Phage therapy in Aquaculture

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Abstract

Phage therapy is a promising alternative to antibiotics in aquaculture, which is facing increasing concerns over antimicrobial resistance (AMR). Bacteriophages are viruses that specifically infect and replicate within bacteria, causing bacterial cell death. They are abundant in the aquatic environment and can be isolated from a variety of sources, including fish, water, and sediment. Phage therapy has been shown to be effective against a wide range of bacterial pathogens which are responsible for causing severe disease outbreaks in aquatic fauna. Phages are highly host specific and therefore they can target and eliminate harmful bacteria while leaving beneficial ones unaffected. As a targeted therapy, phage therapy can be used in delicate environments like the fish hatcheries where the use of broad-spectrum antimicrobials may disturb the microbial equilibrium with adverse effects on the quality of the developing larvae.

However, phage therapy in aquaculture and the aquatic environment faces several challenges. The most important challenge is the rapid development of resistance by the bacteria against phage infection. In addition, a big challenge is the lack of knowledge about the diversity and distribution of phages in aquatic environments. As such, identifying and isolating phages that are effective against specific bacterial pathogens can be time-consuming and resource-intensive especially taking into consideration the strain diversity of several aquatic pathogens like the vibrios. Additionally, the efficacy of phage therapy can be affected by environmental factors, such as temperature and pH and the presence of other microorganisms in the environment which can influence the host-phage interactions. Moreover, the regulatory framework of phage therapy applications but also manufacturing of phage products is still far from being established.

Despite these challenges, the potential benefits of phage therapy in aquaculture are significant. It offers a sustainable and environmentally friendly alternative to antibiotics, which can reduce the risk of AMR and environmental pollution. Moreover, phage therapy can be tailored to specific bacterial pathogens, which can improve treatment efficacy and reduce the risk of non-target effects. Overall, phage therapy has the potential to revolutionize the way bacterial infections are managed in aquaculture, leading to improved health and welfare of farmed aquatic animals and sustainable aquaculture practices.

1. Basic concepts regarding bacteriophages and phage therapy

Bacteriophages, also known as phages, are type of viruses that specifically infect bacteria and are abundantly present in the world. Their immense numbers, estimated to be around 10^{31} in the biosphere, highlight their significance. The existence of phages was initially hypothesized by Ernest Hanbury Hankin, an English microbiologist, towards the end of the 19th century. In 1915, Frederick William Twort, an English microbiologist, published the initial scientific manuscript in Lancet, detailing the behavior of bacteriophages. Nevertheless, credit for the discovery and nomenclature of bacteriophages is commonly attributed to Felix d'Herelle, a French microbiologist based at the Pasteur Institute. It was in 1917 that d'Herelle authored a paper delineating bacteriophages as viruses that infect bacteria. [1]. Bacteriophages were initially employed in the treatment of bacterial infections. It is important to note that this occurred prior to the discovery of antibiotics, when even minor bacterial infections posed a significant risk to human life. The application of bacteriophages for therapeutic purposes is commonly referred to as phage therapy.

Before delving into the possibilities offered by phage therapy in aquaculture, it is essential to grasp some fundamental concepts of phage microbiology. Bacteriophages serve as highly efficient "predators" of bacteria in nature, playing a crucial ecological role in controlling bacterial populations. The structure of phages consists of a protein capsid enclosing their genetic material, which can be DNA or RNA, along with a tail that attaches to the capsid. Tailed bacteriophages, which are the focus here, exhibit various morphologies, with tails that can be long or short, contractile or non-contractile. The tail which in the past played a key role in taxonomy possesses a baseplate, tail fibers, and a spike. The receptor binding proteins at the distal end of the tail fibers interact with specific surface receptors on the bacterial host, while the spike proteins facilitate the degradation of the bacterial surface layer, allowing the phage to bind irreversibly **(Figure 1)**. Once attached, the phage injects its genetic material into the bacterial cell. Subsequently, the phage utilizes the bacterial machinery for propagation, hijacking the cell's resources.

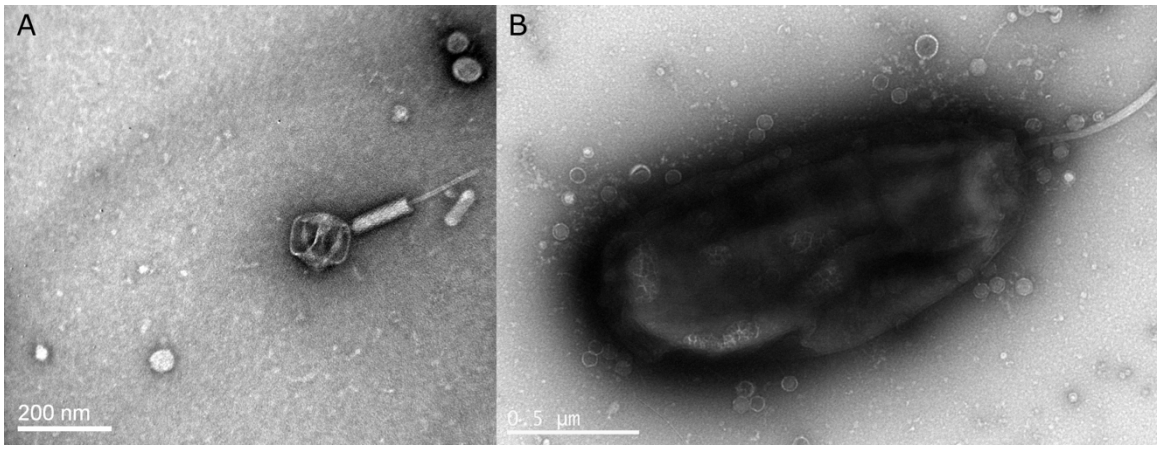

Figure 1. A. Vibriophage of a Myoviridae morphotype with the characteristic contractile tail. **B.** A negatively stained *Vibrio alginolyticus* bacterial cell under bacteriophage attack. TEM micrographs courtesy of Dr. Katharios.

Phages have diverse life cycles, with the two most extensively studied types being the lytic or virulent and the temperate. Upon injection of their DNA into the host cell, lytic phages initiate the production of structural proteins and genetic material, leading to the assembly of progeny virions within the host. Once assembly is complete, lytic enzymes are secreted, causing the degradation of the bacterial cell wall from within, resulting in a burst and the release of phages into the external environment. Temperate phages, on the other hand, may integrate their DNA into the bacterial chromosome upon infection, becoming dormant and replicating along with the bacterium until induced by damage of the host's DNA or environmental cues. Upon induction, the viral DNA excises from the host's DNA, and the phage enters the lytic cycle, releasing new virions. During the temperate phase, the phage genes integrated into the bacterial host can become functional, potentially encoding traits such as toxins and proteins associated with antibiotic resistance. For instance, the cholera toxin serves as a prime illustration, acting as the primary virulence factor of *Vibrio cholerae*. This toxin is encoded within a prophage integrated into the bacterium's chromosome. Similarly, marine pathogenic vibrios like *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, and numerous others harbor prophage-encoded toxins that enhance their virulence compared to non-phage-infected strains. This phenomenon, known as lysogenic conversion, involves the acquisition of new properties that can augment bacterial fitness, with virulence being particularly important. However, it is precisely this attribute that poses the greatest risk when utilizing phages as a therapeutic tool, as it can inadvertently transform non-virulent bacterial strains into virulent ones. Consequently, it is crucial to carefully select lytic phages while excluding temperate ones in phage therapy. Moreover, modern approaches now employ genomic analysis for a more precise selection of suitable phages.

Phages typically exhibit a high degree of host specificity, at times to the extent of infecting a subset of individual strains within a given species. Nevertheless, there are phages that possess a broad host range, capable of infecting different species, most commonly within the same genus. This characteristic sets phage therapy apart from other existing methods for controlling bacteria in aquaculture, providing a more focused and precise treatment strategy. The specificity of phages towards their hosts is influenced by intricate molecular interactions that occur throughout the infection cycle, although these details lie beyond the scope of this chapter. However, one crucial factor contributing to host specificity is the type and diversity of receptor binding proteins located on the phage tail, which mediate the initial interplay between the phage and the bacterium. In Gram-negative bacteria like vibrios, these phage proteins primarily target elements of the outer membrane such as lipopolysaccharides (LPS), which are major virulence factors, as well as flagella and porins that serve as receptors for nutrient uptake from the extracellular environment [2].

Phages and bacteria engage in an ongoing evolutionary battle within their environmental realm. Bacteria face continuous exposure to phage "predation," prompting them to develop defensive strategies against phage infection for survival. Bacterial resistance to phages can emerge rapidly, often involving the sacrifice of receptors to which phages attach. This molecular-level process entails the downregulation of genes encoding these receptor proteins. Resistance can also arise through genetic mutations in these proteins, leading to incompatibility between phage-binding receptors and bacterial cell receptors. Even in the face of incursion of phage genetic material, bacteria wield an array of defense mechanisms that bestow them with resistance. The CRISPR-Cas system, functioning as a bacterial "adaptive immune system," plays a

pivotal role in defense against phages [2]. Conversely, phages also adapt swiftly and develop counter-resistance mechanisms. Bacterial resistance to phage infection often involves the of lipopolysaccharides (LPS), which are important virulence determinants. In many cases, resistant strains exhibit reduced fitness or virulence compared to their wild-type counterparts, underscoring the substantial cost of resistance development for bacteria. The rapid emergence of bacterial resistance poses a significant challenge in phage therapy, a hurdle that is almost certain to arise over time. To overcome this issue, phage therapy requires meticulous design. The utilization of "phage cocktails," which involve combinations of different phages, offers a potential solution. However, the selection of appropriate phages for these cocktails demands expertise and knowledge. Previously, phage cocktails were formulated with phages exhibiting diverse host ranges. Presently, it is understood that an effective phage cocktail should ideally comprise phages that employ different receptors for initial attachment to the bacterial host. This approach mitigates the risk of bacterial resistance, as changes in multiple receptors would impose a greater cost on bacterial viability. Notably, "jumbo" phages with expansive genomes have emerged as promising candidates for phage cocktails due to their broad host range, likely attributed to a wider array of receptor binding proteins present in their tails.

2. Reemergence of phage therapy in the era of AMR

Despite being discovered prior to antibiotics, the clinical and therapeutic utilization of phages did not gain widespread acceptance in the Western world. Initially, their application was confined to countries in the former Eastern bloc, such as the USSR and Poland. The advent of World War II and the subsequent mass production of antibiotics played key role in their extensive adoption, eventually overshadowing the use of phages. Antibiotics, like penicillin, could be manufactured in large quantities with consistent quality and stability, a characteristic not necessarily guaranteed with phages.

The realization of antimicrobial resistance as a significant global crisis unfolded gradually as scientific evidence and observations accumulated over time. Even during the early stages of antibiotic use, scientists noticed that certain bacteria could persist and proliferate despite exposure to these drugs. However, it was during the period between 1990 and 2000 that the issue began to gain recognition, leading to intensified research and surveillance efforts. Esteemed organizations such as the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) began to prioritize antimicrobial resistance as a critical public health concern. In 2014, Jim O'Neill, a renowned British economist, led an international commission to investigate the impact of antimicrobial resistance. This initiative culminated in the publication of the Review of Antimicrobial Resistance in 2016, which stands as the most influential work on the subject and has greatly increased public awareness [3]. Consequently, the pursuit of alternatives has emerged as one of the scientific community's most critical research objectives.

3. Phage therapy applications in Aquaculture

Phage therapy presents a highly appealing approach for the control of pathogens in aquaculture. Bacteriophages are extensively found in aquatic environments, benefiting from the water's aqueous nature, which enhances their dispersal and increases the likelihood of encountering target bacteria. Notably, phages present in the water can traverse the gills and stomach (considering that marine fish consume water) to enter the bloodstream and potentially

reach internal organs. Given that phages naturally inhabit aquatic environments, their medicinal utilization could align with organic farming practices. Unlike antibiotics, phages do not leave behind residues and pose no harm to fish, humans, or the surrounding ecosystem. Being self-replicating agents, phages offer the advantage of theoretically not requiring multiple doses in phage therapy. Furthermore, their high specificity for particular hosts renders them the most suitable option for controlling bacteria in delicate environments, such as fish hatcheries and recirculating aquaculture systems (RAS). Lastly, phages can be employed during the early developmental stages of fish, where vaccination may not be feasible due to the immature state of the immune system [2].

Aquaculture is a fast-growing industry that includes the cultivation of aquatic organisms of different taxa, including fish, crustaceans and mollusks. Unfortunately, aquaculture systems are vulnerable to diseases caused by a plethora of pathogens that pose significant challenges to sustainable production. Bacterial species responsible for common disease outbreaks include *Aeromonas salmonicida, Aeromonas hydrophila, Aeromonas veronii, Edwardsiella ictaluri, Edwardsiella tarda, Yersinia ruckeri, Piscirickettsia salmonis, Flabobacterium psychrophilum, Flavobacterium columnare, Pseudomonas anguilliseptica, Vibrio harveyi, Vibrio anguillarum, Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio splendidus, Photobacterium damselae* subsp. *piscicida, Moritella viscosa, Tenacibaculum maritimum, Lactococcus garvieae, Streptococcus iniae, Streptococcus parauberis, Streptococcus phocae* and *Mycobacterium marinum* [2]. The majority of these bacterial pathogens are naturally present in the aquatic environment, encompassing both freshwater and marine ecosystems and have been widely used as experimental models in *in vivo* phage therapy applications in various aquatic organisms yielding promising results.

For the first time, in 1981, Wu et al. introduced the utilization of bacteriophages as a therapeutic approach in aquaculture [4]. Subsequently, there has been significant interest in utilizing bacteriophage therapy for various aquatic animal species in controlling bacterial diseases. In parallel, evaluation of the safety, stability and environmental impact of phage treatment in aquaculture systems has also gathered some significant attention. However, when it comes to applied phage therapy in the aquaculture setting, many things must be taken into serious consideration in order to attain the desired outcome. Phage selection, phage dosage, phage administration route and treatment duration are key parameters that will be thoroughly discussed hereby.

3.1 Phage Formulation and Delivery

One of the key elements when practically applying phage therapy in aquaculture to attain the desired outcome is the optimal route of phage administration for each bacterial infection. The efficacy of bacteriophage application relies on their ability to reach the host, which may not always be feasible. Depending on the infection's location within the body and considering factors such as penetration effectiveness and the ability to sustain high phage levels, phage preparations can be administered in diverse forms. In the aquaculture field, phages employed to control bacterial diseases can be given orally through feed, administered parenterally (via intramuscular, subcutaneous, or intraperitoneal routes), applied topically to the skin and lesions, introduced via bath treatments, or directly released into the water system.

Phage delivery through the water or orally, incorporating them into the feed, is to a great degree among the most widely used approaches. Oral administration of bacteriophages has proven to be an effective treatment for gastrointestinal infections, particularly those caused by *Vibrio* spp. Interestingly, it has been observed that orally administered bacteriophages can be absorbed into the systemic circulation, similar to the process of bacterial translocation. This suggests that oral administration may also be suitable for treating systemic infections. Several elements come into play when considering the behavior of bacteriophages within the aquatic environment. These factors encompass the concentration of bacteriophages, the existence of distinct sequences within the capsid or tail proteins that engage with receptors on enterocytes, as well as interactions with immune cells in the intestinal milieu, all contributing to the dynamics of bacteriophage transit.

The prevalent method to apply the desired substances, in our case phages, to aquaculture animals is through the water body. It can be done in different ways, ranging from a high concentration of phages with a short exposure time (immersion) to a low concentration with a long exposure time (bath). The application of bacteriophage preparations using this method is popular because it allows the bacteriophages to enter the internal organs of fish directly through the gills while providing additional benefits of environmental cleansing. Moreover, marine fish drink water continuously therefore allowing the entrance of the phages in their intestine. However, in commercial-scale aquaculture, the use of this administration route can pose challenges due to the impractically large volume of water that would require for an effective bacteriophage treatment.

Unfortunately, there is not a universally applicable strategy of bacteriophage delivery. The appropriate approach depends on various factors and should be considered on a case-by-case basis. Administering injections, for example, may not be practical for very small fish or crustaceans and of course is not applicable when a large population is to be treated. Likewise, conducting bathing with a high concentration of bacteriophages can be challenging in large bodies of water, and the immersion method may vary depending on the specific environment, infection characteristics, or properties of the bacteriophages. Each administration method has its advantages and limitations, and the choice depends largely on the bacterial pathogen's nature, the animal species, and its size.

The timing of administration also plays a crucial role. Mortality rates tend to increase significantly when treatment is delayed after infection. Optimal effectiveness is observed through the prophylactic application of bacteriophages before infection, and its sustained use. A study conducted by Jun et al. demonstrated a 75% increase in the survival rate of white shrimp when employing prophylactic bacteriophage immersion, along with a 50% survival rate when using phages in feed [5]. Similarly, Schulz et al. noted a 16% higher survival rate in European eel and a 6% higher survival rate in rainbow trout when administering the bacteriophage preparation one day before infection, in comparison to treatment 24 hours post-infection [6]. This improvement is likely linked to the time required for bacteriophages to multiply to an adequate concentration for effective host population control. Additionally, the prophylactic application of phages assists in regulating the concentration of pathogenic bacteria in water and/or fish, preventing them from reaching levels that could trigger disease outbreaks.

The optimal dosage for effective treatment ideally relies on data obtained from *in vitro* studies. The effective dose is linked to the minimum Multiplicity of Infection (the ratio of bacteriophages to the bacterial host) capable of significantly reducing the bacterial titer in *in vitro* assays. Another crucial factor influencing the dosage is the Burst size, which denotes the number of new virions produced following an infection cycle and indicates the quantity of new phages generated by a single infected bacterial cell. This factor holds significant importance as, based on our personal observations, phage therapy exhibits improved outcomes when the initial concentration of bacterial hosts is higher. This is because phages rely on these hosts for their propagation, thereby naturally adjusting the treatment dosage.

Exploring the influence of timing and the dosage of bacteriophages on the effectiveness of phage therapy through additional research may uncover intriguing findings, as a higher concentration of administration might have the potential to offset the effects of delayed treatment.

3.1.1 Interaction of phages and fish Immune system

Our understanding of the correlation between phage activity and the fish immune system is currently limited, leaving several gaps that require further investigation. Nevertheless, it is acknowledged that the method of phage administration plays a pivotal role in the established interactions between phages and the fish's immune system. *In vitro* studies utilizing cultured cells from mucosal surfaces, as well as *in vivo* experiments involving fish immersion, have provided evidence indicating that elevated levels of phages in the mucus layer play a protective role in preventing bacterial infections in the underlying epithelium [7]. Upon entering the fish's body, phages initially engage with and overcome the cells of the mucosal innate immune system before aiming at their bacterial prey. The survival and presence of phages in fish organs largely rely on the specific administration route, as they encounter the host's phage clearance mechanisms and undergo sensitivity checks, such as such as digestive enzymes, pH changes, immune cells, and antimicrobial substances, during gut transit. The phage clearance mechanism in fish primarily involves the kidney, spleen, and intestine. This is indicated by the phage recovery results following their administration through any route within the fish's body [8].

Remarkably, it has been noted that the majority of phages do not exhibit an outer lipid layer, which is usually targeted by the complement system [9]. This raises the question of how phages elude elements of the adaptive and innate immune system, such as antibodies and phagocytes, in order to infiltrate eukaryotic cells. One partial explanation is transcytosis, which involves either free uptake through endocytosis or crossing via a compromised gut, facilitating passive traversal through the epithelium. Another intriguing mechanism involves the phage being endocytosed by a bacterial host and hidden inside it, ensuring unrecognized access beyond epithelial cell layers. It is important to note that phages are incapable of infecting eukaryotic cells but can penetrate epithelial cell layers and spread throughout several regions inside the organism’s body [10].

Moreover, phages belonging to *Caudoviricetes* class possess the inherent capability to translocate across epithelial and endothelial cell lines [9]. Upon successfully penetrating the

mucosal tissue of fish, a systematic screening process unfolds, encompassing both extracellular and intracellular immune recognition mechanisms. However, it is noteworthy that endocytic recognition processes commence exclusively subsequent to lysosomal degradation within the host cell, where pathogen recognition is facilitated by classical and evolutionarily conserved pattern recognition receptors (PRRs).

3.2 Dosage

In the field of phage therapy, two discernible approaches emerge: active and passive [11]. In active therapy, bacteriophages are administered in a dosage that can reduce the host population through multiple reproduction cycles. In passive therapy, extensive bacteriophage quantities are administered, rendering multiple reproduction cycles unnecessary, as the entire host population is lysed. It's worth noting that the passive approach is costlier; however, it can effectively circumvent bacterial defense mechanisms, including abortive infection.

The optimal ratio of bacteriophages to bacterial concentration required for successful bacteriophage therapy differs greatly, contingent upon factors such as the pathogen, fish species, and specific bacteriophage employed. Experimental investigations and field studies have utilized various doses in their assessments. For instance, in a study by Le et al., different bacteriophage amounts were used in catfish therapy, leading to survival rates that exhibited variances of up to 68% [12]. This highlights the importance of ascertaining the precise bacteriophage dose to ensure the success of phage therapy.

3.3 Monophage Therapy – Phage Cocktails

Numerous strategies for bacteriophage therapy have undergone testing and evaluation. Monophage therapy involves using a single type of bacteriophage. It is primarily used for experimental modeling of bacteriophage therapy and as a means of confirming the concept during testing of preparations. However, monophage therapy necessitates an exact match between the pathogen and the bacteriophage [13].

In the context of aquaculture, the realistic scenario often exceeds the simplicity of a "one pathogen-one disease" situation. Fish can experience concurrent infections caused by multiple strains or species of bacteria, which can impact disease outcomes and pose additional complexities for phage therapy. Consequently, polyphage therapy has become increasingly popular in the field of aquaculture [13]. This approach entails the utilization of a bacteriophage cocktail comprising multiple phages that target various strains of a single bacterial species or even multiple bacterial species. One notable advantage of employing multiple phages is that they provide a more comprehensive treatment approach by effectively targeting a broad spectrum of pathogenic bacterial strains. This results in a more substantial reduction in bacterial titers and faster-acting therapeutic effects. Furthermore, using bacteriophage cocktails that target different receptors of the same bacterium may aid in mitigating the development of resistance, a significant concern in phage therapy practices.

The majority of published articles predominantly center around monophage therapy and emphasizing the discovery of potentially beneficial bacteriophages. In contrast, only a limited

number of articles delve into the use of cocktails or alternative combinations. *In vivo* experiments presented in this context illustrate the efficacy of bacteriophage therapy in the management of aquaculture-related diseases. However, the outcomes tend to vary depending on the specific combination of bacteriophages and bacteria used. Some experiments have yielded complete bacterial elimination with no recorded mortality, while others have exhibited no discernible therapeutic effect. In certain instances, disease progression was delayed, but the ultimate mortality rates did not exhibit significant deviations from those observed in the absence of bacteriophage therapy.

3.4 Phage-Derived Enzymes

The utilization of complete phage particles, including phage cocktails, has gained momentum in the fight against antibiotic resistance. The development of lytic enzymes derived from these viruses has garnered interest in various fields, including veterinary medicine, food science, and technology. Phage-encoded proteins with potential applications are categorized into endolysins, exolysins, and depolymerases. Endolysins primarily degrade the bacterial cell wall from within the cytoplasm of the infected bacteria during the phage lytic cycle. Conversely, exolysins or ectolysins act externally, enabling quicker access to bacteria. These enzymatic proteins have shown promise as antimicrobial agents and have demonstrated efficacy in disrupting preformed biofilms. Furthermore, the engineering of lysins has enhanced their binding and traversing capabilities through the bacterial wall. Depolymerases, another group of phage enzymes, have the potential to combat pathogens and the biofilms they form, as biofilm matrices often consist of different polymers. One significant advantage of phage lytic enzymes is that bacteria are unable to develop resistance against them, which contrasts with the current issue of phage resistance development. Although the focus of phage and lysin studies has been primarily on Gram-positive bacteria, some fish pathogens associated with disease outbreaks in fish farms, such as *Streptococcus agalactiae, Lactococcus garvieae, Renibacterium salmoninarum, Streptococcus iniae,* and *S. dysgalactiae,* have been investigated.

3.5 Success stories and Commercial applications

There are numerous success stories demonstrating the efficacy of phage therapy in combating bacterial infections. The focused approach of phage therapy enables the accurate elimination of harmful bacteria while assuring the presence of beneficial microbial populations. This characteristic significantly reduces the reliance on antibiotics and diminishes the likelihood of resistance emergence. Hereby, we will discuss and provide a summary of the most significant successful cases of applied phage therapy in the aquaculture field, highlighting the key elements that led to the desired outcome (Table 1).

Table 1. *Phage therapy application in aquaculture. Adapted from Schulz et al 2022* [14]

3.5.1 Fish

Phage therapy has emerged as a revolutionary approach in managing bacterial infections in fish species, providing targeted and effective solutions for disease control. For instance, in the case of African catfish infected with *Pseudomonas aeruginosa*, the local application of phages resulted in a remarkable reduction of skin lesions by seven-fold [15]. This highlights the precise eradication capabilities of phages, effectively targeting the pathogen while minimizing harm to healthy tissues. Similarly, when Nile tilapia were intraperitoneally injected with phages to combat *Streptococcus agalactiae* infection, a significant 3-day delay in mortality and a notable 40% reduction in overall death rates were observed [16]. This underscores the potential of phage therapy in allowing the immune system vital time to mount a defense against the pathogen. Moreover, phage therapy trials conducted on gilthead seabream larvae setting showed that phages had a noteworthy impact on the survival of the larvae for a period of five days, leading to significant reduction in mortalities [17, 18]. Another notable success story involves the administration of phages against *Pseudomonas plecoglossicida* in Aju sweetfish, where timing played a crucial role [19]. Depending on the timing of phage administration, mortality reduction ranged from 42.5% in 10 g fish to an impressive 78% in 2.4 g fish. These findings emphasize the immense potential of phage therapy in improving disease management, promoting the health, and enhancing the survival rates of fish species in aquaculture settings.

3.5.2 Crustaceans

Applied phage therapy in crustaceans also yielded intriguing results. For example, in the case of *Vibrio harveyi* infection in brine shrimp (*Artemia franciscana*), the use of immersion-based phage treatments resulted in notable improvements in hatching success rates and enhanced survival rates [20]. Hatching success reached 100% compared to approximately 50% in control groups, showcasing the ability of phages to effectively target and control the pathogen, consequently boosting reproductive success and overall survival of crustacean populations. Additionally, the application of phages against *Vibrio parahaemolyticus* in giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*Litopenaeus vannamei*) has yielded significant outcomes [5, 21–27]. Immersion-based phage treatments led to mortality reductions ranging from 18% to 21% in larvae, while prophylactic use resulted in increased larval survival [21]. These findings underscore the potential of phage therapy in combating bacterial infections in crustaceans, improving survival rates, and promoting sustainable practices in aquaculture.

3.5.3 Mollusks

In the realm of mollusk aquaculture, phage therapy is considered an effective strategy for combating bacterial infections. For instance, in the case of *Vibrio splendidus* infection in Japanese sea cucumber (*Apostichopus japonicus*), the application of phages through feed led to notable reductions in mortality rates, ranging from 32% to 47%, depending on the specific phage used [28]. This demonstrates the potential of phage therapy in enhancing the well-being and survival rates of mollusk populations. Additionally, studies focusing on other mollusk species, such as greenlip abalone (*Haliotis laevigata*), have reported significant reductions in mortality rates following the administration of phages through bathing [29]. Mortality decreased by 70%

compared to the control group, highlighting the efficacy of phage therapy in disease management within mollusk aquaculture. Similarly, in the context of *Vibrio splendidus* infection in turbot (*Scophthalmus maximus*) and Blue mussel (*Mytilus edulus*), phage therapy resulted in mortality reductions ranging from 28% to 58% and the elimination of bacteria to undetectable levels in mollusk tissues, respectively [30, 31]. These findings indicate the potential of phage therapy as an effective tool in mollusk aquaculture, offering valuable solutions for disease management and enhancing the overall health and survival rates of mollusk populations.

Another interesting application of phage therapy is for the depuration of bivalve products. Typically, depuration processes make use of the inherent filtering capability of bivalve mollusks to eliminate their intestinal contents. As a result, the likelihood of transmitting infectious agents to consumers through the consumption of contaminated mollusks is significantly reduced. In this regard, the use of phages against significant human pathogens like *E. coli* and *Vibrio parahaemolyticus* is a new promising approach [32, 33].

3.5.4 Phages as biocides and biocontrol method in aquaculture

The remarkable specificity of phages towards their hosts makes them an ideal approach for management of bacteria populations in delicate environments such as fish hatcheries and recirculating aquaculture systems (RAS). Additionally, phages can be effectively utilized during the early developmental stages of fish when vaccination is not feasible due to the immaturity of the immune system. In a recently published study conducted in Finland, it was observed that phages targeting *Flavobacterium* exhibited a persistence of 14 days within the tanks of a RAS after a single administration. Notably, these phages demonstrated even longer persistence within biofilters, suggesting that the presence could be augmented by the formation of biofilms, consequently contributing to their extended viability. This discovery holds significant importance as it indicates that phages can endure the water treatment processes of RAS and can be proactively harnessed within these systems for the precise management of undesired pathogenic bacteria.

However, it is the utilization of phages as a means of controlling bacteria in hatcheries that holds the greatest potential. Despite the implementation of advanced biosecurity measures in modern marine hatcheries, pathogenic bacteria persistently find their way into fish rearing tanks, leading to increased morbidity, mortality, and inconsistent production performance. The introduction of live feeds serves as a vehicle for bacterial entry. Disinfection of live feeds plays a crucial role in establishing a healthy colonization of beneficial bacteria in the fish gut. Our research group has recently proposed a novel application of phages as a "smart disinfectant" for live feeds. We have developed and utilized phage cocktails with a wide host range that selectively target and reduce vibrios in the live feeds. Through a single administration of vibriophages during the four-hour enrichment process of live Artemia, we observed a remarkable 93% reduction in vibrio load compared to the untreated group [34, 35]. Our ongoing efforts are dedicated to the development of phage-based disinfectants that specifically target vibrios within the Harveyi clade, such as *V. harveyi*, *V. owensii*, and *V. alginolyticus*, which are commonly found in live feeds and are associated with larval enteritis in gilthead seabream, resulting in significant losses in Mediterranean hatcheries. A notable advantage of this approach is that the treatment occurs within the batch cultures of live feeds, substantially minimizing the chances of bacterial resistance

development against phages. Aquatic Biologicals, a spin-off company of the Hellenic Centre for Marine Research, is at the forefront of developing innovative aquaculture health products, including the phage "smart disinfectant".

Similar approaches have been reported in salmon hatcheries, where bacteriophages have been employed as a biocontrol agent against *Yersinia ruckeri*, the causative agent of Enteric Red Mouth disease or Yersiniosis. A Norwegian company, ACD Pharmaceuticals, has achieved significant success authorizing a commercial phage product designed for prophylactic use against *Yersinia* in salmon tanks, specifically within Norway.

4. Phage therapy compared to Antibiotics

Bacteriophage therapy offers several advantages compared to traditional antibiotic therapy. The process of isolating bacteriophages is relatively fast, easy, and cost-effective. Unlike antibiotics, bacteriophage resistance develops at a much slower rate, approximately ten times slower than resistance to antibiotics. This slower development of resistant is attributed to the ability of bacteriophages to evolve and generate new genotypes capable of re-infecting a specific bacterial strain. Bacteriophages retain infectiousness even in challenging environmental conditions and continue to replicate until there is substantial decrease in the population density of the host bacteria. These characteristics suggest that bacteriophage therapy, unlike conventional treatments, may require fewer administrations while delivering equal or superior effectiveness.

Assessing the effectiveness of bacteriophage therapy in comparison to antibiotic therapy holds great importance. In a study conducted by Zhang et al., there were no statistically significant differences in the survival rates of sea cucumbers infected with *V. alginolyticus* when treated with a bacteriophage cocktail compared to antibiotics [28]. However, in another study by Karunasagar et al., shrimps treated with bacteriophages exhibited a 20% higher survival rate compared to those treated with antibiotics, while Vinod et al. achieved a 46% higher survival rate in shrimp after natural *V. harveyi* infection when using bacteriophages compared to antibiotics [21, 26]. These findings suggest that bacteriophage therapy may not only be as effective as antibiotics but potentially even more effective.

Because of the high host specificity of phages, phage therapy is more appropriate as personalized treatment. And this is how it is currently being used in human medicine. Phage therapy centers in Georgia, Belgium and elsewhere curate biobanks of bacteriophages which are being screened against life-threatening antibiotic-resistant strains on a case-by-case basis and treatment is following the magistral preparation scheme. However, it is important to note that this personalized approach is not readily applicable in aquaculture. In the case of acute bacterial infections causing significant mortality in aquaculture production, farmers often require urgent and immediate responses. In such scenarios, a combination of antibiotics and phages is frequently seen as a potent method to combat bacterial infections. Numerous studies have explored the effects of this combination in human medicine, yielding varying results. It should be noted that antibiotics could act antagonistically to phages, either by reducing the hosts in which phage would replicate producing new virions or by altering the physiology of the bacteria affecting phage

infection and replication. However, by carefully adjusting the timing of each treatment—for example, initiating phage therapy first and then introducing antibiotics—a more effective outcome might be achieved.

5. Challenges of phage therapy

Despite over a century of phage research, there are significant challenges that need to be addressed before phage therapy can be widely adopted as an industrial-scale treatment and prevention method. Recently, Culot, Grosset, and Gautier, researchers from INRA, France, have conducted an excellent review on the challenges of phage therapy in commercial aquaculture [36].

The inconsistent outcomes in phage therapy can be attributed significantly to the inadequate design of both phage therapy products and application strategies. Thorough characterization of the phages is a crucial prerequisite for effective and safe phage therapy. As mentioned earlier in this chapter, various elements of phage microbiology need to be meticulously studied in the laboratory. Comprehending factors like burst size, host range, life cycle, and genetics of candidate phages not only establishes their suitability as therapeutic agents but also directs the optimal approach for their application, including dosage and timing.

A significant challenge lies in overcoming bacterial resistance development. As explained earlier, resistance development is a natural outcome of the coevolution between phages and bacteria. Phage cocktails offer a potential solution; however, their formulation remains a challenging task that necessitates advanced knowledge and analytical skills.

The pharmaceutical industry faces a substantial challenge in the large-scale production of phages. Cultivating phages in bioreactors is not a readily standardized process. Additionally, when phages are intended for use as therapeutic agents, stringent quality standards must be adhered to, such as ensuring the absence of endotoxins released during the lysis of Gram-negative bacterial hosts. Although technically achievable, this requirement substantially increases production costs.

However, the most substantial challenge lies in the regulatory barriers encountered in many countries, including the EU and USA. Obtaining licenses for phage products as pharmaceuticals can be a nightmarish process. Phages are not considered conventional pharmaceuticals, which significantly diverges their authorization process from the established norms. Moreover, the components of a phage therapy product must undergo frequent revisions and replacements to address resistance issues. Since every single element of a pharmaceutical product must undergo extensive safety and efficacy testing before being approved as a new component of an authorized product, it becomes apparent that licensing phages will be impractical for the pharmaceutical industry. Moreover, producing phages as pharmaceuticals at the Good Manufacturing Practices (GMP) level is highly demanding and incurs substantial costs. There is a strong advocacy effort pushing regulatory authorities to revise legislation in a manner that makes phage therapy economically viable and safe, especially in the era of antimicrobial resistance where alternative solutions are urgently needed. The new EU legislation has recognized the distinctions between phages and conventional drugs, and efforts are underway to update the regulations concerning phage-based Veterinary Medicinal Products (VMPs). On the other hand,

licensing phages as biocides rather than VMPs is significantly easier and entails a considerably reduced cost for both development and production.

6. Future Aspects

Phage therapy is well-suited to be incorporated into multifaceted strategies aimed at enhancing fish health in aquaculture and represents an auspicious approach in combating the emergence of antibiotic-resistant bacteria. However, caution must be exercised as there is a possibility of phage resistance evolving over time. Despite the potential for resistance, the concept of phage training for therapy, involving experimental coevolution, has recently emerged and warrants further investigation. Moreover, a novel area of research which includes the potential use of phage lytic enzymes and their interaction with the immune system of cultured aquatic organisms, is getting attention. The presence of newly discovered phages with diverse phenotypes and genotypes poses a challenge in this field. Each phage exhibits varying host specificity, efficacy, and mechanisms of action against bacteria. Consequently, it becomes vital to integrate and consolidate knowledge of the specific relationships between phages and pathogens. To tackle this challenge, it is necessary to obtain stronger evidence through *in vivo* studies to assess the efficacy and safety of phage lytic enzymes. These studies will offer more realistic insights into the interaction between phages, their enzymes, and the immune systems of the cultured aquatic organisms, helping to make this approach a well-established biotechnology in aquaculture.

The future of phage therapy in aquaculture shows great potential as scientists continue to explore innovative approaches and advancements in the field. One significant aspect that is likely to shape the future of phage therapy is the practice of phage engineering. By utilizing genetic engineering techniques, researchers can modify phages to improve their effectiveness, expand their ability to target different hosts, and optimize their therapeutic capabilities. Phage engineering enables the deliberate manipulation of phage genomes to introduce desired traits and enhance their performance against specific bacterial pathogens. It focuses on the development of phages with broader host ranges, as naturally occurring phages often have limited effectiveness against a wide range of bacterial strains due to their narrow host range. Through genetic modifications, scientists can broaden the host range of phages, allowing them to target a wider spectrum of bacterial pathogens. This expansion of the host range holds great potential to enhance the applicability of phage therapy in aquaculture, as it enables the effective control of multiple bacterial species known to cause diseases in various aquatic organisms. Another exciting avenue in phage engineering involves enhancing the stability and persistence of phages in aquatic environments. Factors such as temperature, salinity, and UV radiation can impact the viability and survival of phages in aquaculture systems. Through phage engineering, researchers can develop phages that can withstand these environmental stressors, thereby improving their overall effectiveness as therapeutic agents. This may involve modifying phage capsid proteins or introducing protective mechanisms that enhance their stability and prolong their activity in aquatic settings.

Furthermore, phage engineering offers the potential for developing customized phage cocktails to address specific bacterial infections in aquaculture. Phage cocktails consist of mixtures of different phages that target multiple strains or species of bacteria simultaneously. By

combining phages with complementary host ranges and mechanisms of action, researchers can create synergistic effects, increasing the likelihood of successful treatment outcomes. Phage cocktails provide a versatile and robust approach to combat bacterial infections, particularly in cases where bacterial pathogens exhibit high levels of diversity and resistance.

In addition, advancements in the delivery systems for phage therapy in aquaculture may shape its future. Ensuring effective delivery of phages to the infection site is crucial for achieving optimal therapeutic outcomes. Scientists are exploring various methods, such as nanoparticle-based delivery systems, biofilms, and encapsulation techniques, to improve the efficiency of phage delivery and protect them from degradation or inactivation. These delivery systems hold promise in enhancing the precision and effectiveness of phage therapy by ensuring an adequate number of phages reach the targeted pathogens, thereby maximizing their therapeutic impact.

Moreover, continuous investigation into the interactions between phages and their bacterial hosts, as well as the mechanisms underlying bacterial resistance to phages, will play a vital role in advancing the development of highly effective phage therapies. Through acquiring a deeper comprehension of the intricate dynamics between phages and their bacterial hosts, researchers can pinpoint strategies to overcome bacterial resistance and bolster the long-term effectiveness of phage therapy.

In conclusion, the future of phage therapy in aquaculture shows great potential, with the important roles of phage engineering and advancements in delivery systems. Through phage engineering, researchers can enhance key properties of phages, such as host range and environmental stability. Concurrently, the development of targeted delivery systems ensures efficient and precise delivery of phages to the infection sites. Ongoing research in phage-host interactions and resistance mechanisms will further contribute to the development of safe, effective, and sustainable phage-based treatments, supporting the growth and sustainability of aquaculture practices worldwide.

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