REVIEW

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Biofilm models of polymicrobial infection

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Interactions between microbes are complex and play an important role in the pathogenesis of infections. These interactions can range from fierce competition for nutrients and niches to highly evolved cooperative mechanisms between different species that support their mutual growth. An increasing appreciation for these interactions, and desire to uncover the mechanisms that govern them, has resulted in a shift from monomicrobial to polymicrobial biofilm studies in different disease models. Here we provide an overview of biofilm models used to study select polymicrobial infections and highlight the impact that the interactions between microbes within these biofilms have on disease progression. Notable recent advances in the development of polymicrobial biofilm-associated infection models and challenges facing the study of polymicrobial biofilms are addressed.

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Polymicrobial interactions in biofilms

Over the past 2 decades there has been a revolutionary paradigm shift in the field of microbiology with the appreciation that bacteria present in most biological systems exist in biofilms, rather than in a free-living state. This understanding has dramatically changed the way we study bacteria in the laboratory and resulted in the development of many new experimental systems that replicate biofilm environments [1–5]. Studies utilizing these systems have demonstrated time and time again that bacteria behave very differently when in a biofilm than during planktonic growth. In many ways, we now have to relearn everything we thought we knew about bacterial behavior through the view of the biofilm lens.

Similarly, the recent explosion of metagenomic studies has significantly increased our appreciation of the complexity of the microbial populations present in biofilms [6,7]. This is also true for infections, most of which are thought to be biofilm related and inherently polymicrobial, including various species of bacteria, fungi and viruses [8]. It is thought that microbes act in concert to establish biofilms, which in turn can increase tolerance to antimicrobials, exacerbate of the host's immune response and increase persistence at the infection site [7,9–11].

Genetic diversity of microbes within biofilm communities is thought to increase the fitness of the residing community, making them more equipped to survive environmental stresses. In large part, this is due to an expanded gene pool, which can be more easily shared within the confines of a biofilm community [12]. Community composition and interactions within the community can have huge influences on bacterial behavior. Thus, just as the behavior of planktonic versus

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biofilm-associated bacteria is dramatically different, so is that of bacteria in single species versus multispecies systems.

Interactions between microbes are complex and highly dependent on context. They can range from fierce competition for nutrients and niches, manifested by antagonistic behavior, to highly evolved cooperative mechanisms between different species that support their mutual growth in specific environments. While other published articles have provided a detailed review of the studies that have been undertaken in an attempt to understand the mechanisms involved in these complex interactions [1,7,9–11,13–15], our intent here is to provide the reader with information about the models that have been used to study polymicrobial interactions in biofilms. Thus, below we briefly define the major interactions that have been studied (Figure 1), and then discuss the models in which they have been examined in the following sections.

• Synergism

Microbial synergy can be defined as a cooperative interaction between two or more species of microbes that produces an effect not achieved by an individual species alone [1,7,9–14,16] (Figure 1). In biofilms and biofilm-related infections, these ‘effects’ include increased growth, antimicrobial tolerance, virulence and persistence, and enhanced production of exopolysaccharide (EPS) [17–28]. Another classic cooperative interaction is metabolic cross-feeding, or syntrophy, where one species makes a metabolic byproduct which enhances the growth of a neighbor [29].

• Antagonism

Microbial antagonism, also called antibiosis, can be defined as the suppression of one microbial species by another (Figure 1). Antagonistic mechanisms include: production of factors that kill or inhibit the growth of neighbors, production of chemical signals that can interfere with or disrupt the behavior or physiology of neighbors, or hoarding of nutrients that starves neighbors [15,30–33]. ‘Surface blanketing’ occurs when one species occupies all the attachment sites on a surface, thereby preventing the attachment of another [15,34,35]. Investigating antagonistic relationships between microbes not only provides insight into bacterial social behavior, but also may hold keys to disrupting biofilm structure and composition, potentially leading to novel treatments for biofilm-related infections.

• Community structure

All biofilm dynamics, including interactions between microbes, will depend on community structure (Figure 1). This includes the composition and spatial distribution of members within the biofilm population. Thus, many polymicrobial models focus on observing and imaging spatial structure, measuring the physical, mechanical and chemical properties of the biofilm, and determining how species composition, including changes in environmental parameters, alter these characteristics [36–38].

• Polymicrobial biofilm-related infections

There are several different types of infections that are often, if not always, caused by a multispecies population of microbes, which are thought to be biofilm-associated. As will be discussed below, most *in vitro* and *in vivo* models are designed with intent to recapitulate the natural host environment of these infection sites. The most common types of polymicrobial, biofilm-associated infections that have been studied are those occurring in the lung, inner ear, urinary tract, oral cavity, in wounds and those that are device or foreign body-related. Generally, it is believed that biofilms present at these sites negatively affect the host and potentiate the infection by: causing a chronic inflammatory state that results in collateral damage to the surrounding host tissue; protecting the microbes from antimicrobials and host immune factors and/or acting as a mechanical barrier to healing. Although most of the focus on medical biofilms is on their pathogenic potential and role in disease, it is worth noting that biofilms likely also play a protective role *in vivo* as well. For example, probiotic bacteria have recently been a hot topic for their potential as prophylactic and therapeutic agents against opportunistic and commensal-turned pathogens. And it has recently been put forth that *Lactobacillus* biofilms in the vagina protect against the acquisition of bacterial vaginosis, and likewise *Lactobacillus casei* gut biofilms offer immunomodulatory benefits [39,40].

Biofilm models of polymicrobial infections

Multispecies biofilms are typically studied from one of two perspectives: the first is to study the consortia of microorganisms within the biofilm as a single unit, and the other is to investigate the effects of one species on another (or others). Initial observations that prompt the study are

Figure 1. Polymicrobial interactions are an important aspect of biofilm-related infections. These interactions are complex and dynamic. Community composition and spatial distribution are central to the biofilm population and will likely influence all of its physical properties; however, very little is yet understood about the mechanisms that control these factors during infections. Most published studies have focused on revealing mechanisms of synergy (green lines) or antagonism (red line) between two species within an infection. These interactions can either be direct (indicated by a straight line), such as an evolved synergetic relationship between two species where each produces a substance that benefits the other, or indirect (indicated by curvy line), such as the production of an enzyme that inactivates an antibiotic, subsequently protecting the producer, as well as neighboring microbes.

usually made either in the laboratory (e.g., the observation that one species is killed by another) or by a clinical observation (e.g., a coinfection with two specific species is associated with a worse prognosis or increased likelihood of mortality). Whether it is from the bench-side or bed-side, using the appropriate model to study polymicrobial interactions is of utmost importance to preserve the phenotype of interest.

• ***In vitro* models of polymicrobial, biofilm-related infection**

In vitro models are extremely important for exploring fundamental questions about biofilms, acquiring empirical data and laying a foundation upon which confirmatory *in vivo* testing may be pursued. There are many benefits to using *in vitro* biofilm models such as low-cost, high-throughput potential, relative ease in replicability and flexibility to modify conditions as desired. However, the obvious drawback of *in vitro* models is their oversimplification of the

in vivo environment, most notably their lack of the host immune response.

Many *in vitro* models of biofilm-associated infections have been established. While most of these have been used to study single bacterial species [2,3,9], there are virtually infinite combinations of microbes that can be adapted into these existing models, allowing for the potential to study a higher level of complexity that is not well understood. The major challenge in this lies in culturing microbes that are found together in natural environments, *in vitro*. Very often, co-culturing different species of microorganisms results in the undesired killing of one or more species, even though they coexist stably in their natural environment. Challenges include finding media and conditions that simultaneously support multiple species, so that growth rates are normalized and one species does not unintentionally outcompete or inhibit the others. Furthermore, isolating or visualizing individual species once they have been intermingled

in a biofilm are additional challenges to using established *in vitro* monospecies biofilm models for multiple species.

Lastly, the ecological aspect of *in vivo* biofilms should also be considered when designing *in vitro* biofilm models and experiments with existing models. Biofilms found *in vivo* typically do not display the morphological characteristics of those grown *in vitro* (e.g., mushroom structures and towers), are often not surface-associated, and are usually composed of far fewer cells than biofilms grown *in vitro*. The host cell to bacterial cell ratio, host EPS versus bacterial EPS volume and number of bacterial cells encompassed within the biofilm are all important parameters that undoubtedly affect bacterial behavior.

Static models

Static or closed-system models are the most reported, typically least expensive and technically challenging, and are conducive to high-throughput analyses. While they are probably the most basic method of evaluating numerous combinations of species, they may also be the most limited in translation to *in vivo* conditions. The major variables in static model design are growth media and substratum, both of which can be modified to optimize biofilm adhesion. The major limitation of static models is their finite nutrient supply, which can quickly become exhausted, making it impractical to conduct experiments over a long time period. It should also be noted that many static models rely on plastic polymers as the surface to which microbes adhere. These surfaces are not typically used in biomedicine and therefore are not particularly relevant to infection. However, other more relevant surfaces can be incorporated into the experimental design of static systems, of which a few examples are described below.

Microtiter plate assay

The microtiter plate (MTP) assay is currently the model used most often to study polymicrobial biofilms [2] (Figure 2A). A multi-well plate is typically used to measure many properties of biofilms such as attachment, maturation, biomass, metabolism, antimicrobial tolerance, matrix quantification and/or to evaluate microbe–microbe interactions in a static environment, independent of the natural substrates associated with infection. While the surface material of most MTPs is not relevant to biomedical applications, variable coupons composed of a

variety of materials (e.g., metals or polymers) can be included in the well, and removed at any point during the experiments for analysis. Then, rather than studying biofilms attached to the MTP itself, attachment to medically relevant surfaces can be examined. Overall static systems are easy to set up, inexpensive and highly conducive to high-throughput screening [41]. The major restriction of this assay is the limitation of growth medium, which becomes exhausted and can prevent the full maturation of biofilms [42].

Calgary biofilm device

The commercialized Calgary biofilm device (CBD) assay is based on the 96-well MTP assay but also includes a pegged lid on which biofilms can adhere (Figure 2B). The pegs allow transfer of the adhered biofilm to a new well/environment, such as fresh medium, an antimicrobial solution or media containing other microorganisms, with less disruption of the biofilm than in the MTP [43,44].

Other static models & biofilm quantification

Other static biofilm models are used to grow polymicrobial biofilms, simultaneously or staggered, and to monitor species interactions. Agar-based static models involve growing bacteria and visualizing changes in phenotype, such as the colony biofilm assay (Figure 2C) or cross-streak assay, where neighboring interactions between species may be visualized on agar by comparing changes in colony color or size and noting indications of swarming or lysis. There are also innovative products to aid in harvesting biofilms, such as with the commercialized Biofilm Ring Test, where the formation of biofilm occurs on inert magnetic beads that are later collected via magnetic force for quantification [2,3].

Aside from conventional plating, advanced molecular techniques and imaging approaches, there are a variety of simple methods to quantify biofilm and its components, most of which are optimized for MTP assays. Biofilm biomass can be quantified using crystal violet, a basic dye that binds to all negatively charged surface molecules and EPS polysaccharides, or Syto9, a nucleic acid stain that binds to all DNA. Dimethyl methylene blue is another dye that ‘stains’ the biofilm matrix by forming a complex with sulphated polysaccharides. The reduction of dyes, such as of 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), resazurin and fluorescein

Figure 2. Common examples of static *in vitro* biofilm systems. (A) The microtiter plate assay is easy, inexpensive and conducive to high-throughput screening. It is frequently used to measure attachment, maturation, biomass, metabolism, antimicrobial tolerance, matrix quantification and/or to evaluate microbe–microbe interactions. Crystal violet is often used to stain adhered biofilm and can provide a semi-quantitative measure of biomass. (B) The Calgary biofilm device provides the benefit of a transferable surface area, where adhered biofilm cells can easily be moved to a new well/environment with little disruption to the biofilm. (C) Colony biofilm assays are simple, and can reveal potential polymicrobial interactions, such as the production of an inhibitory compound by one species.

diacetate, are used to distinguish living versus dead cells based on metabolic activity [41,45].

Notable polymicrobial studies in static systems

While countless combinations of microorganisms can be grown using static models, currently the most commonly reported interactions are of two-species biofilms, either bacteria–bacteria or fungi–bacteria. The most extensively reported class of bacterial–bacterial interactions involves the oral microbiota. Due to the outstanding number of bacterial species comprising the oral flora, the MTP assay provides the easiest method with which to screen for synergistic or antagonistic interactions between numerous oral species [46]. For example, Soares *et al.* grew a consortium of 35 different oral bacterial species involved in chronic periodontitis in a CBD to investigate the effects of different combinations of antimicrobials, whereby azithromycin and metronidazole were synergistic in killing compared with a single antibiotic alone [47].

Fungal–bacterial biofilms are common in several different types of infections. For example, *Candida albicans* and *Escherichia coli* or *Staphylococcus aureus* are frequently found dwelling together on medical devices such as endotracheal tubes and urinary catheters, and *Aspergillus fumigatus* and *Pseudomonas aeruginosa* can co-infect the lungs of cystic fibrosis (CF) patients [48–50]. However, developing polymicrobial biofilms with fungi and bacteria can present unique challenges. For example, the co-culture of *A. fumigatus*

and *P. aeruginosa* initially killed *A. fumigatus*, but Manavathu *et al.* were able to prevent this, and form stable polymicrobial biofilms, by co-inoculating with varying densities of *P. aeruginosa* and staggering the inoculation of *P. aeruginosa* into a pre-established *A. fumigatus* biofilm [48]. Likewise, successful co-culturing of *C. albicans* with *E. coli* or *S. aureus* in a compatible nutrient medium and selecting the most synergistic strains were also solutions to avoiding unwanted antagonism [49,50].

Dynamic models

Dynamic or open-system models are those that provide a continuous supply of nutrients to the microbes in the biofilm (Figure 3). These models are typically not as cost-efficient as closed systems and are more difficult to use for high-throughput experiments. The goal of dynamic systems is to simulate the natural environment by constantly replenishing nutrients and providing shear conditions; the rationale being that some microorganisms require certain flow displacement conditions to thrive in a polymicrobial community and to form biofilms. In addition, the shear forces generated from circulating materials can also be used to define the physical and chemical properties of biofilms, while replenishment of nutrients allows the biofilm to persist for extended periods of time. While most dynamic systems are probably more relevant to environmental biofilms (e.g., those in aquatic environments), they can also simulate infections where biofilms are exposed to the flow of biological fluids (e.g., oral cavity and urinary tract).

Flow cells

A typical flow-cell system is constructed of plastic polystyrene material, with the continuous flow of media controlled by a peristaltic pump (Figure 3). This allows for the control of biofilm growth, by means of nutrient delivery and flow rate, over time. Typically, the cell is mounted on a microscope slide, allowing for visualization of fluorescent strains of bacteria within the biofilm with confocal laser scanning microscopy in real-time. There are commercially available systems or they can be self-assembled. Improvements and optimizations of these systems are continually being made, but common drawbacks to flow-cell systems include the confinement of microbial growth to the polymer surface, outgrowth into the inlet tubing and the investment of time, which impedes high-throughput experiments [51,52]. The shear forces generated by liquid in flow cells can be beneficial when studying biofilm-associated infections of the vascular system (e.g., infective endocarditis), oral cavity and urinary tract [36,42,53,54].

Robbins device

With a Robbins device, biofilm is formed on removable plugs, which are unilaterally exposed

to flowing liquid. One common method of using a Robbins device is to allow planktonic cells to adhere with low- to no-flow conditions, and then once an initial biofilm has formed, the device is flipped over and the flow rate increased [55]. With this technique, the flow can be altered during different phases of biofilm maturation, although nutrients are not evenly distributed across the device due to the gradient of fresh nutrients at the input and waste produced at the output [2].

Drip flow reactor

Drip flow reactors utilize dripping liquid to intentionally create continuous, low-shear fluid forces down an angled substratum where biofilms form [2]. Uneven distribution of dripping liquid nutrients and exposure to air or gases at the air-liquid interface promotes biofilm heterogeneity, potentially shifting the dynamics of the maturing biofilm. Some organisms may respond differently to varying types of fluid and flow combinations, including the drip rate of the liquid, material of the substratum, exposure to gases (or lack thereof) and experiment length. Ceramic or metal substrates can be used when simulating the oral cavity, and catheter,

Figure 3. Basic schematic of a dynamic biofilm system. The goal of dynamic systems is to simulate the natural environment by replenishing nutrients and providing shear conditions. Typically, fresh media from a receptacle is pumped through a flow-cell and into a waste receptacle. The speed at which the media flows through the system will dictate the amount of shear force to which the biofilm cells are exposed. An alternative to the common peristaltic pump setup is to allow media to flow through the system by gravity, as in the drip flow reactor model. This generates much lower shear forces, which flow down an angled substratum. Other variations on this basic experimental setup include using different flow-cell apparatuses, such as a Robbins device, or the use of different substrates or surfaces within the flow-cell. Once a biofilm has developed within the flow-cell it can be imaged, either in real-time or after removal from the flow-cell or other apparatus. The main advantages of dynamic systems include the ability to visualize biofilm development in real-time under flow conditions, and for longer periods of time due to the replenishment of nutrients.

endotracheal tube lumens, and other biomedically relevant polymer substrates, are all potential surfaces that can be used with drip flow reactors [35,56].

Microfluidic platforms

Other dynamic models include microfluidic platforms, which allow for the controlled determination and visualization of a maturing biofilm in real time over extended periods. Customizable chips allow full control of environmental conditions, such as hydrodynamic variables, establishment of chemical gradients and differing properties of substratum [38,57]. Some microfluidic chips are also being adapted into high-throughput commercialized platforms [58].

Microfluidic platforms are very good for studying the fine details of several biofilm properties including the role of bacterial motility in establishing and maintaining a biofilm, the interplay of bacteria with different types of substrata, the dynamics of signal exchange (quorum sensing) within the biofilm and the mechanical characteristics of biofilms (viscoelasticity). The major perks for these systems are the significantly reduced amount of reagents they require, the versatility of design options, the ability to dictate experiment length and the ease of automated systems that accompany the chips. The major drawbacks are limitations to chip materials (glass, silicon, other polymers and some metals) and complicated logistics in designing custom chips, often requiring extensive knowledge of fluid engineering. Additional potential drawbacks include expensive and/or technically challenging accessories that may be required for constructing the microfluidic chambers and/or performing experiments (e.g., design software, photomask, fluid pumps, rheometer, imaging and analysis platforms).

Notable polymicrobial studies in dynamic systems

Blanc *et al.* developed a two-step, *in vitro* oral biofilm model for the formation of multispecies biofilms that closely simulated the flow conditions of the oral cavity [55]. Several different bacterial species, including *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, were grown planktonically in co-cultures under anaerobic conditions in a continuous-flow bioreactor. The cultures were then pumped into a

modified Robbins device where multispecies biofilms formed on ceramic calcium hydroxyapatite discs coated with sterile saliva. This experimental setup simulated the temperature, pH, flow and anaerobic conditions of the oral cavity and included a tooth surface (hydroxyapatite) for attachment. The investigators used this model to investigate interactions between primary, intermediate and late colonizers during the progression of oral infections.

Due to the varying oxygen content found in chronic wounds, an *in vitro* wound model using a drip flow reactor and 5% bovine serum was used to investigate the role of strict anaerobes in wound-like environments [59]. Common wound pathogens *P. aeruginosa* and methicillin-resistant *S. aureus* and the obligate anaerobe *Clostridium perfringens*, were used to establish a unique anaerobic environment that allowed for the growth of *C. perfringens* under otherwise prohibitory conditions. In addition, Curtin *et al.* used a drip flow reactor to establish biofilms within catheter lumens and saw a reduction in the adherence of *Staphylococcus epidermidis* cells on those pretreated with bacteriophage in comparison to those coated with blood proteins [35].

Pathogens *P. aeruginosa* and *Flavobacterium* sp. were grown together in a planar flow cell under varying flow gradients over several days to evaluate the effect of shear on biofilm properties and multispecies interactions [37]. Under low-shear with minimal media, *Flavobacterium* outcompeted *P. aeruginosa* in obtaining nutrients by orienting itself above *P. aeruginosa*, thus restricting it to a nutritionally depleted niche. Conversely, high-shear, nutrient-abundant conditions revealed a synergistic relationship between these two species, manifested by stronger adherence of the biofilm to the surface and optimal spatial orientation for each species to obtain nutrients. Similarly, a mixed-species biofilm, consisting of *Pseudomonas* sp. and *Klebsiella pneumoniae*, was grown in a flow-cell and observed under varying flow rates of antimicrobial solutions to determine how this influenced species composition and spatial distribution [36].

Because there is a continuous replacement of nutrients during experiments in flow cells, studies can be carried out for long time periods. For example, *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia*, which all play an integral role in the progression of chronic periodontitis, were grown together under low flow rates

of gingival crevicular fluid to simulate their interaction within the periodontal pocket [42]. The investigators observed that, while these bacteria were poor biofilm formers in a static, time-limited model, they were able to form mature biofilms in flow cells [42,54]. This is a perfect example of how shifting compositional dynamics in an experimental setup can change polymicrobial interactions.

Microcosms

Microcosms are a step closer to simulating an *in vivo* environment by mimicking *in situ* conditions or utilizing *ex vivo* tissues. Microcosms can be static or dynamic, but what differentiates them are the use of nutrient sources and surfaces that are relevant to the site of infection.

Oral microcosm

Many models have been developed in an attempt to simulate the oral cavity [55,60–62]. Innovative strategies have been incorporated, such as using a salivary medium and/or teeth as the substratum, to more closely simulate the *in vivo* environment. For example, Lundstrom *et al.* developed an *ex vivo* translational model, using bovine incisors treated with mucin as the substratum for a dental biofilm. Different species of oral bacteria were inoculated into the model in three distinct colonization phases to assess the efficacy of bactericidal irrigation treatments during different phases of biofilm development [62].

Wound microcosm

Several strategies have been used to simulate the wound environment *in vitro*. Approaches typically include the addition of blood components, and/or the incorporation of host tissue substrate components, including artificial skin and collagen [38,63–66]. For example, Sun *et al.* developed the Lubbock Chronic Wound Model (LCWM) to mimic the wound environment using a combination of bovine plasma, Bolton Broth and laked horse red blood cells [65]. After inoculation with a coagulase-positive bacterial species (such as *S. aureus*), the plasma components of the media clot due to the action of staphylocoagulase, which transforms the liquid media into a solidified mass (Figure 4A). Bacteria then adhere to insoluble fibrin strands and create a biofilm within the host-derived matrix. Alternatively, if a coagulase positive species is not included, a sterile pipet tip, or other type of surface can be added for biofilm adherence.

The LCWM has been used to determine how the growth of different combinations of common wound pathogens affects tolerance to different classes of antibiotics, and to determine how interactions between species are altered in the wound environment [25,26,65]. For example, DeLeon *et al.* observed that while *P. aeruginosa* completely eradicated *S. aureus* after co-culture in standard laboratory media, the two species established stable co-cultures for up to 7 days in this wound model. Furthermore, growing the bacteria together in this environment significantly increased their tolerance to some classes of antibiotics [26].

To simulate the diffusion of chemicals through tissue, a microfluidic chip made of an agarose gel substrate and channels of polydimethylsiloxane was designed to simulate the wound environment [38]. This model was used to study the spatiotemporal properties of polymicrobial biofilms and chemotactic behaviors in real time. In this example, bacteria traveled up created gradients of amino acids and were measured for chemotaxis. This polydimethylsiloxane chip provides the flexibility to utilize any desired fluid medium, making it versatile for studying the impact of different environmental parameters on the spatiotemporal aspects of biofilms.

The Zurich Burn Biofilm model was developed to observe the polymicrobial interactions of common burn pathogens within biofilms, including assessing their collective antimicrobial susceptibility prior to transfer into an *in vivo* burn model [67]. With this model, biofilms are grown on a gauze surface with Gram-positive bacteria, establishing a biofilm prior to the addition of Gram-negative bacteria. The gauze may then be transferred onto an animal's burn wound. However, a completely *in vitro* polymicrobial model that accurately simulates the burn environment has yet to be reported.

Urinary tract microcosm

An easy and common method of studying catheter-associated urinary tract infections (CAUTI) *in vitro* involves establishing uropathogenic bacterial biofilms on catheter surfaces in an artificial urine medium (AUM) [68]. Utilizing these types of models, it has been reported that *C. albicans* and the Gram negatives *E. coli* and *P. aeruginosa* suppressed fungal biofilm formation in AUM that was enriched with glucose [30]. This finding could be clinically relevant, as diabetics tend to have higher rates of urinary tract infections [30,69].

Figure 4. A wound microcosm model. Microcosms are models that incorporate aspects of the *in vivo* environment into an *in vitro* system and can be either dynamic or static. In this example, a wound microcosm has been created with the addition of plasma and red blood cells to a rich, meat-based medium. In this model, *Staphylococcus aureus* clots the plasma components of the media due to the action of staphylocoagulase, which transforms the liquid media into a solid mass. (A) Liquid media is inoculated with different bacterial species in a tube. After 24 h, the media has coagulated and can be removed from the tube. Within this coagulated plasma, bacteria adhere to insoluble fibrin strands and are surrounded by a combination of microbial and host-derived matrix components. This model has been used to support the polymicrobial growth of several bacterial species. (B) Sectioning and subsequent staining with hematoxylin and eosin reveals the adjacent positioning of *S. aureus* and *Pseudomonas aeruginosa* clusters within the coagulated host-matrix material. (C) Intense concanavalin A staining can be seen around clusters of *S. aureus* cells within the wound model.

(B) Reproduced with permission from [26].

Another CAUTI-based model attempted to simulate the bladder environment by growing polymicrobial biofilms in sterile AUM encased in a water jacket, and to develop catheter encrustation from mineral deposits from the urinary tract infection environment [70,71]. *Proteus mirabilis*, in conjunction with other CAUTI-relevant pathogens, were tested for their ability to form crystalline bacterial biofilms in the lumen of the catheter. The investigators observed that some combinations of microbes promoted catheter blockage while others inhibited it.

For prevention of CAUTIs, catheter segments were pretreated with a combination of *E. coli* biofilm and anti-pseudomonal bacteriophage, inoculated with *P. aeruginosa*, and then incubated in human urine to determine the pathogen colonization. The investigators observed that the blanket of bacteria and phage across the catheter surface prevented *P. aeruginosa* adherence [34], suggesting a potential therapeutic probiotic/phage approach to prevent CAUTI.

• *In vivo* models of polymicrobial, biofilm-related infection

Polymicrobial biofilm-associated infections involve interplay not only between microorganisms within the biofilm, but between the microbes and the host. While the host

environment can be simulated, it is impossible to completely replicate the conditions that microbes encounter during infection. Host responses to polymicrobial infections can help predict evolutionary selection of bacterial species and strains, and highlight the importance of the immune response in shaping the competitive or synergic environment [72]. Therefore, it is important to validate results obtained using *in vitro* models *in vivo* to help determine their translatability into clinical settings.

Invertebrate models

Mammalian model systems often can be expensive, necessitate specialized training and/or require lengthy authorization processes to conduct experiments. This is why other, non-mammalian animal model organisms have been adapted to study many different infectious processes, including biofilm-associated infections. Nonetheless, their biggest challenge is translatability to mammalian *in vivo* models and relevance to humans.

There are a variety of invertebrate models, each with unique characteristics including reproduction, lifespan, infection tolerance and growth conditions. Unlike mammalian models, most invertebrate models share low cost, easy maintenance and high manipulability. Invertebrates including *Drosophila melanogaster*

(fruit fly), *Caenorhabditis elegans* (nematode), *Galleria mellonella* (greater wax moth) and *Danio rerio* (zebrafish) are increasingly being used in biofilm, pathogenesis and immunology research [73–76]. Many of these alternative models were originally used to study monospecies biofilm-associated infections, but are now being adapted to study polymicrobial infections [3,7,73].

Drosophila melanogaster, fruit fly

Drosophila melanogaster is a low-cost, easy maintenance model conducive to utilization for high-throughput assays. The fruit fly has served as a key model contributing to our understanding of human development, anatomy and the innate immune response [7,75,77,78]. Relative to the number of reported polymicrobial studies, there are vastly more reports investigating single species infections in *D. melanogaster*. While bacteria, fungi and viruses have all been studied [77], the observance of biofilms in fruit flies has only recently been reported [78].

The *in vivo* environment harbors natural flora that may cause unintentional polymicrobial interactions between inoculated and commensal microbes. For instance, in *D. melanogaster*, coinfection with Gram-positive commensal organisms and *P. aeruginosa* significantly increased *P. aeruginosa* virulence and fly mortality [79]. Eradicating Gram positives present in the environment via antibiotics prior to the inoculation of *P. aeruginosa* decreased virulence, indicating a polymicrobial interaction that aggravated the infection process. This observation was further defined by feeding antibiotic-treated flies with peptidoglycan and *P. aeruginosa* and noting the same increase in virulence, demonstrating that *P. aeruginosa* senses the greater peptidoglycan component of Gram-positive microbes [79].

Recently, *D. melanogaster* was categorized, explicitly, as a biofilm-associated infection model [74,77,78]. After inoculation into fruit flies, *P. aeruginosa* hyperbiofilm-forming strains were less virulent than the non-biofilm-forming strains due to the decreased host immune response, bringing to light a new interplay between microbe and host that is not well understood in *D. melanogaster* [78].

Caenorhabditis elegans, nematode

Similarly to the fruit fly, *C. elegans* is also a model organism requiring little maintenance and for which there are vast genomic and molecular tools available. It also has the added benefit of a

transparent body, allowing for direct visualization of infecting microbes. It is well known for its use in developmental, genomic and host–pathogen interaction studies, and its incorporation into high-throughput assays [75,80].

Monoinfection with *C. albicans* is lethal to *C. elegans* due to hyphae protrusion into the organs. However, coinfection with *Enterococcus faecalis* and *C. albicans* does not cause mortality, as *E. faecalis* inhibits *C. albicans* hyphal morphogenesis [31]. *In vitro* MTP biofilm studies demonstrated that the supernatant of *E. faecalis* inhibited *C. albicans* hyphal and biofilm formation, and then confirmed that inhibition occurred due to quorum sensing disruption [31]. *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhimurium similarly inhibited *C. albicans* filamentation by the production of quorum sensing inhibitors [32,33].

To study the effects of infection on lifespan, a stepwise polymicrobial infection was initiated by immunochallenging *C. elegans* with *S. aureus* and subsequently infecting with the opportunistic pathogen *P. mirabilis*. Alone, *P. mirabilis* did not kill the host; however, in conjunction with immune suppression by *S. aureus*, the *C. elegans* lifespan was reduced by 80% in response to *P. mirabilis* coinfection [81]. *Caenorhabditis elegans* has also been used to study monospecies biofilm formation with either *Yersinia* sp. [82,83] or *Staphylococcus* sp. [84]; however, published polymicrobial infection studies in *C. elegans* have not focused on biofilms.

Other invertebrate models

Other invertebrate models such as *Galleria mellonella*, greater wax moth and *Danio rerio*, more commonly known as the zebrafish, have potential to be utilized for polymicrobial biofilm infection studies. Although most studies involve monoinfections and their effects on immunomodulation and survivability of the host, there have been studies that use previously established models to investigate dual-species interactions in these invertebrates.

Unlike many other invertebrate models, *Galleria mellonella* (greater wax moth) larvae can be incubated at 37°C and are easy to handle due to their large size. However, they do not have a fully sequenced genome, or the array of mutants and molecular tools available for other invertebrate models [75]. Vilela *et al.* investigated the inhibitory effects of probiotic bacteria, specifically *Lactobacillus acidophilus*, against

pathogenic *C. albicans* filamentation using the *G. mellonella* larvae model. Injecting *L. acidophilus* into *C. albicans*-infected larvae increased the survival of the larvae from candidiasis. The prophylactic or therapeutic inoculation of *L. acidophilus* decreased the fungal load of *C. albicans*, attributing to the decreased mortality [85].

Danio rerio (zebrafish), a freshwater cypriid and well known model for developmental biology and immunology, is recently being utilized for modeling host–pathogen interactions. Rendueles *et al.* used germ-free zebrafish to evaluate the protective effects of 37 potentially probiotic bacteria against an extremely virulent *Edwardsiella ictaluri* infection. The investigators noted that one particular strain of *E. coli* protected zebrafish from fatal *E. ictaluri* infections likely by colonization inhibition (either by surface blanketing or direct competition) [76].

Mammalian models

Mammalian models offer the most similar environment to that of the human host, but major challenges still lie in developing mammalian models that are truly reflective of human disease. For example, some microbial species are not able to colonize certain animals; therefore, known human pathogens may not cause infection in animals [27,86]. Also, a pre-existing condition, such as diabetes, or preinfection with one microbe, such as a virus, can influence the incidence of infection, virulence and/or colonization of another microbial species. Therefore, a good animal model should accurately reflect the infection sequela seen in human infection. Many of the models described below were originally developed for monospecies biofilm-associated infection studies, but have subsequently been adapted for polymicrobial infections. Although other biofilm infection models exist for each category of infection, we are limiting the discussion to those that have been widely used to study polymicrobial infections.

Oral infection

Biofilm-related infections frequently occur in the oral cavity, causing periodontitis, dental caries or even abscesses that can lead to sepsis. The cascade of oral biofilm development is well characterized [87]; ergo, the microbial complexity of caries ranges from 70 to 400 different species, but can differ dramatically between individuals and even between lesions of the same individual [88].

Many studies utilize the rat oral cavity to study polymicrobial interactions during oral infections [89]. For example, Kesavalu *et al.* mixed multispecies inoculums of *P. gingivalis*, *T. denticola*, *T. forsythia*, and in a second group included *F. nucleatum*, before administering by oral gavage. The resulting infected area was swabbed for PCR analysis, and maxillae and mandibles were retrieved for alveolar bone resorption analysis [90]. *T. forsythia* and *F. nucleatum* caused more severe alveolar bone loss than other combinations of microbes tested, which the investigators had predicted due to their synergistic ability to form larger biofilms *in vitro* [91].

A rat pup model was used to demonstrate a synergistic interaction between *Streptococcus mutans* and *C. albicans* during oral infection [92]. Pups were first screened and cleared of any endogenous *S. mutans* and *C. albicans* colonization, then infected with *S. mutans* for 23 days, followed by *C. albicans* for an additional 2 weeks. Plaque biofilms were then harvested, sonicated and plated for growth, and the teeth were analyzed for the progression of dental caries [92]. Coinfected pups had larger biofilms and more virulent infections, which more severely eroded their enamel than did pups given monospecies infections.

As microbes present in the normal oral flora can complicate the interpretation of polymicrobial interactions, antibiotics and mouth rinses are frequently used to clear the native oral flora prior to inoculating [90,91]; alternatively, a murine abscess model has been utilized to study interactions between oral microbes, in the absence of the oral flora [29]. Utilizing a murine thigh abscess model, Ramsey *et al.* co-cultured the oral commensal species *Streptococcus gordonii* and the opportunistic oral pathogen *A. actinomycetemcomitans*, and demonstrated that metabolite cross-feeding between the two species correlated with virulence. The investigators observed that *A. actinomycetemcomitans* required L-lactate, a *S. gordonii* metabolite, for establishment of coinfection [29].

Otitis media

Otitis media is an infection of the middle ear and is commonly associated with upper respiratory infections. Recurrent acute otitis media and chronic otitis media with effusion are often associated with biofilms [93,94]. The three most

common animals used to model these infections are chinchillas [95,96], rats [97] and mice [98–100]. Chinchillas have a 15-year lifespan, large ears, which provide easy access to the ear canal, and similar ear anatomy to humans. However, mice and rats have the advantage of lower cost, easier care and the availability of genetic variants, including those for specific host inflammatory responses.

Haemophilus influenzae, *Streptococcus pneumoniae* and *Moraxella catarrhalis* normally reside in the upper respiratory tract, but can become otopathogens and create polymicrobial biofilms in the middle ear [72,101]. Mice and chinchillas were intranasally co-inoculated with *S. pneumoniae* and *M. catarrhalis* to evaluate nasopharyngeal colonization, ascension of the Eustachian tube and, ultimately, increased antibiotic resistance. It was determined that quorum sensing with AI-2 played an important role during these coinfections [27].

Based on an *in vivo* chinchilla model of polymicrobial otitis media involving nontypeable *H. influenzae* and *S. pneumoniae*, Mukherjee *et al.* developed an *in silico* approach to characterize the ecological interactions between these species and the host [72,102]. In the presence of nontypeable *H. influenzae*, *S. pneumoniae* formed localized biofilms in the chinchilla middle ear, rather than systemic infections.

It is thought that upper respiratory viral infections, like those with influenza A virus, increase the chance of bacterial invasion into the middle ear and enhance virulence [103–105]. To investigate the interactions between viral and bacterial pathogens in upper respiratory infections, Wren *et al.* infected mice intranasally with influenza A for 4 days, and then infected with *S. pneumoniae*. When a weak biofilm-forming variant of *S. pneumoniae* was given alone, it displayed impaired colonization. However, when the weak biofilm variant was coinfecting with influenza A, the investigators found increased biofilm formation and inflammation in the nasopharynx and middle ear [104]. While this is an excellent example of how a pre-existing viral infection can affect bacterial biofilm formation, there are very few animal models used to study viral–bacterial interactions. Presumably this is because of the challenges of infecting nonhuman species with human viruses, many of which do not infect, or do not cause symptoms characteristic of human infection, in animals [86,105,106].

Lung infection

Biofilm-related lung infections can manifest as acute pneumonia, as seen in ventilator-associated infections, or in chronic lung infections that commonly occur in patients with respiratory disorders such as chronic obstructive pulmonary disorder or CF. Organisms commonly associated with lung infection are also native nasopharyngeal microbiota, including *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, *Staphylococcus* sp. and *P. aeruginosa*, with different organisms presenting depending on the level of disease severity [105,107–109].

S. aureus typically colonizes the lungs of CF patients during childhood, whereas *P. aeruginosa* dominates later in life. An acute pneumonia mouse model was used to evaluate *in vivo* competition between *S. aureus* and *P. aeruginosa* and showed that a *P. aeruginosa* wild-type strain outcompeted *S. aureus*, as calculated by the competition index, or the ratio of *P. aeruginosa* to *S. aureus* [110]. In a similar murine lung infection model, intratracheally co-inoculated carbapenem-resistant *A. baumannii* (CRAB) demonstrated synergy by protecting susceptible organisms, such as *E. coli*, against treatment with antibiotics [28].

Another strategy often used to simulate CF lung infections is to suspend bacterial cultures in agar or alginate, to mimic the presence of excess mucus, which is characteristic of CF lungs. In one study, *P. aeruginosa* and *Burkholderia cenocepacia*, two species frequently found together in severe CF infections, were suspended together in agar-bead solutions, and then inoculated into the trachea of mice [111]. The coinfection showed increased biofilm formation and inflammation, possibly due to interactions between the two opportunistic species.

Skin & soft tissue infections

Many mammalian wound infection models have been developed such as, but not limited to, those that mimic human chronic and burn wound infections, and human pressure ulcers [112,113]. While many species of mammals have been used to model wound infections, rodents are by far the most common due to the benefits previously mentioned (e.g., cost and availability of genetic knockout strains). However, it is important to note that there are many differences in the anatomy and physiology of the dermis between different mammalian species, which can greatly affect healing. For example, rodents heal much

faster than do humans due to contractile wound closure [114]. Thus in order to slow healing and more closely replicate chronic wounds, contraction is prevented with some kind of mechanical barrier that physically holds the wound open. Typically, some type of stint or transparent dressing is used for this purpose [114].

One murine model for chronic wound infection involves surgical excision of a defined area of skin on the dorsal surface, followed by the inoculation of bacteria straight onto the wound bed (Figure 5). Using this model, Dalton *et al.* grew four wound pathogens, *S. aureus*, *P. aeruginosa*, *E. faecalis* and *Finnegoldia magna*, in the LCWM (refer to *in vitro* microcosm models) and then transferred sections of the resulting microbial population onto mouse wounds [25]. The investigators reported that this 'biofilm transplant' effectively preserved community structure and population distribution, whereas simply infecting wounds with planktonic cultures led to one species (*P. aeruginosa*) quickly taking over the population. The investigators also reported that they were only able to grow the obligate anaerobe, *F. magna*, in wounds in the presence of the other species, which were all facultative anaerobes.

Chronic infections with multispecies biofilms are a major problem in the diabetic population. Diabetes adds a significant immunological impairment that affects the clearing of infection and rate of healing. Therefore, animal models that are able to include co-morbidities, such as diabetes, are important for the study of host-microbe interactions. In one chronic mouse model, *db/db* mice (diabetic mice that have a spontaneous mutation in the leptin receptor, resulting in insulin tolerance) were given a full thickness excision wound, and over time were infected with bacterial species considered to be part of the mouse native skin flora: *S. aureus*, coagulase-negative *Staphylococcus* sp., *Enterococcus* sp., *Enterobacter cloacae* and *Pseudomonas* sp. [115]. The microbes developed biofilms on the wound and notably impaired wound healing compared with the noninfected mice, thus creating a chronic infection [116].

In an inner thigh abscess model, diabetic obese mice with spontaneous mutations in the leptin receptor were infected with combinations of *E. coli*, *Bacteroides fragilis* or *C. perfringens* [22]. By measuring bacterial loads and inflammatory responses at different time points, the investigators were able to identify bacterial-bacterial

and host-bacterial interactions, where *E. coli* and *B. fragilis* were synergistic, but *C. perfringens* was antagonistic. The investigators also observed an overall higher bacterial load in the diabetic mice than their normal counterparts.

The rabbit ear wound model has been used to study biofilm-related infection caused by *P. aeruginosa* and *S. aureus*. Full thickness dermal wounds were administered, and bacteria were inoculated onto the rabbit ear. To maintain a 'biofilm-only' phenotype, antibiotic ointment was administered and antimicrobial absorbent dressings were applied to mature wounds to eliminate planktonic bacteria. The dual species-infected wounds demonstrated impaired healing and increased cytokine expression in comparison to monospecies infected wounds; however the investigators did note that *P. aeruginosa* tended to dominate the infection [117].

Pig skin anatomy and physiology has long been considered to be most similar to that of humans, outside of primates [113]. Pigs also have the added advantage of a large surface area on which several small wounds can be created, providing the opportunity to examine multiple variables and controls within the same animal. A porcine burn model was used to investigate the long-term effects of mixed species biofilms on immune function [118]. Full thickness burns were administered with a novel microprocessor-controlled, electrically heated, burn device and coinfectd with *P. aeruginosa* and *A. baumannii*. Although there was no gross variation in wound closure, barrier skin function was compromised, as measured by transepidermal water loss, resulting in the failure of tight junctions, which could lead to sepsis [118].

Conclusion & future perspective

Our world is filled with diverse consortiums of people that we interact with every day. Sometimes these interactions are friendly and cooperative and sometimes conflicts arise; sometimes communication is fluent and sometimes a barrier is presented. But no matter the nature of our interactions with the people around us, they constantly shape our behavior and development. This is also true for microorganisms, and the 'community view' of microbial life has become the subject of intense study, with the realization that the ways in which bacteria respond to each other may dramatically affect the progression and outcome of infection. For example, it would be important to know that the presence of a specific consortium

Figure 5. Chronic wound mouse model for polymicrobial biofilm infection. (A) Surgical excision of the dermis can be performed on mice to create a full thickness wound. Bacteria are then inoculated directly onto the wound bed. (B) An adhesive dressing is applied to prevent contractile healing, and help protect the wound from contaminants. (C) Wound closure can be measured over time, and tissue can be removed to visualize the biofilm. (D) If the wound has been infected with bacterial strains producing fluorescent proteins, confocal microscopy can be used to directly image bacteria from fresh tissue.

in the lung caused one species to excrete an exoproduct that resulted in edema, or if the adhesion of one specific species onto an orthopedic implant facilitated the colonization of a pathogen, or the synergistic interactions of multiple species resulted in an increased resistance to an antibiotic that normally killed each when alone.

The inadequacies and inaccuracies of studying microbes in the ultra-artificial laboratory environment (e.g., monoculture planktonic growth in rich media) have been brought to light and investigators are investing more time and effort to develop translational biofilm-based *in vitro* and *in vivo* models. Every attempt is being made to more fully replicate the natural conditions that microbes encounter during infection; however, the percentage of studies that have

incorporated multiple species of microorganisms into these models remains relatively small.

The presence of another microbial species will alter the behavior of both, or many, in any given environment. While the importance of these polymicrobial interactions is not lost on investigators, incorporating diverse sets of microbes into experimental models can be challenging. Strategies such as staggering colonization, selecting compatible nutrients and substrata, altering physical environmental forces, and capitalizing on mutant strains, have worked well to establish stable communities in which the polymicrobial interactions between a few select species have been studied. Yet, we are still far away from replicating and deciphering the dynamic and complex web of interactions that occur in natural systems.

Future efforts will be spent improving these models, including new and more powerful methods of analysis, such as genetic tools (RNA sequencing, the use of transposon library sequencing) and imaging (confocal microscopy and advancements in fluorescence microscopy), as well as more efficient methods of data and image processing. And as we progress toward more sophisticated models with which to study polymicrobial interactions in biofilms, there are several questions that need to be addressed for each model of infection: Do ordered communities consistently arise? What governs community composition and spatial

distribution between different species? Are there specific consortia or bacterial loads that result in much worse infection outcomes? As new mechanisms of interaction are discovered, we should eventually be able to model predicted outcomes based on the identity and quantity of different microbes present in an environment, as well as present more accurate diagnoses and potential treatments for polymicrobial infections.

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EXECUTIVE SUMMARY

Polymicrobial interactions in biofilms

- Polymicrobial interactions are just as likely to influence bacterial behavior as any other environmental parameter.
- Synergistic and antagonistic interactions are most commonly studied when modeling polymicrobial biofilm communities.
- Community composition and structure will influence all aspects of biofilm dynamics.

Biofilm models of polymicrobial infections

- While many models of biofilm-related disease have been developed, relatively few have incorporated multiple species of microbes.
- *In vitro* models can be static or dynamic and are indispensable for exploring fundamental questions about biofilms, acquiring empirical data and laying a foundation upon which confirmatory *in vivo* testing may be pursued.
- *In vitro* design strategies that strive to reflect the *in vivo* environment, such as microcosms, have been successful in overcoming many of the obstacles involved in growing diverse species together.
- *In vivo* biofilm ecology should be an important consideration when designing *in vitro* models to mimic infection.
- While the host environment can be simulated, it is impossible to completely replicate the conditions that microbes encounter during infection *in vitro*. Therefore, it is important to validate results obtained using *in vitro* models *in vivo* to help determine their translatability into clinical settings.
- Invertebrates can provide a low cost *in vivo* environment that is more feasible than mammalian models.
- A good mammalian model should accurately reflect the infection sequela seen during human disease.

Future perspective

- Insight into the pathogenic mechanisms of polymicrobial biofilm consortia are likely to help in treating future lung, ear, oral, urinary tract, device-related and wound infections, among others.
- Future polymicrobial biofilm models will likely include the application of more powerful and efficient genetic tools, imaging and data processing.
- Future questions to be addressed include: Do ordered communities consistently arise? What governs community composition and spatial distribution between different species? Are there specific consortia or bacterial loads that result in much worse infection outcomes?
- Modeling predicted infection outcomes based on the identity and quantity of different microbes present in an environment could eventually provide more accurate diagnoses and potential treatments for polymicrobial infections.
- Determining how polymicrobial communities influence the antibiotic resistance and susceptibility of each of the members may eventually be used to better predict the development and spread of antibiotic resistance and improve diagnostics.

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