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Understanding the Complex Phage-Host Interactions in Biofilm Communities

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Abstract

Bacteriophages and bacterial biofilms are widely present in natural environments, a fact that has accelerated the evolution of phages and their bacterial hosts in these particular niches. Phage-host interactions in biofilm communities are rather complex, where phages are not always merely predators but also can establish symbiotic relationships that induce and strengthen biofilms. In this review we provide an overview of the main features affecting phage-biofilm interactions as well as the currently available methods of studying these interactions. In addition, we address the applications of phages for biofilm control in different contexts.

1. INTRODUCTION

Most of the bacteria found in nature live in microbial communities called biofilms (1), where the microbial cells are attached to a surface and encased in a self-producing matrix of extracellular polymeric substances (EPSs) that confers on them an environment protected from hostile conditions (2, 3). In turn, bacteriophages (phages), the natural predators of bacteria, are considered the predominant biological entities on earth and can be found in almost all ecological niches where their host bacteria are present (4, 5). Although phages and biofilms have coexisted in natural environments for millions of years, the complex interactions between them are far from fully understood. So far, most phage-bacteria studies are conducted in suspended cultures; in addition, the biofilm studies that have been reported are usually performed under specific conditions that are unable to mimic the high complexity of real biofilms found in nature or in health contexts (6). This makes it difficult to draw conclusions about the impact biofilms may have had on phage evolution over the years, as well as the role of phages in shaping the bacterial diversity in these particular niches. Combining in vitro, ex vivo, or in vivo biofilm infection assays with computational simulations can, however, help us to uncover and better understand the phage-biofilm interactions. In this review, we address the mechanisms underlying phage-bacteria interactions in biofilm communities, the possible methods of studying these interactions, and the potential applications of phages for biofilm control in different contexts.

2. PHAGE-BIOFILM INTERACTION FROM MOLECULAR, EVOLUTIONARY, AND ECOLOGICAL PERSPECTIVES

As biofilms are ubiquitous in nature, so are phages (1, 7). The coevolution between phages and bacteria has been an important driver for the huge phenotypic and genotypic diversity found in these microbial populations (8). Although biofilms can protect bacteria from harsh environmental conditions and phage predation, phages can encode in their genomes EPS-degrading enzymes, such as depolymerases, to obtain an advantage against these complex structures (9). On the other hand, bacteria within biofilms have coevolved to find new counter-defense mechanisms, leading to an endless arms race between phages and bacteria (8, 10–12). There is also growing evidence that phages can promote biofilm formation and bring benefits to their bacterial hosts (13). All of these aspects regarding phage-biofilm interactions are discussed below and are represented in **Figure 1**.

2.1. How Biofilm Composition and Architecture Affect Phage Infection

It is well known that biofilm structure and composition can pose limitations on phage predation (6). In addition to the biofilm matrix that contributes to impairment of the diffusion of phages and their propagation, other factors such as the low metabolic activity of biofilm cells, the presence of secreted molecules that may act as phage decoys, or even the presence of more than one microbial species in the biofilm may also contribute to an inefficient phage infection (6, 14).

Flemming & Wingender (15) estimated that in most biofilms, the EPS matrix accounts for more than 90% of the biofilm dry mass, whereas the microbial cells account for less than 10%. This EPS matrix—composed of polymeric substances and other secreted products including enzymes, proteins, lipids, or nucleic acids—contributes to the cohesion of biofilms (15) and can cause phage entrapment, acting as a physical barrier to phage diffusion and access to the bacterial cells, and consequently preventing an efficient infection (16).

González et al. (17) studied the parameters that affected the diffusion and propagation of two phages in *Staphylococcus* spp. biofilms. Although the authors confirmed that both phages could

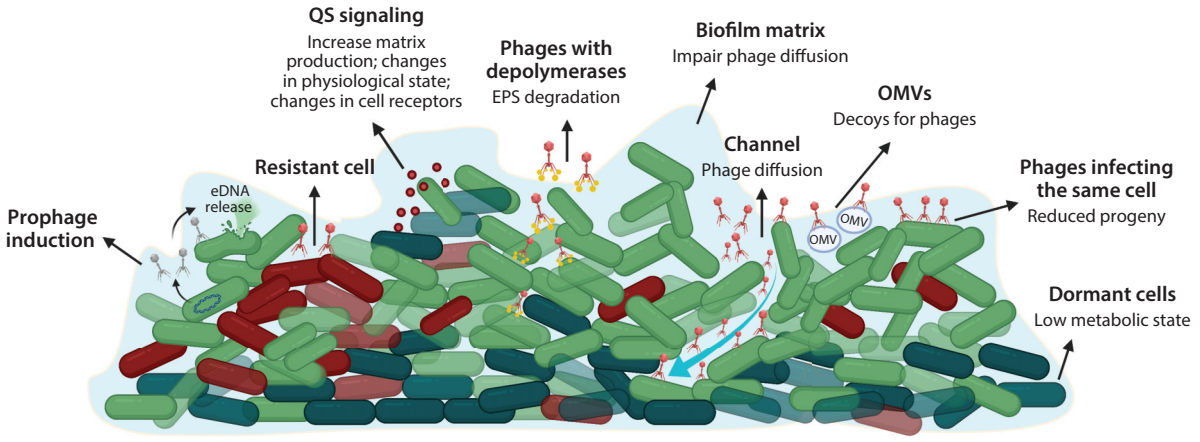

Figure 1

Schematic representation of the features affecting phage-biofilm interaction. The biofilm is composed of the EPS matrix (*light blue*), known to impair phage diffusion through the biofilm, and different types of bacterial cells: phage-susceptible and metabolically active cells (*green*), dormant cells (*dark green*), and phage-resistant cells (*red*). The close proximity of biofilm cells may lead to more than one phage infecting the same bacterial cell, resulting in reduced progeny. The secretion of OMVs or other molecules by the bacterial cells may act as a decoy for phages that will bind to these molecules instead of the bacterial receptors. The release of signaling molecules by bacteria can also influence the phage-host dynamics in the biofilm, as it can result in increased matrix production, changes in the physiological state of the bacterial cells, and changes in cell wall receptors. The presence of clusters of resistant cells in the biofilm may protect phage-sensitive cells from phage predation, and the induction of prophages may contribute to biofilm formation through the release of biofilm-promoting agents (e.g., eDNA). However, phages may encode EPS-degrading enzymes in their genomes (e.g., depolymerases) or use biofilm channels to reach the target cells. Abbreviations: eDNA, extracellular DNA; EPS, extracellular polymeric substance; OMV, outer membrane vesicle; QS, quorum sensing. Figure adapted from images created with BioRender.com.

diffuse through all the different biofilms tested, their data suggested that the diffusion rates of phages within biofilms were influenced by several factors: the amount of biofilm biomass, the susceptibility of the bacterial strains to the phages, the phage concentration, and the composition of the biofilm matrix that might contain phage-inactivating enzymes or components able to anchor the phages (17). For instance, it is known that the outer membrane vesicles (OMVs) secreted by some bacterial species can mediate phage entrapment in biofilms. These OMVs may contain phage receptors, as observed for both *Escherichia coli* and *Vibrio cholerae* species (18, 19), which may contribute to an irreversible binding of phages that will not be available to infect the biofilm cells.

The protective role of the biofilm matrix to phage predation was clearly demonstrated in a recent study by Melo et al. (20) that assessed the interaction of a *Staphylococcus epidermidis* phage with different biofilm-associated host cell populations. The observations of this study were corroborated by confocal laser scanning microscopy (CLSM) data, which demonstrated that phage-infected cells appeared only in certain regions of the biofilm where lower amounts of matrix were present, evidencing that the biofilm matrix can serve as a shield to protect the embedded bacteria from viral attack (20). In fact, the spatial organization of the biofilm can be a determinant to the success of phage infection, as it may lead to limited mobility of cells that tend to organize in localized niches with different nutrient availability (21). While the proximity of the cells in these clusters might contribute to a decreased number of progeny phages as a result of multiple phages infecting the same host cell (22), the nutrient gradients often lead to cells under different metabolic states, including dormant or persister cells (6). It is known that phages require an active machinery of the host to propagate, and consequently, their replication is strongly influenced by

the physiological state of the host cell (23, 24). Therefore, so far only a few phages were reported to have the capacity of infecting stationary-phase cells (25, 26). The number of biofilm cells with reduced metabolic activity is expected to increase with biofilm age; consequently, older biofilms (frequently found in nature) will be less susceptible to phages than younger biofilms (6).

Another important feature that also affects phage diffusion through the biofilm structure is the presence of more than one microbial strain or species. It is estimated that most biofilm communities found in nature are composed of a variety of microorganisms instead of a single one (27). Testa et al. (28) demonstrated that the outcome of phage infection is influenced by both the spatial structure of the biofilm and the presence of more than one strain. The interaction of phages with multispecies biofilms is a rather complex process due to the higher diversity of polymeric substances and heterogeneity of the biofilm (14). Although these biofilms are expected to be less susceptible to phage predation, more studies are needed to understand their interaction with phages in real habitats.

2.2. How Phages Have Adapted to Infect Bacterial Biofilms

The long coevolution between phages and bacteria in nature has led them to evolve mechanisms that facilitate their access to the bacterial cell surface, which might be particularly useful in biofilms, where the bacterial cells are encased within the EPS matrix. In fact, it is known that a large number of phage genomes encode enzymes capable of degrading polymeric substances including capsular polysaccharides, exopolysaccharides, or lipopolysaccharides (9, 29). These phage-derived enzymes, called depolymerases, are mostly found as part of the tail fiber or tail spike proteins of phages and are responsible for the depolymerization of bacterial capsules, facilitating phage adsorption (29). Phage depolymerases may also play an important role in phage-host interaction within biofilms by promoting matrix disruption and a consequent easier diffusion of phages through the biofilm structure to the target bacterial cells (6).

In 1998, Hughes et al. (30) reported an *Enterobacter agglomerans* phage displaying depolymerase activity that was capable of disrupting biofilms, a characteristic that was attributed to the combined effect of EPS degradation caused by the enzyme and the subsequent cell lysis caused by the phage. Similarly, studies by Cornelissen et al. (31) showed that although a *Pseudomonas putida* phage encoding a polysaccharide depolymerase revealed biofilm-degrading properties, phage amplification had a major role in biofilm degradation, as the experiments using phage depolymerase alone did not cause biofilm disruption. However, some studies have highlighted the role of depolymerases in biofilm degradation and dispersion, even when these enzymes are applied alone. For instance, Gutiérrez et al. (32) reported that an EPS depolymerase derived from a *S. epidermidis* phage was able to prevent and disperse staphylococcal biofilms when applied alone, although the response was dose dependent. In a similar way, Wu et al. (33) expressed a depolymerase encoded by a *Klebsiella pneumoniae* phage and applied it in mature biofilms, which revealed the biofilm-dispersion ability of the enzyme. The antibiofilm properties of depolymerases may also be enhanced by other phage-encoded enzymes, such as endolysins [lytic enzymes responsible for peptidoglycan degradation and host cell lysis (34)], as described by Olsen et al. (35) in a study targeting *Staphylococcus aureus* biofilms.

It is also important to highlight that phages can find other ways to penetrate the biofilm structure and reach the bacterial cells. In a study by Vilas Boas et al. (36), a fluorescence molecular probe designed to target the messenger RNA of a phage major capsid was used to track phage-infected cells within a biofilm population. The authors demonstrated that phage diffusion through the biofilm may be mediated by the channels that can be found in some biofilms, as the phage-infected cells were primarily located close to the edges of these structures (36).

2.3. How Bacteria Evolved to Escape from Phage Predation

To date, several studies have reported the fast proliferation of bacteriophage-insensitive mutants (BIMs) after biofilm treatment with phages (37–41). Although the mechanisms underlying phage resistance in these studies are not always clear, the genotypic analysis of BIMs frequently reveals mutations in genes encoding phage receptors (39, 41). Nonetheless, other mechanisms can be used by bacteria to counterattack phage evasion, namely in biofilm mode, which include signaling systems or CRISPR-Cas systems.

It is known that bacterial communication relies on signaling molecules, known as autoinducers, which regulate gene expression in response to variations in population density by a process called quorum sensing (QS) (42). Because QS plays an important role in controlling the gene expression of virulence factors and biofilm development (43), this communication system is also relevant to understand the phage-host dynamics in biofilm populations. In fact, QS can be extremely useful when bacterial cells are under phage predation; consequently, it has been linked to increased phage resistance in several ways (44, 45). One example is that QS signals can regulate the genes involved in the production of biofilm matrix (46, 47), which was described above as one of the major factors impairing phage infection. Additionally, QS can modulate the expression of phage receptors in bacterial cell surface as described by Høyland-Kroghsbo et al. (48). Using a model system of *E. coli* and phage λ, the authors found that the bacterial host reduced the numbers of cell surface receptors in response to QS signals, which resulted in a reduction in phage adsorption rate (48). Similar observations concerning the QS regulation of antiphage mechanisms were also reported by Tan et al. (49) in *Vibrio anguillarum*. In addition, QS can also influence phage infection by affecting the physiological state of the host cell population, as observed for *Pseudomonas aeruginosa* (50). There is also increasing evidence that QS can control the regulation of CRISPR-Cas systems of several bacterial species, such as *P. aeruginosa* (51) or *Serratia* spp. (52). CRISPR-Cas systems are widely distributed across bacterial genomes and provide them with adaptive immunity against invasive genetic elements, including phages (53). Many other antiphage systems have been described over the past few years (reviewed in 54, 55). These systems result from the long-term evolutionary adaptation of bacteria to survive the constant offense of phages in natural environments. Overall, QS contributes to maintaining population stability when phage densities are relatively high. Other density-dependent mechanisms, such as superinfection immunity, make important contributions for the equilibrium of biofilm populations. This has been explained by the Piggyback-the-Winner (PtW) theory, which proposes that the phenotypic advantages of lysogeny are favored at high host abundances (56).

Because of all these defense mechanisms, and similar to what happens under lab conditions, the presence of phage-resistant bacteria is also expected in biofilms found in natural contexts. However, it is not clear how these resistant populations will interact with phages in biofilms. To better understand the dynamics of a phage-resistant population within biofilms, Simmons et al. (57) set up an experimental model of mixed *E. coli* biofilms with resistant and susceptible hosts subjected to T7 phage attack, which was analyzed by confocal microscopy. According to the authors, the biofilm structure promotes the coexistence of both phage-resistant and phage-susceptible bacteria. When phage-resistant cells are initially rare in the biofilm, the susceptible cells are cleared by phage and the number of phage-resistant cells will increase and form clusters in the empty space; however, when phage-resistant cells are initially common (at least 60% of the population), the relative fraction of resistant and susceptible bacteria will not substantially change after phage treatment, as the susceptible cells are protected from phage exposure through immobilization of phages in clusters of resistant cells, resulting in a more structured biofilm composed of both populations (57).

2.4. How Phages Can Modulate and Trigger Biofilm Formation

Although several studies have highlighted the potential of phages for biofilm control, not all phages have this ability, and studies have shown evidence that some phages can modulate biofilm formation and even increase biofilm levels (44). This can be explained by the selective pressure caused by phages that results in fast propagation of phage-resistant cells or by the induction of prophages that contributes to a release of biofilm-promoting molecules.

Hosseinidoust et al. (58) studied whether a phage treatment can lead to enhanced biofilm formation in consequence of resistant cells or spatial refuges. To address this question, the authors exposed single-species biofilms (*P. aeruginosa*, *Salmonella enterica*, and *S. aureus*) to specific phages (as a pretreatment or post-treatment) and observed that some phage treatments resulted in increased biofilm formation with levels above the control (58). In a study by Henriksen et al. (59), where different phage treatments against *P. aeruginosa* flow-cell biofilms were evaluated, the authors observed that repeated phage treatments (three phage doses every 24 h) did not improve the antibiofilm efficacy of phages, resulting in a significant increase of microcolonies, which provide protection from phages, as well as increased biofilm thickness. Tan et al. (60) studied the effect of two vibriophages in the biofilm formation of *V. anguillarum* and observed different effects depending on the phage used: While one of the phages was able to control biofilm formation, the other one stimulated biofilm development. The authors of the study explained the different behaviors of the phages by the presence of spatial refuges formed by some strains, which can promote the coexistence of phages and bacteria, as already mentioned above. The authors also highlighted the diversity of phage-host interactions even within the same bacterial species (60). Similarly, Fernández et al. (61) showed that the exposure of *S. aureus* biofilms to subinhibitory doses of phages can promote biofilm formation and protect cells from complete eradication.

Although these studies were performed with lytic phages, prophages are also known to directly affect biofilm formation. In fact, prophage induction during biofilm development might mediate a release of biofilm-promoting components as observed by Carrolo et al. (62). In this study, the authors reported that the lysis of *Streptococcus pneumoniae* host cells mediated by spontaneous induction of prophages into the lytic cycle contributed to extracellular DNA (eDNA) release, which favored biofilm formation by the remaining pneumococcal population (62). This is not surprising because eDNA is a key component of the biofilm matrix of most bacterial species, and it is known to have a major role in biofilm development by promoting adhesion to surfaces and maintenance of the structural integrity of biofilms (reviewed in 63). The enhanced biofilm formation in consequence of phage-induced lysis was also reported by Gödeke et al. (64). While the cell lysis mediated by three prophages harbored in the genome of *Shewanella oneidensis* MR-1 promoted biofilm formation, a bacterial mutant devoid of prophages revealed impaired biofilm formation ability (64). Similar observations related to the ability of prophages to trigger biofilm formation were also reported for *Actinomyces odontolycus* (65). In addition to these studies, it is important to highlight that the *P. aeruginosa* filamentous phages (Pf-like) have also been revealed to play an important role in the life cycle and structural integrity of *P. aeruginosa* biofilms (66, 67). Another interesting example of how phages can modulate biofilm formation was reported by Ojha et al. in *Mycobacterium* (68). In this study, the authors observed that the integration of the *Mycobacterium smegmatis* temperate phage Bxb1 led to the inactivation of gene *groEL1*, which contains the *attB* site for phage integration. Although Bxb1 integration did not affect the planktonic growth of bacteria, it prevented biofilm maturation, as the *groEL1* gene is involved in the synthesis of mycolic acids, namely during biofilm formation.

Although some of the studies described above established a link between prophage induction and biofilm formation, the cell lysis mediated by spontaneously induced prophages may also lead

to biofilm dispersion. For instance, Rossmann et al. (69) demonstrated that high levels of the QS molecule Al-2 produced by *Enterococcus faecalis* induced the dispersal of bacterial cells from established biofilms due to prophage release. In a recent study by Tan et al. (70), the authors also highlighted the role of QS signaling in coordinating phage-host interaction and biofilm formation in *V. anguillarum*; however, in this study an H_2O-like prophage stimulated the host's biofilm formation, although its induction was repressed by QS. In a study using *P. aeruginosa* PA14, Zegans et al. (71) observed that lysogeny by phage DMS3 inhibited biofilm formation and swarming motility of the strain. According to the authors, this inhibition was explained by a concerted action of the phage and the CRISPR system of the host (71).

3. METHODS OF STUDYING PHAGE-BIOFILM INTERACTION

Although numerous methods of biofilm formation have been described in the literature, there is still a lack of standardized and appropriate protocols to simulate real biofilms under laboratory conditions. The type of device used for biofilm formation, the culture media, and the presence of external stresses (e.g., shear forces) will directly influence the biofilm structure, which will have a major impact on the outcome of phage treatment. Below we present an overview of the experimental setups that are most commonly used to form biofilms, as well as the different methods that have been implemented to study phage-biofilm interactions (**Figure 2**).

3.1. Experimental Setups for Biofilm Formation

The choice of an adequate platform for biofilm experiments can determine the outcome results. Numerous factors can influence biofilm formation, structure, and composition and consequently impact phage interaction with biofilm cells.

3.1.1. In vitro models and the influence of biofilm formation conditions. The majority of in vitro biofilm studies involve the use of microtiter plates as experimental setups (72). The main advantages of using these devices are their low price and the possibility of performing high-throughput studies (72). There are several studies reporting the efficacy of phages against biofilms formed in microtiter plates, and a compilation of them was previously reviewed (6). However, it is with high difficulty that the results obtained using different microtiter devices can be translated to the reality found in different biofilms of environmental, clinical, food industry, or veterinary contexts. The biofilms formed in real conditions face several stresses, namely shear forces under continuous liquid flow that static devices cannot mimic. Therefore, for a better understanding of phage-host interactions, the use of more sophisticated biofilm dynamic models is recommended. Examples of dynamic devices are flow cells, drip flow reactors, modified Robbins devices, and rotary biofilm devices (72). In a study by Rieu et al. (73), time-lapse CLSM was used to characterize the structural dynamics of *Listeria monocytogenes* growth in static (stainless steel chips on petri dishes) and dynamic (flow cell BST FC 81) conditions. In static conditions, thin unstructured biofilms were observed, while when biofilms were grown under dynamic conditions, they were highly organized with microcolonies surrounded by a network of knitted chains (73). More recently, Yang et al. (74) used nitrogen sparging to induce shear stress on biofilms formed on cubic dual-chamber air-cathode microbial fuel cells with a cation exchange membrane. Using electrochemical impedance, the authors observed that a shear stress–enriched anode biofilm showed a low-charge transfer resistance in comparison with the unperturbed enriched anode biofilm. Moreover, CLSM micrographs clearly indicated that the shear stress–enriched biofilms were entirely viable, in opposition to unperturbed biofilms that exhibited a viable outer layer with a high proportion of dead cells in the inner layers of the biofilm (74). Taken together, these results emphasize the

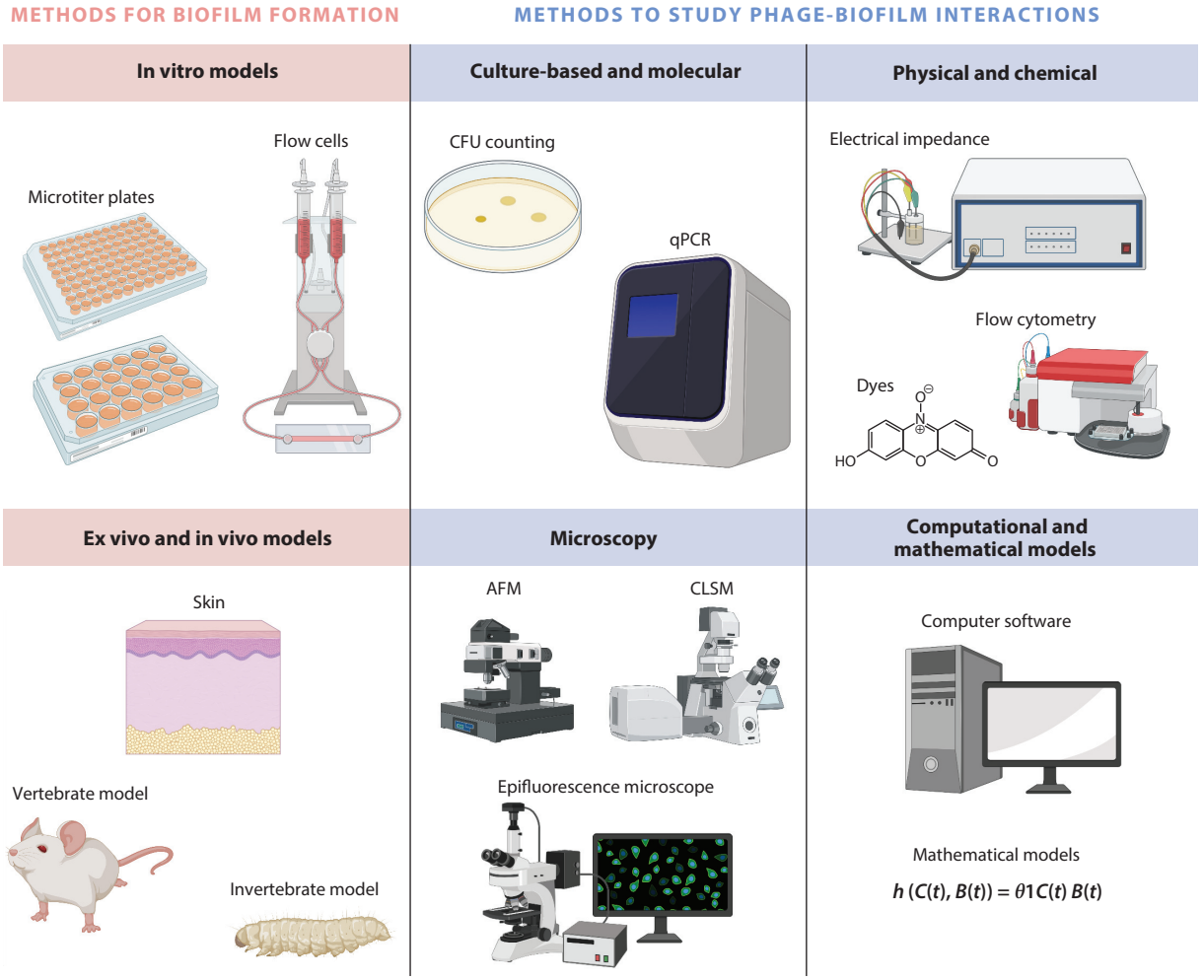

Figure 2

Representation of the methods used for biofilm formation and studying phage-biofilm interactions. Abbreviations: AFM, atomic force microscopy; CFU, colony-forming unit; CLSM, confocal laser scanning microscopy; qPCR, quantitative PCR. Figure adapted from images created with BioRender.com.

importance of shear stress conditions on the biofilm formation outcome, which ultimately affects interaction with phages.

Another important feature of biofilm studies is the effect of culture media on the biofilm structure and cells. Most biofilm studies are performed with bacteria growing in rich media. Jones et al. (75) used CLSM to compare the structure of *Proteus mirabilis* biofilms formed in Luria–Bertani broth and artificial urine. The authors observed that while biofilms formed on rich media displayed the typical mushroom structure with water/nutrient channels, biofilms formed using artificial urine exhibited a flat structure almost deprived of channels (75). Different phage-biofilm interaction studies were assessed on dynamic biofilms using simulated body fluids. In a study using microtiter plates, a phage cocktail containing two enterococci phages successfully reduced the bacterial load after three hours of infection in a medium simulating wound conditions (76). In two

other studies (77, 78), phage cocktails were successfully applied on sections of Foley catheters to reduce biofilms grown in artificial urine.

Both biofilm-formation devices and conditions used, such as culture media, highly interfere with biofilm structure and composition. As previously discussed, this has a huge influence on the way phages interact with biofilms.

3.1.2. Ex vivo and in vivo models. The majority of studies mimicking real conditions are usually performed to simulate phage therapy against infectious biofilms. Lebeaux et al. (79) discussed the applicability of ex vivo models as an interesting alternative approach to the use of in vivo models. Ex vivo models have reduced alterations of natural conditions, as they involve the use of tissue derived from a living organism in an artificial environment. In comparison to in vivo models, they also allow a more controlled experimental setup, with reduced ethical concerns. For example, phages were applied in porcine skin to simulate wound treatment of infections caused by different pathogens (80, 81). Despite the advantages of using this type of model, the lack of host (human or animal) response and the short duration of experiments are still some hurdles to the widespread implementation of ex vivo models. In that sense, in vivo models are the best choice for studies that intend to understand the pathology of infection. Recently, a comprehensive review of the most relevant in vivo studies accomplished in the past decade was published, and different routes of phage administration, dosage effect, and different animal models of distinct types of infections were compared (82). It is important to highlight that the in vivo studies performed in biofilm models usually represent acute infections, in opposition to real biofilm infections that are usually characterized by their chronicity and recalcitrance (83).

3.2. Methods of Studying Phage-Biofilm Interactions

Phage-biofilm interactions can be studied by a set of approaches that assess biofilm biomass and/or cell viability. These approaches can be divided into culture-based, molecular, physical, chemical, microscopy, and computational and mathematical models (**Figure 2**). The advantages and disadvantages of the majority of these methods have been thoroughly reviewed (72).

3.2.1. Culture-based methods. The determination of the number of colony-forming units (CFUs) is the most widely used technique to assess the efficacy of phage killing in biofilms. This technique is based on serial dilutions of bacterial suspensions; it is a straightforward and universally used method. But despite these advantages, CFU determination usually underestimates the number of biofilm cells. Biofilms are composed of a subpopulation of viable but nonculturable cells that normally are not detected by CFUs (84). In addition, the presence of biofilm aggregates also dramatically interferes with cell counting (85).

3.2.2. Molecular methods. In an alternative to culture-based methods, PCR- or molecular-based methods can be used to study biofilm communities. These approaches allow the quantification of the number of viable cells, usually assessed by quantitative (q) PCR. Unlike CFU determination, regular qPCR frequently overestimates the number of viable cells, as the results are influenced by the presence of eDNA and dead cells (86). To overcome this limitation, Magin et al. (87) used PEMAX® dye, which detects only metabolically active cells, to study the effect of phages against *P. aeruginosa* biofilms.

Whole-transcriptome analysis has also been used to study the effect of phages on biofilm cells. Fernández et al. (61) showed that when *S. aureus* biofilms were exposed to low doses of phage vB_SauM_phiIPLA-RODI (phiIPLA-RODI), the cells entered a unique physiological state that

can benefit both prey and predator. This happens because, under phage predation, biofilms are thicker and have higher amounts of eDNA. In addition, RNA sequencing data evidenced that infected biofilms activate a stringent response that can delay phage infection progression, helping both populations tend to an equilibrium (61).

3.2.3. Physical methods. The aforementioned limitation on biofilm cell counting accuracy can be overcome by using flow cytometry in combination with bacterial cell staining with viability fluorophores (88). In addition to a very quick and precise cell counting, using an appropriate dye, this methodology also allows an evaluation of the physiological state of cells (88). This methodology has been suggested as a very promising approach to study, in almost real time, phage-biofilm interactions (89).

Other physical methods can be used to assess biofilm biomass, such as wet or dry weight measurements. Sillankorva et al. (90) used the dry weight method to calculate the amount of biofilm biomass reduction caused by a *Pseudomonas fluorescens* phage when interacting with biofilms of different stages of maturity.

In the past decade, a more sensitive method based on electrical impedance has also been applied to study the effect of phages against biofilms. This methodology allows a real-time analysis of different electric parameters that can be used to assess phage efficacy against biofilms (91) or to measure physiological modifications of matrix composition after phage challenge (92).

3.2.4. Chemical methods. Chemical methods allow indirect measurement of biofilm components, through the use of dyes or fluorochromes that can adsorb or bind to cells or matrix components, or assessment of the cellular physiology of biofilms. For example, resazurin (93) and XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] (94) have been used to determine the effect of phages against biofilms. Despite the widespread use of these methods, there is a lack of reproducibility associated with them. In addition, the fact that a standard protocol is not available makes the comparison of results among different studies difficult.

3.2.5. Microscopy methods. Numerous microscopy-based imaging modalities are available to analyze biofilms; their pros and cons have been widely discussed elsewhere (72). Several of these approaches have already been used to examine phage-biofilm interactions, namely epifluorescence microscopy, CLSM (95), scanning electron microscopy (SEM) (96), field emission SEM (90), and atomic force microscopy (97). An optical system that allows simultaneous imaging of individual bacterial cells over a 36-mm^2 field of view was recently developed (98). With this system, *E. coli* biofilms were observed in a detail never seen before, and new intracolony channels with an approximately 10-μm diameter were discovered (98).

For fluorescence microscopy, biofilm elements need to be marked with fluorescence probes. Microbial cells are usually stained with DAPI or LIVE/DEAD for viability. Components of the biofilm matrix can be marked with fluorescence-labeled lectins such as wheat germ agglutinin conjugated with different fluorophores. Recently, different fluorescence-based approaches were designed to study phage-biofilm interactions. For instance, Akturk et al. (99) designed bacteria-specific fluorescent probes based on phage proteins to discriminate between *S. aureus* and *P. aeruginosa* on dual-species biofilms. Another elegant approach is based on the use of fluorescence in situ hybridization (FISH). Although phageFISH was designed to detect *Pseudoalteromonas* using polynucleotide probes (100), more recent techniques using locked nucleic acid probes as an alternative to DNA probes proved to be very successful when applied on biofilms. These probes allow the discrimination of phage-infected cells and the visualization of their spatial distribution within single-species (20) or multi-species biofilms (36).

3.2.6. Computational and mathematical models. Mathematical models hold great potential for the quantitative description of the population dynamics in a biofilm following phage predation. For example, Heilmann et al. (101) used stochastic spatial models to study the degree of bacterial susceptibility to phage predation. The authors showed that bacterial density or biofilm formation can produce refuges and edges in a self-organized manner (101). Laboratory experiments performed by Li et al. (102) demonstrated that, when phages find motile hosts, a well-delimited lysis zone is formed; when the authors applied a mathematical model, they observed that the lysis pattern was a consequence of local nutrient depletions and inhibition of bacterial and phage motility. In a similar approach, Ping et al. (103) showed that phage mobility requires virus particles to hitchhike with moving bacteria, which can simulate what happens on biofilms.

A mathematical model developed by Eriksen et al. (104) predicted that biofilm microcolonies formed only by phage-sensitive bacteria have the ability to survive due to the bacterial growth throughout the microcolony, which can exceed the rate at which the cells are being killed by phage action. Using mathematical models and a computational framework, Simmons et al. (105) developed simulations that led to the conclusion that the equilibrium state of interaction between phages and biofilms is largely affected by the nutrient availability of biofilm cells, the infection likelihood per encounter, and the capacity of phages to diffuse through the biofilms. The authors also concluded that the biofilm matrix has a role in controlling these interactions by governing the extent to which prey and predator can coexist in the environment (105). In another study, a computer simulation of phage-host dynamics during biofilm development was applied based on experimental data obtained using *S. aureus* and the virulent phage phiIPLA-RODI (106). The results demonstrated that even small differences on pH evolution can dramatically affect the course of biofilm infection, suggesting that phage-host interactions can be tightly coordinated by different environmental signals (106). Very recently, Hartmann et al. (107) developed BiofilmQ, which is an innovative image cytometry software tool that allows automated and high-throughput quantification, analysis, and visualization of numerous biofilm properties. This tool is able to provide quantitative data from data analysis by scientists without programming skills to study biofilms and will provide new insights into phage-biofilm interaction.

4. APPLICATIONS OF PHAGES FOR BIOFILM CONTROL

Phages can access and kill sessile bacteria causing biofilm destruction through mechanisms that were already discussed. This feature has boosted the development of several phage-based strategies to control biofilms in a variety of anthropogenic contexts where biofilms are harmful. The use of phages to combat biofilm-associated infections is usually referred to as phage therapy, while the application of phages to control environmentally detrimental biofilms is considered phage biocontrol.

In this section we present the latest developments related to the application of phages as therapeutics and biocontrol agents, and we discuss the challenges and pathways for future developments (**Figure 3**).

4.1. Phages to Control Clinically Relevant Biofilms

Biofilms are associated with a variety of chronic and difficult-to-treat infections. They can be formed on human tissues, causing tissue-related infections (e.g., endocarditis, lung infections, periodontitis, rhinosinusitis, osteomyelitis, chronic wounds, meningitis, and kidney infections), and on indwelling materials, triggering device-related infections (108). The serious implications of biofilms on human health and the renewed interest in phage therapy have motivated the

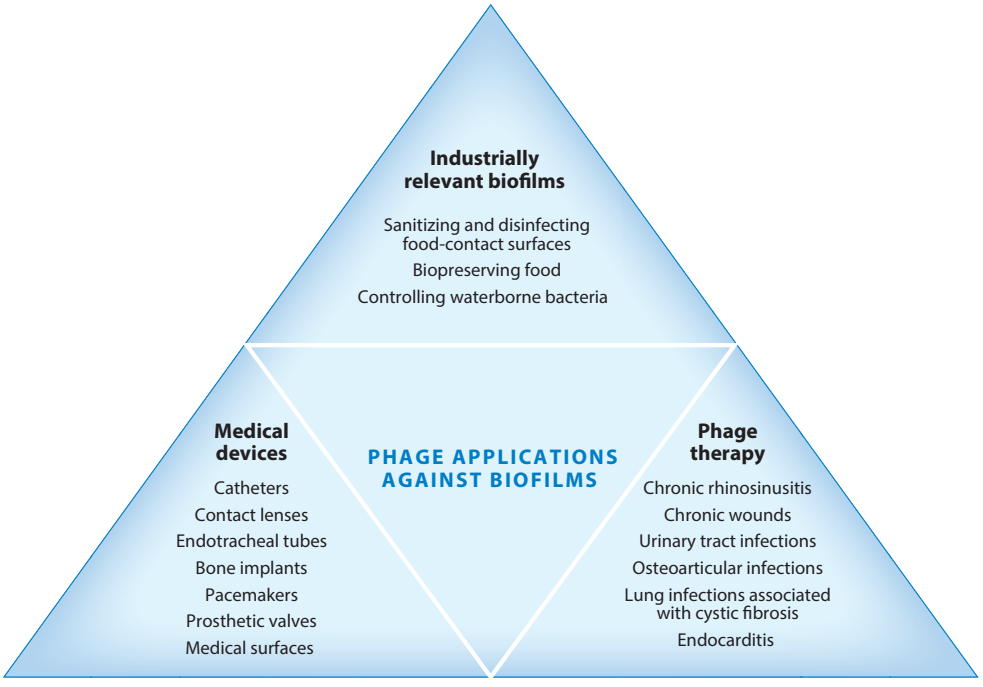

Figure 3

Schematic representation of the different applications of phages against biofilms. Figure adapted from images created with BioRender.com.

investigation of phage-biofilm interactions toward the development of phage therapy against infectious biofilms. Phages have also been proposed as antimicrobial coatings or sanitizing agents to prevent and control device-associated infections.

4.1.1. Phage therapy against infectious biofilms. While many studies have addressed phage therapy against infectious biofilms in vitro and in vivo, the number of case reports and clinical trials is still scarce. Despite the already-mentioned limitations of using in vitro studies, they have been important to describe the dynamic of phage-biofilm interaction and to identify the features that can contribute to impair the efficacy of phages against biofilms.

One of the main limitations of phage therapy is the rapid emergence and proliferation of BIMs. Pires et al. (41) reported *P. aeruginosa* biofilm regrowth 6 h after application of a single phage treatment in vitro, which was attributed to the proliferation of BIMs lacking phage receptors. The same has been reported for phage-treated biofilms of *K. pneumoniae*, where a rapid regrowth was observed following the initial lysis, suggesting that phage-resistant variants were selected in the host populations (109).

The clinical significance of BIMs remains unclear. Several studies have shown that phage resistance may diminish fitness or virulence of these bacterial variants and therefore facilitate clearance by the immune system (110). Olszak et al. (111) demonstrated that a *P. aeruginosa* biofilm population that survived PA5oct jumbo phage treatment became sensitive to the immune system due to the reduced virulence of BIMs. Despite the fact that resistance can be associated with decreased bacterial virulence, phage resistance should not be underestimated and efforts should be made to develop methodologies for preventing it.

The application of cocktails composed of phages that target different cell receptors has been suggested to improve phage therapy by extending host range and reducing resistance (112). This is particularly important in biofilms, in which the application of cocktails rather than a single phage can delay (41) or even prevent the emergence of bacteria-resistant variants. Morris et al. (113) evaluated the therapeutic effect of a phage cocktail for treating peri-prosthetic joint infections caused by *S. aureus* in rats and demonstrated that the bacterial isolates recovered from the infected knee of the animals that received phage therapy remained susceptible to the five-phage cocktail.

Another way to prevent phage resistance is combining phages with antibiotics. Verma et al. (114) prevented the emergence of phage-resistant variants during treatment of *K. pneumoniae* biofilms by combining ciprofloxacin with phages. The application of phages with antibiotics, simultaneously or sequentially, has been described as particularly effective against biofilms. Synergism may occur because phage-associated bacterial lysis releases nutrients that can reactivate the metabolic activity of the growth-arrested cells, which become sensitive to antibiotics. Cell lysis also causes a dispersion of the EPS, enhancing the diffusion of the antibiotic to the inner matrix layers, whereas the oxygen availability increases the drug activity (108). In some cases, phage-resistant cells might be more susceptible to antibiotics (115).

Besides resistance, the efficacy of phage therapy against biofilms can be compromised by the deficient phage penetration into the biofilm matrix, as already mentioned. Nevertheless, mechanical or enzymatic disruption of the biofilm can facilitate phage infection, which was already proven both in vitro and in vivo. Melo et al. (20) reported poor antibiofilm activity by a sepunavirus, despite its high activity against planktonic cells at different growth stages. It is noteworthy that the authors demonstrated that after mechanical disturbance, the biofilm becomes susceptible to phage attack. In a study by Seth et al. (116), the application of a phage treatment in *S. aureus* biofilm wounds had no effect on healing; however, when the phage was administered after sharp debridement, wound healing parameters assessed by histological analysis improved significantly and bacterial counts were reduced.

In clinical contexts, mechanical debridement has also been applied as a routine care procedure before phage application. Patey et al. (117) summarized the outcomes of 15 compassionate phage therapy treatments (from 2006 to 2018) in patients suffering from osteoarticular infections caused predominantly by *S. aureus* monospecies biofilms and, more rarely, polymicrobial infections with the presence of *P. aeruginosa* and *E. coli*. The results of the treatments were very satisfactory, with 12 of 15 patients completely recovered. The therapeutic procedure consisted of a prior debridement and cleaning of the infectious foci, followed by the application of the phage preparation (117).

4.1.2. Phages to control biofilms in medical devices. Biofilm formation in medical devices (e.g., catheters, cardiac pacemakers, implants, contact lenses, endotracheal tubes, and others) is a common cause of serious infections, which are responsible for a high number of deaths in health care settings (118). In this context, phages may play an important role in preventing or even controlling device-related infections in clinical environments.

Because one of the major challenges in health care settings is prevention of catheter-associated infections, Curtin & Donlan (119) used an in vitro system to study the efficacy of phages as a pretreatment of hydrogel-coated silicon catheters to prevent *S. epidermidis* biofilms. The authors observed a significant reduction of biofilm formation in phage-treated catheters, suggesting that this may be a promising approach to prevent device-associated infections. Using a similar in vitro model, Fu et al. (38) developed a phage cocktail to prevent *P. aeruginosa* biofilm formation. Although the phage pretreatment significantly reduced biofilm formation on catheters, phage-resistant variants were isolated during the experiment. The potential of phages to prevent or

control biofilms in catheters has also been widely studied against *P. mirabilis*, the leading cause of catheter-associated urinary tract infections, and promising results were reported in these studies (77, 78, 120).

Another interesting application is the use of phages for biofilm prevention or control in biomaterial surfaces. In a recent study, Bouchart et al. (121) assessed whether the Remus phage loaded on a calcium phosphate–based ceramic device was able to prevent biofilm colonization. The authors reported that the phage was able to not only prevent *S. aureus* biofilm initiation but also destroy established biofilms formed on microtiter plates. In addition, they observed that Remus phage was safe for osteoblastic cell proliferation, leading them to conclude that the phage-loaded material could be a good strategy to prevent bacterial infections in bone and joint surgery (121).

4.2. Phages to Control Industrially Relevant Biofilms

The formation of biofilms in industrial settings represents a great challenge for industries, particularly the food industry. Biofilms tend to accumulate on surfaces in industrial settings, causing corrosion, loss of efficiency of certain equipment (e.g., heat exchangers), and contamination of food products. Chemical disinfection usually fails to efficiently sanitize food-contact surfaces where biofilms have accumulated, due to the high tolerance of biofilms to disinfection.

4.2.1. Phages to control foodborne biofilms. Phage biocontrol is increasingly accepted as a natural and green technology for targeting bacterial pathogens in various foods and food-contact surfaces (122). Many phage preparations (e.g., ListShield™, Listex™ P100, EcoShield™, SalmoFresh™, Finalyse™) have been granted Generally Recognized as Safe designation by the Food and Drug Administration to be used as food additives and/or food-processing agents against many foodborne pathogens. These products have been tested on contaminated foods but not specifically on biofilms; nevertheless, it is most likely that they also have antibiofilm properties. It is noteworthy that Listex P100 and ListShield have been assessed for *L. monocytogenes* biofilm removal on different food-contact surfaces and lettuce, and promising results were reported (123–126). Biofilms formed by *Salmonella*, another important foodborne pathogen, have also been the subject of many phage biocontrol studies. For example, a cocktail of two phages proved to be very effective in removing *Salmonella* spp. biofilms from stainless steel, rubber, and lettuce surfaces (127). Another interesting example is the control of dual-species biofilms formed by *Salmonella* and *E. coli* (128). Milho et al. (128) observed that the biofilms formed by the two species were less susceptible to phage predation than the monospecies ones, raising awareness of the difficulty of controlling multispecies biofilms on industrial surfaces. González et al. (129) also characterized the interaction of an *S. aureus* phage with dual-species biofilms formed by combining the *S. aureus* host with different bacterial species. The results suggested that the effect of phage treatment on *S. aureus* mixed biofilms varies depending on the accompanying species and the infection conditions (129). These results highlight the need to study the effect of phage biocontrol on microbial communities that reflect more realistic conditions.

Phages did not always exhibit good killing properties against foodborne biofilms. Many studies have reported moderate to low killing efficacies that are not sufficient for an efficient surface sanitation. This is the case with a cocktail of three phages that failed to destroy established *Vibrio parahaemolyticus* biofilms (130). Nevertheless, the phages demonstrated a great ability to prevent biofilm formation (130). This feature is extensively reported in many phage-biofilm studies. Even if the phage is not able to reduce the cell population of a mature biofilm, it can prevent the biofilm from further proliferation (131). For example, Endersen et al. (132) demonstrated the successful use of a phage cocktail targeting *Citrobacter sakazakii*, an important pathogen involved in the contamination of infant formula, to prevent biofilm formation.

Several strategies have been suggested to improve phage efficacy against foodborne biofilms. As described previously for clinical purposes, the use of a cocktail of phages against foodborne biofilms is also highly recommended to limit the emergence of BIMs. Other strategies are based on the combination of phages with other sanitizing agents (that do not inactivate phages) (133, 134) or essential oils (135). An interesting work by Li et al. (136) demonstrated the potential of using phages attached to magnetic colloidal nanoparticle clusters that facilitate biofilm penetration under a relatively small magnetic field, which led to approximately 90% biofilm removal of *P. aeruginosa* and *E. coli* biofilms within 6 h of treatment.

4.2.2. Water transport and treatment systems. Water systems are among the industrial devices most affected by biofilms. Pipes and water-cooling systems are usually colonized by biofilms that can induce corrosion and equipment damage. Most importantly, the biofilms formed in these systems are often a reservoir for pathogenic bacteria (*V. cholerae*, *Helicobacter pylori*, *Legionella* spp.) (137–139). Phages are very specific and therefore cannot match the broad-spectrum capabilities of antimicrobial chemicals used in water disinfection, but they can be used to specifically target dangerous or problematic bacteria present in water transport and treatment systems. For example, Naser et al. (140) tested the effect of three vibriophages against *V. cholerae* biofilms and concluded that one of the phages could degrade the biofilm matrix of *V. cholerae* and increase the concentration of planktonic *V. cholerae* in water, whereas the other two phages could effectively kill planktonic *V. cholerae* cells, suggesting that a possible combination of diverse phages can be effective in controlling waterborne pathogens. Other possible applications of phages in water treatment processes were discussed by Mathieu et al. (141).

Another industrial application of phages is in water treatment plants as a means to control antibiotic-resistant bacteria (ARB), as proposed by Yu et al. (142). In this study, the authors used a cocktail of polyvalent *E. coli* phages to suppress the proliferation of ARB in activated sludge microcosms, and they observed that the phages were able to reach high densities and significantly decrease ARB.

The impact of the extensive application of phages in the environment is still controversial due to the question of if this could lead to widespread phage-resistant bacteria, compromising the future of phage therapy. There is no definitive answer; however, as phages are naturally coevolving with bacteria, it seems improbable that the arms race between phages and their bacterial hosts will come to an end.

5. SUMMARY AND FUTURE PROSPECTS

Phage efficacy in controlling biofilms formed either in industrial settings or on human and animal surfaces is limited by the intrinsic biological properties of phages and the protective shield of the biofilm. Phages are unquestionably powerful weapons to combat undesirable biofilms, but they have limitations. It is important to understand the factors that hamper phage efficacy in order to design effective phage-based biocontrol strategies. The many strategies that have been suggested are already discussed in other reviews and mostly rely on combining phages with chemical, enzymatic, or physical treatments or rely on the use of genetically engineered phages. Regardless of the strategy used to coadjuvate phages, it is important to remember that biofilms are dynamic structures that vary in composition and structure in response to environmental conditions and that phages respond differently to different biofilms. Therefore, the complexity and diversity of phage-biofilm interactions limit broad conclusions and call for more research in this area. Particularly, there is a need to establish standardized methods for assessing phage-biofilm interactions in different contexts of application, which will allow for rigorous testing of phages for either therapeutic purposes or biocontrol against biofilms.

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