160120152704

Scheman, L., Janota, M., & Lewin, P. (1941). The production of experimental osteomyelitis; preliminary report. *The Journal of the American Medical Association, 117*, 1525–1529.

Schuster, M., Joseph Sexton, D., Diggle, S. P., & Peter Greenberg, E. (2013). Acyl-homoserine lactone quorum sensing: From evolution to application. *Annual Review of Microbiology, 67*, 43–63.

Sharma, A., Gupta, P., Kumar, R., & Bhardwaj, A. (2016). dPABBs: a novel in silico approach for predicting and designing anti-biofilm peptides. *Scientific Reports, 6*, 21839. https://doi.org/10.1038/srep21839

Sidrim, J. J., Amando, B. R., Gomes, F. I. F., do Amaral, S. M. S. G., de Sousa, P. C. P., Ocadaque, C. J., Brilhante, R. S. N., Cordeiro, R. A., Rocha, M. F. G., & Castelo-Branco, D. S. C. M. (2019). Chlorpromazine-impregnated catheters as a potential strategy to control biofilm- associated urinary tract infections. *Future Microbiology, 14*, 1023–1034. https://doi.org/10.2217/fmb2019-0092

Singhal, D., Baker, L., Wormald, P. J., & Tan, L. (2011). Aspergillus fumigatus biofilm on primary human sinonasal epithelial culture. *American Journal of Rhinology & Allergy, 25*, 219–225. https://doi.org/10.2500/ajra.2011.25.362

Sissons, C. H., Anderson, S. A., Wong, L., Coleman, M. J., & White, D. C. (2007). Microbiota of plaque microcosm biofilms: Effect of three times daily sucrose pulses in different simulated oral environments. *Caries Research, 41*, 413–422. https://doi.org/10.1159/000104801

Snowden, J. N., Beaver, M., Smeltzer, M. S., & Kielian, T. (2012). Biofilm- infected intracerebroventricular shunts elicit inflammation within the central nervous system. *Infection and Immunity, 80*, 3206–3214.

Srivastava, G. N., Malwe, A. S., Sharma, A. K., Shastri, V., Hibare, K., & Sharma, V. K. (2020). Molib: A machine learning based classification tool for the prediction of biofilm inhibitory molecules. *Genomics, 112*, 2823–2832. https://doi.org/10.1016/j.ygeno.2020.03.020

Stokes, J. M., Yang, K., Swanson, K., Jin, W., Cubillos-Ruiz, A., Donghia, N. M., et al. (2020). A deep learning approach to antibiotic discovery. *Cell, 180*, 688–702.e13. https://doi.org/10.1016/j.cell.2020.01.021

Storz, M. P., Maurer, C. K., Zimmer, C., Wagner, N., Brengel, C., de Jong, J. C., … Hartmann, R. W. (2012). Validation of PqsD as an anti-biofilm target in *Pseudomonas aeruginosa* by development of small-molecule inhibitors. *Journal of the American Chemical Society, 134*(39), 16143–16146.

Sulaeman, S., Le Bihan, G., Rossero, A., Federighi, M., De, E., & Tresse, O. (2010). Comparison between the biofilm initiation of Campylobacter jejuni and Campylobacter coli strains to an inert surface using BioFilm Ring Test (R). *Journal of Applied Microbiology, 108*, 1303–1312. https://doi.org/10.1111/j.1365-2672. 2009.04534.x

Sun, Y., Dowd, S. E., Smith, E., Rhoads, D. D., & Wolcott, R. D. (2008). In vitro multispecies Lubbock chronic wound biofilm model. *Wound Repair and Regeneration, 16*, 805–813. https://doi.org/10.1111/j. 1524-475X.2008.00434.x

Sztukowska, M. N., Roky, M., & Demuth, D. R. (2019). Peptide and non-peptide mimetics as potential therapeutics targeting oral bacteria and oral biofilms. *Molecular Oral Microbiology, 34*, 169–182. https://doi.org/10.1111/omi.12267

Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G., & Ausubel, F. M. (1999). *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 2408–2413.

Thebault, P., Lequeux, I., Jouenne T. CNRS UMR 6270, Laboratoire "Polymères, Biopolymères, Surfaces", Faculté des Sciences, Université de Rouen, France.

Thebault, P., Lequeux, I., & Jouenne, T. (2013). Antibiofilm strategies. *Journal of Wound Technology, 21*, 20–23.

Thomann, A., de Mello Martins, A. G. G., Brengel, C., Empting, M., & Hartmann, R. W. (2016). Application of dual inhibition concept within looped autoregulatory systems towards anti-virulence agents against *Pseudomonas aeruginosa* infections. *ACS Chemical Biology, 11*, 1279–1286.

Thomsen, R., & Christensen, M. H. (2006). MolDock: A new technique for high-accuracy molecular docking. *Journal of Medicinal Chemistry, 49*(11), 3315–3321.

Tolker-Nielsen, T., & Sternberg, C. (2014). Methods for studying biofilm formation: Flow cells and confocal laser scanning microscopy. *Methods in Molecular Biology, 1149*, 615–629. https://doi.org/10.1007/978-1-4939-0473-0_47

Van Delden, C., & Iglewski, B. H. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerging Infectious Diseases, 4*(4), 551.

Verardi, G., Cenci, M. S., Maske, T. T., Webber, B., & Santos, L. R. (2016). Antiseptics and microcosm biofilm formation on titanium surfaces. *Brazilian Oral Research, 30*, e30. https://doi.org/10.1590/1807-3107BOR-2016.vol30.0030

Vila, T. V., Rozental, S., & de Sa Guimar∼aes, C. M. (2015). A new model of in vitro fungal biofilms formed on human nail fragments allows reliable testing of laser and light therapies against onychomycosis. *Lasers Medical Science, 30*, 1031–1039. https://doi.org/10.1007/s10103-014-1689-y

Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P., & Pesci, E. C. (2005). Regulation of Pseudomonas quinolone signal synthesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology, 187*(13), 4372–4380.

Wilson, M. (2001). Bacterial biofilms and human disease. *Science Progress, 84*(3), 235–254.

Woodworth, B. A., Tamashiro, E., Bhargave, G., Cohen, N. A., & Palmer, J. N. (2008). An in vitro model of *Pseudomonas aeruginosa* biofilms on viable airway epithelial cell monolayers. *American Journal of Rhinology, 22*, 235–238. https://doi.org/10.2500/ajr.2008.22. 3178

Yang, L., Nilsson, M., Gjermansen, M., Givskov, M., & Tolker-Nielsen, T. (2009). Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginosa* biofilm formation. *Molecular Microbiology, 74*(6), 1380–1392.

Yang, Q., Phillips, P. L., Sampson, E. M., Progulske-Fox, A., Jin, S., Antonelli, P., & Schultz, G. S. (2013). Development of a novel ex vivo porcine skin explant model for the assessment of mature bacterial biofilms. *Wound Repair and Regeneration, 21*, 704–714. https://doi.org/10.1111/wrr.12074

Yang, K., Swanson, K., Jin, W., Coley, C., Eiden, P., Gao, H., et al. (2019). Analyzing learned molecular representations for property prediction. *Journal of Chemical Information and Modeling, 59*, 3370–3388. https://doi.org/10.1021/acs.jcim.9b00237

Yan, X., Gu, S., Cui, X., Shi, Y., Wen, S., Chen, H., et al. (2019). Antimicrobial, anti-adhesive and anti-biofilm potential of biosurfactants isolated from *Pediococcus acidilactici* and *Lactobacillus plantarum* against *Staphylococcus aureus* CMCC26003. *Microbial Pathogenesis, 127*, 12–20. https://doi.org/10.1016/j.micpath.2018.11.039

Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: The microbial "protective clothing" in extreme environments. *International Journal of Molecular Sciences, 20*(14), 3423.

Zhang, S., Gao, X., Xiao, G., Lu, C., Yao, H., Fan, H., & Wu, Z. (2018). Intracranial subarachnoidal route of infection for investigating the roles of Streptococcus suis biofilms in meningitis in a mouse infection model. *Journal of Visualized Experiments, 137*, e57658.

Ziegler, A., Gonzalez, L., & Blikslager, A. (2016). Large animal models: The key to translational discovery in digestive disease research. *Cellular and Molecular Gastroenterology and Hepatology, 2*, 716–724.

https://vina.scripps.edu/.

https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/.

https://www.gromacs.org/About_Gromacs.