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REVIEW

Bacteriophage therapy in aquaculture: current status and future challenges

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Abstract

The escalation of antibiotic resistance has revitalized bacteriophage (phage) therapy. Recently, phage therapy has been gradually applied in medicine, agriculture, food, and environmental fields due to its distinctive features of high efficiency, specificity, and environmental friendliness compared to antibiotics. Likewise, phage therapy also holds great promise in controlling pathogenic bacteria in aquaculture. The application of phage therapy instead of antibiotics to eliminate pathogenic bacteria such as *Vibrio*, *Pseudomonas*, *Aeromonas*, and *Flavobacterium* and to reduce fish mortality in aquaculture has been frequently reported. In this context, the present review summarizes and analyzes the current status of phage therapy in aquaculture, focusing on the key parameters of phage application, such as phage isolation, selection, dosage, and administration modes, and introducing the strategies and methods to boost efficacy and restrain the emergence of resistance. In addition, we discussed the human safety, environmental friendliness, and techno-economic practicability of phage therapy in aquaculture. Finally, this review outlines the current challenges of phage therapy application in aquaculture from the perspectives of phage resistance, phage-mediated resistance gene transfer, and effects on the host immune system.

Keywords Phage therapy · Aquaculture · Efficacy · Phage resistance

Introduction

Bacteria-derived diseases are the leading cause of high fish mortality and tremendous economic losses in aquaculture. Antibiotics have been mainly used for therapeutic and prophylactic purposes in aquaculture (Vaseeharan and Thaya 2014). However, antibiotics may enter into the environment by leaching from uneaten feeds, the excreted fraction with manure since antibiotics are mainly administered through the dip-coated feed (Robinson et al. 2007). To the present knowledge, the residues of antibiotic components and the spread of antibiotic resistance genes (ARGs) are universal in aquaculture and pose significant health threats (Fang et al. 2019; Gao et al. 2018; Guo et al. 2019; Han et al. 2020). Furthermore, broad-spectrum antibiotics can jeopardize the indigenous microorganisms and beneficial microbiota. Recently, studies have shown that the addition of antibiotics, even below the dose approved by FDA, can irreversibly influence the diversity and community structure of the gut microflora of fish (Saenz et al. 2019).

In recent years, advances in antibiotic alternatives for fish disease control have emerged. Some novel vaccines (Vinay et al. 2018; Yan et al. 2021; Zhang et al. 2021a)

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have been trialed in aquaculture, and promisingly, a vaccine injection of salmonids in particular is performed on a large-scale. However, given the complexity of pathogens and the difficulties of vaccine administration in aquaculture (Micoli et al. 2021), vaccination is still debatable. Probiotics, live microbial food additives for regulating host gut homeostasis (Fuller 1989), appear to be an effective biological control agent owing to their salient nature of positively affecting host metabolic activity and increasing host resistance to pathogens (Dawood et al. 2019). Nonetheless, the rapid colonization and proliferation of probiotics in a given environment have always been an insurmountable gap due to the competition for niches and nutrients with another microbiota in the environment (Van Hien et al. 2021). These impediments, coupled with the escalating issue of antibiotic resistance, have made it urgent to explore novel and efficient antibiotic alternatives in aquaculture.

Bacteriophages (phages), the most abundant organisms on the planet (Suttle 2007), are known as natural killers of bacteria (Domingo-Calap and Delgado-Martinez 2018). The phage-based biocontrol method, or phage therapy, is characterized by high efficiency, specificity, and environmental friendliness (Choudhury et al. 2017; Sieiro et al. 2020) and has gradually become the protagonist of a post-antibiotic era (Altamirano and Barr 2019). Nowadays, phage therapy has been heavily explored in medicine (Onsea et al. 2019; Ooi et al. 2019), agriculture (Kahn et al. 2019; Svircev et al. 2018), food (Clavijo et al. 2019; Luiz Vaz et al. 2020), and environmental fields (Ayyaru et al. 2018; Sun et al. 2019). Similarly, phage therapy has great potential in aquaculture against pathogenic bacteria (Le and Kurtboke 2019). Recently, many successful cases of phage therapy for preventive and therapeutic purposes in aquaculture have been reported, using phages targeting *Vibrio* sp. *Va-F3* (Chen et al. 2019), *Vibrio coralliilyticus* (Chen et al. 2019; Kim et al. 2019a), *Vibrio alginolyticus* (Tuan Son et al. 2020), *Pseudomonas aeruginosa* (Cafora et al. 2019), *Aeromonas hydrophila* (Cao et al. 2020; Tuan Son et al. 2018), and *Flavobacterium columnare* (Laanto et al. 2015) and other fish pathogens.

Although phage therapy in aquaculture has made great progress in laboratory research, there are still many problems to be solved before practical application. Due to the complexity of application scenarios, some of the key parameters in phage therapy, such as phage selection, dose, and administration method, are often customized, but a systematic review and analysis of relevant content are currently lacking. In addition, it is necessary to introduce some novel approaches that may be used to improve the efficacy of phage therapy in aquaculture. Furthermore, the environmental health, food safety, and techno-economic practicability of phage therapy in aquaculture, which is an ongoing concern for us, has also been rarely mentioned in other literature. In such a context, this review spotlights these issues and presents some challenges and prospects of phage therapy in aquaculture, intending to provide new insights into phage therapy and accelerate its application in aquaculture.

Rationale for phage therapy in aquaculture

The earliest cognition of phages can be dated back to "the cholera period." Ernest Hanbury Hankin, a British bacteriologist, believed that there was a mysterious substance that could kill *Vibrio cholerae* in the Ganges River, India, and Frederick W. Twort first confirmed the existence of phages in 1915. However, scientific data on phage-based pathogen control is limited at the time. In particular, the discovery of penicillin, a broad-spectrum antibiotic, in 1928, exacerbated the long stagnation of phage therapy research. Until recently, it was gradually revived and received widespread attention with the advent of the antibiotic crisis.

Phages can replicate through the lytic, lysogenic, chronic, and pseudolysogenic cycles (Mirzaei and Maurice 2017), but chronic and pseudolysogenic cycles remain poorly described. In the lytic cycle, phage utilizes the substrates within the host to produce progeny phages after invading the host and, hence, leading to host lysis and death. As for the lysogenic cycle, phages typically integrate their DNA into the genome of the host and replicate synchronously with the host replication but ultimately leading to the death of the host as well. Phage therapy, as a means of preventing or treating diseases, mainly exploits the lytic effect of virulent phages to reduce the density of pathogenic bacteria and thereby reduce or avoid the incidence of disease occurrence. Although cases of lysogenic phage for therapy are still relatively rare, lysogenic phages in the natural environment can enter into the lytic cycle induced by temperature (Chu et al. 2011), pH (Miller-Ensminger et al. 2020) and UV (Zhang et al. 2020) to regulate microflora. Notably, unlike chemical bacteriostatic agents such as antibiotics, phages can lyse hosts continuously to produce progeny phages to maintain phage titers in the environment.

There is an order of magnitude more phages than bacteria on the planet, namely, 10^{31} or more (Guemes et al. 2016; Mokili et al. 2012), widespread in seawater (Ignacio-Espinoza et al. 2020; Wu et al. 2020), marine sediments (Engelhardt et al. 2014, 2015; Lachnit et al. 2019), soils (Jin et al. 2019; Kuzyakov and Mason-Jones 2018), and artificial ecosystems (Brown et al. 2019). Additionally, phages for therapeutic use in aquaculture can stably survive in a wide range of pH (5–9), temperature (4–37 °C), and salinity (0.1–3.5%) (Chandrarathna et al. 2020; Kim et al. 2019a; Nikapitiya et al. 2020a), covering common aquaculture environments. Of particular interest is the fact that these phages can survive for extended periods even

used in vivo in aquatic economic organisms. For example, phages can maintain survival activity in the complex biological environment of the rainbow trout intestines and spleens for at least 4 days (Christiansen et al. 2014). Strong environmental tolerance reduces the frequency of phage administration, facilitating their practical application. Compared with other biochemical agents, phage therapy has the following unparalleled advantages: (1) high efficiency against drug-resistant bacteria; (2) strong specificity to hosts; (3) low direct toxicity to eukaryotes; and (4) flexible administration modes.

Pre-application preparation

Since the antibiotic crisis, especially since the twenty first century, the number of phage therapy-related publications has been increasing dramatically with each passing day, with a rate of > 1400 publications/year in the last decade (Fig. 1A, B). Inappropriate choice, poor preparation, and decay of phages before application are recognized as the three main factors in the failure of phage application (Gill and Hyman 2010). Furthermore, the possibility of phage formulation determines the potential of phage application in moving

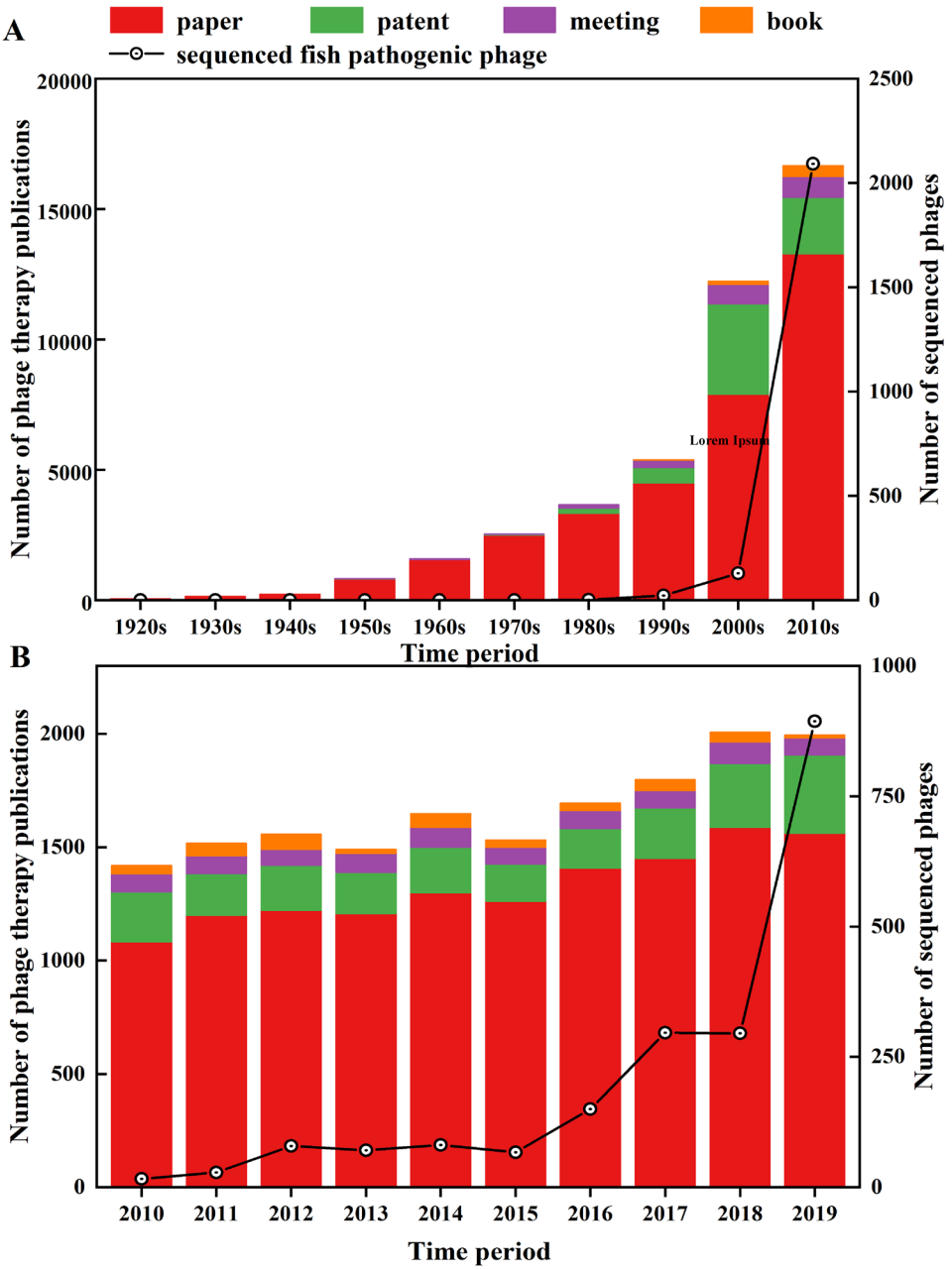

Fig. 1 A number of phage therapy-related publications and sequenced phages targeting fish pathogenic bacteria in the last 100 years (**A**) and in the last decade (**B**) demonstrate widespread interest in phage therapy. The bar indicates the number of Web of Science searching for the publications related to phage therapy (TS = (phage therap* OR bacteriophage therap* OR phage control* OR bacteriophage control* OR phage cure* OR phage treat*)) and NCBI released complete phage genomes associated with fish pathogens (*Aeromonas*, *Edwardsiella*, *Flavobacterium*, *Francisella*, *Photobacterium*, *Piscirickettsia*, *Pseudomonas*, *Tenacibaculum*, *Vibrio*, *Yersinia*, *Lactococcus*, *Renibacterium*, *Streptococcus*, *Acinetobacter*, *Shewanella*, *Plesiomonas*, *Stenotrophomonas*, and *Kocuria*) from 1920 to 2019

from small trails to field applications. This section reviews and summarizes the preparation steps for phage application in aquaculture (Fig. 2), aiming to provide theoretical support for phage application.

Phage isolation

Phages and their corresponding hosts tend to concomitance, so it is common to isolate phages from sources with high host abundance (Kim et al. 2020; Richards et al. 2021). For example, Richards et al. (2021) isolated 16 novel *Vibrio coralliilyticus* and *Vibrio tubiashii* phages from seawater. Also, Liu et al. (2020) isolated 5 *Aeromonas hydrophila* phages from fish ponds and polluted rivers, not from sewage. These suggest that the source of isolation is critical. By 2019, over 2,000 phages targeting fish pathogens have been isolated, and over 90% of these phages were isolated in the last decade (Fig. 1A, B) (complete genomes of phages targeting fish pathogens released by the National Center Biotechnology for Information).

For phage isolation, the most classical method is the double-layer agar (DLA) method. This method places a semi-solid medium containing the host bacteria and phages on top of a solid plate to visualize phage plaques. Clear plaques are most likely to be formed by virulent phages and appropriate for subsequent applications, while turbid ones are mostly formed by temperate phages. Interestingly, broad-spectrum phages are more common in environments with low bacterial concentrations owing to their ability to infect more potential hosts, while high host concentrations stimulate the growth of phages with strong host specificity (Gill and Hyman 2010). Accordingly, this survival strategy of phages undoubtedly promotes the selection for narrow-spectrum phages due to the high host density in the DLA method.

Several novel methods for phage isolation have been developed in recent years, such as ultrafiltration- or adsorption-based separation methods, and concentration methods by enrichment from water, soil, and sediment (Nair et al. 2021). Nafarrate et al. (2020) used Bolton selective broth to enrich phages for 48 h at 42 ℃ to isolate *Campylobacter*-specific phages without shaking. This method achieved a low detection limit and high recovery rate compared to other isolation methods with different parameter settings, demonstrating this method to be a simple, reproducible, and efficient approach for the isolation of *Campylobacter* phages. In addition to typical strong-specific phages, polyvalent phages (Ngiam et al. 2021; Yu et al. 2016) and macrophages (Saad et al. 2019) can also be selectively isolated by modified isolation methods.

Currently, almost all phage isolation methods are host-dependent. However, a typical paradigm that only 1% of bacteria are cultivable (Whitman et al. 1998) suggests that the vast majority of hosts in the natural environment are unculturable, let alone corresponding phages. *Vibrio*, *Flavobacterium*, *Aeromonas*, and *Pseudomonas* are the hosts with the most isolated phage counterparts, as well as the pathogens with the most phage therapy applications in aquaculture (Culot et al. 2019). Fortunately, cultivation techniques for

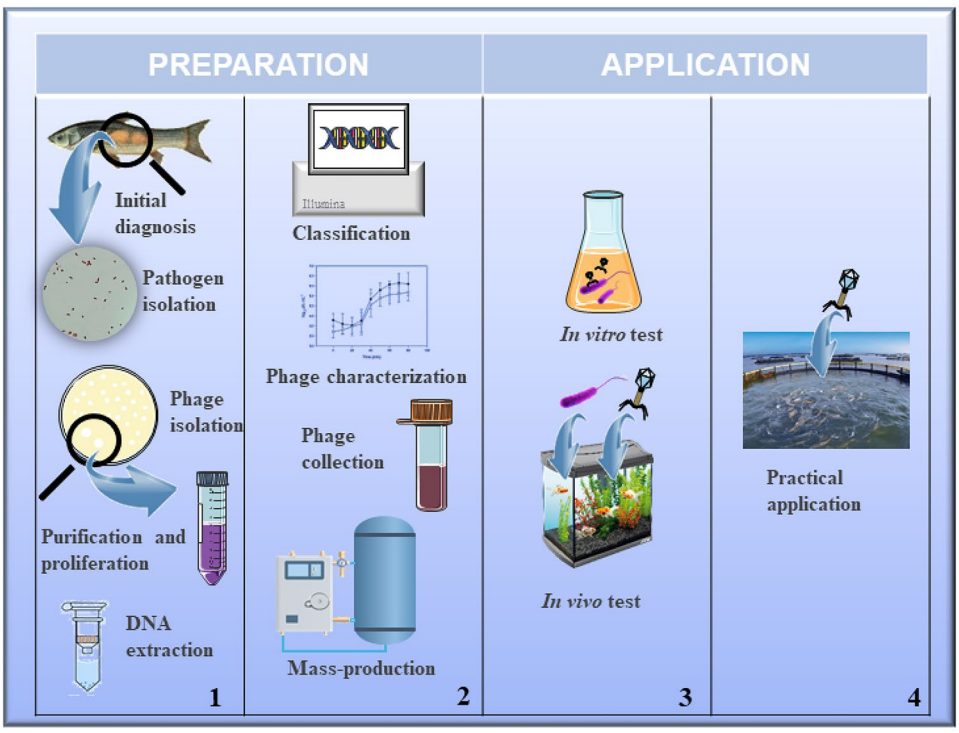

Fig. 2 General procedures of phage therapy in aquaculture

uncultured pathogenic bacteria are relatively well-developed (Lewis et al. 2021), which is conducive to phage-based disease control in aquaculture.

Phage characterization and selection

Clarifying the fundamental properties of phages is a prerequisite for screening phages as safe biocontrol agents. Some basic but critical features, such as phage morphology, host range, one-step growth curve, the optimal multiplicity of infection (MOI), environmental tolerance, detailed genome analysis, and in vitro lysis effect, are closely related to phage selection. For example, optimal MOI can guide the phage-to-host ratio in actual production, and in vitro lysis effect can guide phage dosage. In fact, studies have shown that there are significant differences in the protective effects of different MOIs on fish (Kim et al. 2019a; Laanto et al. 2015). More detailed selection criteria of phages for therapeutic use have been discussed in several literatures (El Haddad et al. 2019; Gill and Hyman 2010; Luong et al. 2020).

Theoretically, an ideal phage candidate for therapy should be strictly lytic, polyvalent, environment-tolerant, and free of antibiotic resistance and virulence genes and can coexist with other phages (to form phage cocktails). The ability of phages to infect bacteria in biofilms should also be considered, since biofilm is a common form of aggressive resistance of pathogenic bacteria to therapeutic agents and thus a challenging aspect for treatment. Some phages can destroy the biofilm formed by single- or multi-species bacteria effectively (Forti et al. 2018; Gonzalez et al. 2017; Kim et al. 2019b), while others may promote biofilm formation by increasing aggregation, surface adhesion, and production of bacteria fimbria to create barriers (Hansen et al. 2019; Lacqua et al. 2006). Although phage selection is a critical step to the success of phage therapy trials, uniform standards have not yet been met, and much work remains to be done.

Phage preservation

To maintain the phage activity for a long-time, a suitable preservation method should be selected. In general, the phage titer slightly decreases over several months with commonly used protective agents such as chloroform and SM buffers, as well as with cryopreservation. For example, Srinivasan and Ramasamy preserved the isolated *Vibrio vulnificus* phages VV1–VV4 in chloroform (7%) or DMSO (7%), which showed that the infectivity of all phages was not affected at − 40 °C for up to 30 days in both preservation methods (Srinivasan and Ramasamy 2017). González-Menéndez et al. (2018) compared the titer of *Staphylococcus aureus* phages phiIPLA-RODI and phiIPLA-C1C under different preservation conditions. They found that lower temperatures (− 80 °C and − 196 °C) or lyophilization allow phages to exhibit good viability, and encapsulation facilitates preservation and transportation, but repeated freezing and thawing cycles will inactivate part of the stored phage particles (González-Menéndez et al. 2018). Other than this, the results of Leung et al. (2017) showed that a powder containing ≥ 40% trehalose exhibited good phage PEV2 storage performance within 12 months. In another analogous study, pullulan–trehalose encapsulated phages can remain infectious for up to 3 months at ambient conditions (Leung et al. 2018).

Phage preparation

Most phages currently isolated that target fish pathogenic bacteria are more poorly tolerated at low pH owing to the nature of their protein capsids (Le Thanh et al. 2021; Tan et al. 2021; Xu et al. 2021), while the pH values in the gastrointestinal tract of fish vary from 2 to 7 (Kihara et al. 2012). Therefore, for intestinal disorders, it is particularly essential to coat or encapsulate phages to minimize titer loss as the phages pass through the digestive tract when administrated orally. Huang and Nitin (2019) developed a phage-based edible whey protein isolate (WPI) coating on fish feed; the results demonstrate that this feed loaded with coated phages enhances phage stability and reduces host bacteria concentration in a simulated gastrointestinal environment compared to feed loaded uncoated phages (validation with T7 phage and *Vibrio* phage and their corresponding hosts). In a similar study, *Salmonella* phage SPT 015 microencapsulated with a 3: 1 ratio of WPI and trehalose can survive over 90% of exposure to pH 1.5 for 5 h, while non-encapsulated phages could not survive at the same conditions (Petsong et al. 2021). Furthermore, studies on liposome- or alginate/$CaCO_3$-encapsulated phages can enhance the tolerance of phages under a low pH environment compared to non-encapsulated phages which has also been reported (Colom et al. 2017, 2015).

Customized phage therapy

Several parameters, such as dosage and mode of phage administration, are crucial in phage therapy, and optimizing such parameters allows to reduce the costs as well as to enhance the therapeutic efficacy and accelerate the widespread use of phage therapy in aquaculture. The following section discusses the latest researches on these parameters. For reference, laboratory findings under different parameters are listed in Table 1.

Table 1 Current status of phage therapy research under different parameters

Phage names	Fish and/or disease	Dosage	Administration modes	Duration of administration or the weight of fish	Outcomes	Reference
pVco-5	Pacific oyster	1.25×10^5 to 1.25×10^7	Directly add phage suspension	Larvae	Cumulative mortality rates of pacific oyster larvae were below 11%, which was significantly lower than the phage-untreated control group	(Kim et al. 2020)
pVco-7	Pacific oyster	1.44×10^7	Directly add phage suspension	Larvae	Survival rates was 26.32 ± 8.14%, but the in vivo preventive efficacy was not effective as pVco-5	(Kim et al. 2020)
pVco-14	Pacific oyster	MOI = 0.1, 1, 10	Directly add phage suspension	Larvae	The phage treated group exhibited 21.77 ± 5.41% and untreated control group exhibited 81.44 ± 7.3% mortality	(Kim et al. 2019a)
Vibrio phage vB_VspP_pVa5	Skin ulceration syndrome	MOI = 1, 10, 100	Directly add	Unreported	Almost completely eliminate bacterial growth for 8 h for in vitro lysis assay	(Katharios et al. 2017)
Vibrio phage VEN	External hemorrhagic ulcers and gill rot disease	MOI = 0.1, 1, 10, 100	Directly add phage stock dilutions	Unreported	The higher the MOI, the more effective the inactivation	(Kokkari et al. 2018)
Vibrio phage KVP40	Cod and turbot larvae/hemorrhagic septicemia and body surface ulceration	$0.5–8\times10^8$ PFU/mL OR MOI = 5–100	Directly add	Eggs and larvae	Effectively infect *V. anguillarum* and reduce or delay the mortality of the cod and turbot larvae challenged with *V. anguillarum*	(Rorbo et al. 2018)
Vibrio phage CHOED	Atlantic salmon	MOI = 10	Directly add	20–25 g	The survival rate was 100% after add phages within 20 days, while phage-free group was 60%	(Higuera et al. 2013)
Phage AS-A	Solea senegalensis	10^{10} PFU/ml OR MOI = 100	Directly add phage suspension	Juveniles ~ 30 mm	The average mortality of fish + AS + AS-A group was 1.1% after 72 h treatment, far below than fish + AS group with 35.66%	(Silva et al. 2016)

Table 1 (continued)

Phage names	Fish and/or disease	Dosage	Administration modes	Duration of administration or the weight of fish	Outcomes	Reference
MJG	Rainbow trout	3.2×10^6 PFU/fish or 3.2×10^6PFU/ml	Intraperitoneal injection, immersion, and oral feeding	Approximately 20 g	MJG could provide protection via various delivery methods	(Cao et al. 2020)
Akh-2	Loach (*Misgurnus anguillicaudatus*)	1×10^8 PFU/ml	Immersion	10 g	The survival rate of Akh-2 treated group was significantly higher than the non-treated group	(Akmal et al. 2020)
ETP-1	Zebrafish	9.85×10^8 PFU/ml	Bath exposure	0.55 ± 0.5 g	Higher cumulative survival of 68% in the ETP-1 exposed group compared to the control group of 18%	(Nikapitiya et al. 2020a)
Flavobacterium phage FCL-2	Zebrafish and rainbow trout/columnaris disease	MOI = 1,100	Directly add single phage	Unsexed, adult fish	In the phage treatment, 100% of the zebrafish and 50% of the rainbow trout survived (survival rates were 0 and 8.3% in the phage-free treatment, respectively)	(Laanto et al. 2015)
vB_Pd_PDCC-1	Longfin yellowtail (*Seriola rivoliana*)	2×10^8 PFU/ml	Directly add phage suspension	Eggs	Hatching rate of treated with phage vB_Pd_PDCC-1 was close to 80%, significantly higher than untreated control challenged with *P. damselae*	(Veyrand-Quiros et al. 2020)
Phage FCP1	Indian catfish	4.55×10^6 PFU/ml 9.15×10^6 PFU/ml	Immersion	20–25 cm	Lysed the targeted bacterium in relatively shorter period of time make it entity below threshold level	(Prasad et al. 2011)
Phage FCP1	Indian catfish	4.55×10^6 PFU/ml 9.15×10^6 PFU/ml	Oral	20–25 cm	Decrease of host number and recovery of columnaris disease	(Prasad et al. 2011)

Broad-spectrum phages or phage cocktails

Aquaculture environments contain multiple species of bacteria that are pathogenic to aquatic organisms, and the same species of bacteria also include several pathogenic serotypes, which impedes the widespread use of a single narrow-spectrum phage. In such a context, more efforts have been dedicated to broad-spectrum phages and phage cocktails.

Broad-spectrum phages, phages that can infect multiple distinct hosts within one genus or in multiple genera (de Jonge et al. 2019), can efficiently lyse not only host bacteria but also background pathogens that are ubiquitous in farming and natural environment (Kalatzis et al. 2016; Rorbo et al. 2018). For example, *Vibrio* phage KVP40, a broad-spectrum phage, was able to not only postpone death or reduce mortality in the challenge group but also lower background mortality in the non-challenge control group by lysing background pathogenic hosts (Rorbo et al. 2018), highlighting the prevention use of phage KVP40 in aquaculture. Phages capable of lysing multiple serotypes have also been reported. Phage PH669 can lyse multiple strains of O3 and O4 serotypes in *Vibrio parahaemolyticus* (Hu et al. 2021), while the broader host range of phage vB_SPuM_SP116 and phage vB_SalS-LPSTLL can lyse 9 (Bao et al. 2019) and 11 (Guo et al. 2021) different types of serotypes in *Salmonella*, respectively. However, the acquisition of broad-spectrum phages remains challenging due to the differences in phage survival strategies (see part 3.1) and imperfection of isolation methods (Ngiam et al. 2021; Yu et al. 2016).

Phage cocktails, the mixture of multiple phages, can expand the host range and reduce the frequency of bacterial resistance development (Peters et al. 2020), as bacteria that are resistant to certain phages can also be killed by other phages. In general, the efficacy of phage cocktails is more potent than that of a single phage (Chen et al. 2019, 2018; Forti et al. 2018; Mateus et al. 2014). For example, a phage cocktail containing phage VP-1, VP-2, and VP-3 delayed the formation of phage resistance and improved the effect of inactivation against *Vibrio parahaemolyticus* compared to single phage therapy (Mateus et al. 2014). Moreover, Chen et al. (2019) isolated five broad-spectrum phages with strong lytic capacity from aquaculture wastewater and the intestinal tract of a diseased shrimp to construct a phage cocktail for inactivation of *Vibrio* sp. *Va-F3*. The inhibitory effect of the phage cocktail on *Vibrio* sp. *Va-F3* was significantly better than any single phage in vitro, and the in vivo protection of shrimp can be comparable to antibiotics (survival rates reach 91.4 and 91.6% in 7 days, respectively) (Chen et al. 2019). Encouragingly, phage cocktails containing phage PVP1 and PVP2 were as effective as antibiotics in controlling *Vibrio parahaemolyticus* in sea cucumber *Apostichopus japonicus* at doses of MOI = 10 and MOI = 100 (Ren et al. 2019). These highlight the advantages of phage cocktails in the control of bacterial diseases in aquaculture (fish and other aquatic organisms). However, it has been shown that cocktails containing excessive species of phages constructed for a wider host range may produce new host specificity (Essoh et al. 2013), and the optimal phage cocktail formulation is modifiable and should not be overly complex (Chan et al. 2013).

Dosage

Although it is challenging to determine the MOI in the actual disease context, the determination of the optimal phage dosage to be administrated can ensure therapeutic efficiency while saving costs in the laboratory study. Underdose of therapeutic phages may decrease the efficacy or induce phage resistance in bacteria (Kim et al. 2012), while overdose may cause hosts to resist phage infection by forming aggregates or biofilms and increase costs (Tan et al. 2015). Water flow and environmental stresses in the aquaculture ecosystem are responsible for the loss of therapeutic phages, while phages can hijack hosts to continuously produce progeny phages. These factors, together with the administration dosage, co-determine the phage titer in the aquaculture environment.

Typically, MOI provides an essential reference for the dosage administered. Dang et al. (2021) studied the protective efficacy of different MOIs of phage PVN02 against hemorrhagic septicemia in striped catfish *Pangasianodon hypophthalmus* via oral administration. They found that the mortality rate of striped catfish reduced by 60% at the phage dose of log 6.2 ± 0.09 compared to the phage-free group and striped catfish mortality was negatively correlated with phage dosage (Dang et al. 2021). A similar pattern that the higher the phage dose, the lower the mortality was also observed in the control of acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus* in the shrimp aquaculture industry (Ding et al. 2020). In contrast, phage pVco-14 can inactivate *Vibrio coralliilyticus* favorably at low MOI (MOI = 0.1) for oyster larvae protection (Kim et al. 2019a). Interestingly, in our previous study of phage therapy for Edwardsiellosis in zebrafish, the optimal MOI for phages against pathogenic bacteria in vitro and in vivo would be different since the fish gastrointestinal tract is a complex environment containing multiple microorganisms and chemical substances (unpublished data). This further emphasizes the indispensable line of clinical validation of phage therapy in aquaculture. The dose of phage administrated tends to be personalized due to the influence of water quality, fish species, and other factors and requires extensive field trials for ongoing optimization.

Administration mode

The mode of phage administration is also a crucial factor affecting the efficacy of phage therapy. Several modes of administration, such as oral administration by soaking feed; directly adding phages in the culture system, intraperitoneal or intramuscular injections; and immersion, have been reported for phage therapy in aquaculture (Donati et al. 2021; Kokkari et al. 2018; Prasad et al. 2011).

In a flow-through experimental system close to the real-life rearing environment, bath treatment achieves the optimal protection for rainbow trout when the first symptoms of columnaris disease were observed (Kunttu et al. 2021). However, the volume of water requiring phage treatment in practical aquaculture is generally quite large. Pre-infection administration for prophylactic purposes is currently the prevailing approach in aquaculture, particularly for injection administration to avoid secondary hurt to the fish, but cases of phages for post-infection treatment have also shown good results (Kunttu et al. 2021; Wu et al. 2021). This administration method resembles vaccine injection, with a single mode of dosing and difficulty in operation. Other than this, oral phage has been demonstrated to be more efficient than the injection route in the control of skin syndrome of sea cucumber caused by *Vibrio cyclitrophicus* (Li et al. 2016). Despite the fact that gastric acid is a negative factor for orally administered phages, as far as the literature is concerned, most phages can reach the intestine through the gastric barrier (Donati et al. 2021; Xue et al. 2020). Donati et al. (2021) adopted three different modes of administration, namely, oral, bath, and injection, to study the phage efficacy against *Flavobacterium psychrophilum* and migration rate in different organs of rainbow trout. The *Flavobacterium psychrophilum* phage can be detected consistently in the intestine and determined sporadically in the spleen, brain, and kidney via oral administration (Donati et al. 2021). This finding broadly provides evidence for previous observations that phages can be detected in the intestine, spleen, brain, and kidney post-oral administration (Christiansen et al. 2014). Of great concern is that phages loaded onto the feed will be re-released into the aqueous environment (Huang and Nitin 2019), which is advantageous for oral administration because some of the pathogens are freely in the water or can form biofilms on rearing equipment.

Upgrading phage therapy to boost the efficacy

Most current methods aimed to boost the phage efficacy and avoid the emergence of phage resistance broadly follow at least one of three main strategies. They either rely on combination with other antimicrobial drugs for co-inhibition (phage–antibiotic synergy), or enhance the efficacy of phage therapy by customized modification of the phage (genetically engineered phages), or utilize its gene expression products (phage lysis proteins). Methods that fall into these three categories are reviewed and discussed in the sections below (Fig. 3).

Phage–antibiotic synergy

The phenomenon that phage–antibiotic combinations can enhance therapeutic efficacy was discovered as early as 1945 (Himmelweit 1945) but failed to receive sufficient attention. Until 2007, Comeau et al. (2007) observed plaque expansion in β-lactam antibiotics, and the phenomenon was then officially designated as phage–antibiotic synergy (PAS) and gradually regained attention.

Phage–antibiotic synergy has been shown to effectively inhibit multiple antibiotic-resistant bacteria (Jo et al. 2016a, 2016b; Ryan et al. 2012). The co-existence of a sub-lethal dose of antibiotics with phages and hosts may cause cocci swelling or bacilli filamentation and, simultaneously, increase the content of mRNA encoding phage polymerase and delay the lysis time after infection to obtain a greater phage burst size and a larger plaque (Kim et al. 2018). Likewise, Kamal and Dennis (2015) mixed *Burkholderia cepacia* complex (Bcc) and Bcc phages KS12 and KS14 with antibiotics and found that the host cells developed a chain-like arrangement, an elongated morphology, as well as a clustered arrangement, and the size of the plaque and phage titer increased with increasing antibiotic concentration. Although

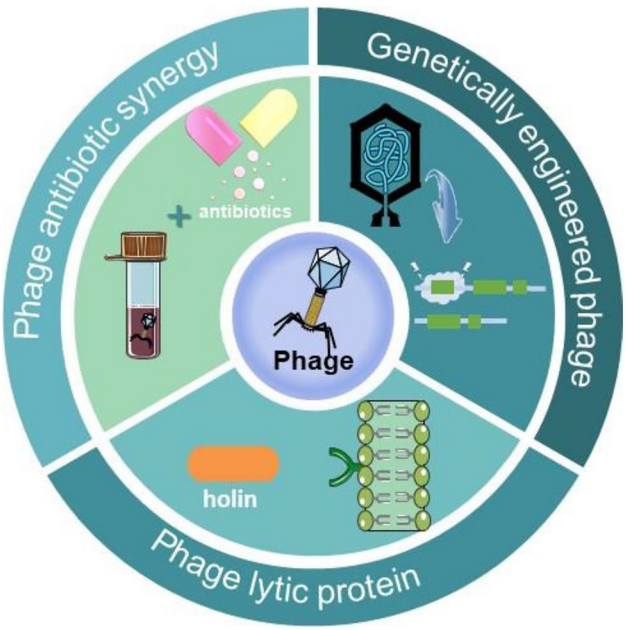

Fig. 3 Methods to circumvent the possible defects in the application of phage therapy

to our knowledge there are currently no studies reported on the application of phage–antibiotic combination in aquaculture, the remarkable results of PAS in other fields herald its great potential in aquaculture. For example, in the treatment of Bcc infected *Galleria mellonella* larvae, the survival rate of Bcc phage KS12 combined with low concentrations of the meropenem group was significantly higher than that of KS12 or antibiotics alone (Kamal and Dennis 2015). However, discordant opinions have also appeared that the combination of sub-MIC streptomycin with phages not only enhances antibiotic resistance in bacteria but also leads to the extinction of phages (Cairns et al. 2017). Although this anomalous result may be due to the fact that streptomycin is a common mutagen, this finding still remains alarming. In particular, when combining phages with antibiotics against pathogens in aquaculture, the selection of phages and antibiotics and their mixing ratio, and even the order of addition, should be fully considered (Tagliaferri et al. 2019).

Genetically engineered phages

Genetic engineering as an emerging technology allows the insertion of antibiotic sensitive genes into the genome of temperate phages and further integration of these genes into resistant bacteria using their lysogenic properties and hence restores antibiotic sensitivity of bacteria (Edgar et al. 2012) or customizes the tail recognition structure of virulent phages to broaden their host range (Mahichi et al. 2009). Yehl et al. (2019) altered and expanded the host range by modifying complementarity-determining regions (CDRs) in the phage tail fiber, wherein phage pB10 was effective in inhibiting pathogen growth in a mouse skin infection model without phage resistance. Notably, genetically engineered phages for human therapy were first reported with good results in 2019 (Abbasi 2019). Despite the fact that the world is currently conservative on genetic engineering and genetically modified phages are still confined in the laboratory, it may be a potential tool against pathogenic bacteria in aquaculture.

Phage lysis protein

A method for direct utilization of phage lysis proteins, mainly virion-associated phage hydrolase proteins and cell envelope digesting proteins, has been proposed in recent years to broaden the host range (Drulis-Kawa et al. 2015). These proteins can also synergize with other antimicrobial agents to reduce the risk of virulence factor transfer and resistance mutations in bacteria (Duarte et al. 2021; Grabowski et al. 2021; Letrado et al. 2018; Linden et al. 2021; Schmelcher et al. 2012). Virion-associated peptidoglycan hydrolase plays a major role in bacterial surface receptor recognition in the first step of the infection. Cell envelope digesting proteins, mainly holins and endolysins, disrupt the cell structure after completing the assembly of progeny phages, thereby releasing the progeny phages. Although the cell envelope digesting proteins destroy cellular structure from inside the cell, it has been shown that externally administered endolysins can still lyse Gram-negative bacteria slowly by osmosis (Briers and Lavigne 2015). LysVPMS1, endolysins with a broad lytic activity, has been proven to be effective biocontrol agents (Angelica Zermeno-Cervantes et al. 2018). In addition, although phage PSP01 lyses the host bacteria *Plesiomonas shigelloides* specifically, the combination of HolPSP and LysPSP-1, holin and endolysin of phage PSP01, exhibited a synergetic effect on the lysis of *E. coli* (Zhang et al. 2021b). Notably, protein therapy is particularly powerful toward Gram-positive bacteria such as *Staphylococci* and *Streptococci* (Linden et al. 2021). However, phage lysis proteins may generate neutralizing antibodies to compromise their efficacy due to their proteinaceous nature (Fischetti 2010). Furthermore, whether these proteins are sufficiently tolerated in complex aquaculture environments and the acidic digestive system of the fish are also questions worth investigating in depth.

Food safety, environmental benefits, and techno-economic analysis of phage therapy

Food safety

There is currently no well-developed methodology to assess the safety of phage therapy in food-related industries, but theoretically, it is harmless to human health for the following reasons. Firstly, phages are abundant and ecologically important in aquatic ecosystems (Kavagutti et al. 2019) and widespread in the digestive tract of mammals (Letarov et al. 2010) and are part of the normal microbiota of water environments and human guts. Secondly, phages have been widely used in the food industry for foodborne pathogen detection, active food packaging, postharvest, and processed foodstuff biopreservation (O'Sullivan et al. 2019) and have a significant role in facilitating the development of the food industry. Thirdly, the phages used in aquaculture specifically target only pathogenic bacteria, not the normal human microbiota, and should not pose a threat to human health.

Actually, since the product that anti-*Listeria*, i.e., Listex™, was first generally recognized as safe (GRAS) by the US Food Drug and Administration (FDA) in 2006, an increasing number of phage-related products have been GRAS in recent years. To date, at least seven biotechnology companies have devoted to phage therapy for pathogenic bacteria control in aquaculture and have developed several safe, simple, and effective commercial phage products

to target and destroy pathogenic bacteria (for instance, CUSTUS®$_{YRS}$ for *Yersinia ruckeri* control, LUXON for *Vibrio parahaemolyticus* control). More detailed information is available at https://www.bacteriophage.news.

Environmental friendliness

Pathogenic bacteria are prevalent in intensive marine aquaculture systems and may influence the initial microbial communities and threaten the safety of fish in natural water bodies (Wang et al. 2018). However, a common chemical disinfectant can disturb the bacterial communities in aquaculture even at low concentrations (Saenz et al. 2019; Teitge et al. 2020). Given this situation, the utilization of phages in aquaculture may be a promising method to prevent the disruption of normal microbiota in farmed and further natural water bodies due to its strong specificity. Notably, it has been previously demonstrated that phage therapy has less environmental impact than chemotherapy in fish farming plants (Almeida et al. 2009). On the other hand, a large number of ARGs, such as *sul1*, *tetG*, *intl1*, *tetX*, and *tetW*, have also been detected in large-scale freshwater farming systems, forming a huge reservoir of ARGs (Shen et al. 2020). Theoretically, the use of phage therapy in aquaculture can reduce antibiotic consumption, thereby lowering the frequency of ARG production in aquaculture water and reducing the spread of ARGs in natural environments. In fact, polyvalent phage YSZ-1 alone or in combination with biochar can significantly reduce the abundance of antibiotic-resistant pathogenic bacteria *E. coli* K-12 and *Plesiomonas aeruginosa* PAO1 and their carried ARGs (*tetM*, *tetQ*, *tetW*, *ampC*, and *fosA*) in a soil–lettuce system (Ye et al. 2018). Our present study also found that phage treatment for Edwardsiellosis can significantly reduce the abundance of *floR* gene in zebrafish excreta and associated feeding water environment compared to florfenicol treatment (unpublished data).

Technically efficient and cost-saving

The discovery of novel antibiotics at the current stage is increasingly difficult, requiring a combination of new strategies such as molecular biology, genomics, and metabolomics, and the speed of development remains severely lagging the pace of the emergence of drug-resistant bacteria especially in aquaculture, which consumes substantial antibiotics. By contrast, phage-based antimicrobial agents are renowned for their technical simplicity and economical saving, which greatly reduces the difficulty of development and shortens the application cycle of phage therapeutic agents. For example, the estimated cost of large-scale phage production and cost of biopolymer phage coating formation containing 5% WPI and 2.5% glycerol was $\$4.41 \times 10^{-13}$ per phage particular (Krysiak-Baltyn et al. 2018) and 0.001 cents per fish feed pellets (Huang and Nitin 2019), respectively. Regarding antibiotics, oxytetracycline (OTC), the most widely used in aquaculture, costs about 1.5 cents per day per kg fish body weight (calculated at \$200/kg OTC and 75 mg OTC/d/kg fish body weight). Therefore, phage therapy can be technically simpler and economically more economical than antibiotic therapy in aquaculture.

Challenges and perspectives

Bacterial resistance to phages

Phages, like antibiotics, can also cause bacteria to develop resistance mutations in their interaction with the host, although ten times slower than antibiotics, which may lead to serious therapeutic concerns (Kaur et al. 2021) and thus a major barrier to successful phage therapy (Borin et al. 2021). Bacteria have evolved multiple defense mechanisms to prevent phage infection, including (i) the adsorption-blocking mechanisms; (ii) the super infection exclusion system; (iii) the R-M and CRISPR-Cas system; (iv) the abortive infection (Abi) systems and some chemical defense; or (v) the cyclic oligonucleotide-based anti-phage signaling system (Bernheim and Sorek 2020; Hampton et al. 2020; Labrie et al. 2010). Likewise, phages have also evolved counter-defense mechanisms such as anti-CRISPR proteins, evading Abi system, modification of restriction sites, and adapting host receptors (Harrington et al. 2017; Safari et al. 2020; Shin et al. 2017), to reinfect bacteria in a long arms race with bacteria. One idea of "training" phages to combat resistance by exploiting their natural property of co-evolution with the host has been proposed and exhibited an enhanced suppression effect on the host and delayed resistance mutations, demonstrating its feasibility for robust phage therapy (Borin et al. 2021). On the other hand, multiple studies have shown that pathogenic bacteria that are resistant to phages can gradually reduce their adaptability to the environment or diminish pathogenicity when mutations occur in cell surface structures that act as both bacterial virulence factors and phage adsorption receptors (Geisinger and Isberg 2015; Kortright et al. 2019; Leon and Bastias 2015). In addition, the emergence of phage resistance can also restore the sensitivity of antibiotic-resistant bacteria to antibiotics (Chan et al. 2016; Gordillo Altamirano et al. 2021).

Phage-mediated resistance gene transfer

Phages as reservoirs of ARGs in the environment can promote ARG transmission across strains through transduction (Calero-Caceres et al. 2019; Chevallereau et al. 2021; Larranaga et al. 2018; Yang et al. 2018). It has been shown that phage-mediated resistance gene transfer is prevalent in

full-scale hospital wastewater treatment plants (Manoharan et al. 2021) and that phage-mediated resistance gene transfer had a comparable frequency to that of plasmid under certain circumstances in a laboratory study (Sun et al. 2021). In the aquaculture environment, for the current studies, only a limited mobilization from bacteria to phages or vice versa in aquaculture plants (Colombo et al. 2016) and a significantly lower content of antibiotic resistome in phages than in plasmid in prawn mariculture environment were observed (Zhao 2021). The impacts of using phage therapy in aquaculture on the abundance of ARGs and the changes in the contribution of phage-mediated HGT in fish excreta and associated feeding water environment are even more completely unknown. It is worth noting that the control of resistance gene flow in the environment through phage intervention has also been reported (Parmar et al. 2017). In such a context of unclear phage ecology role in an aquaculture environment, extensive experiments to verify the contribution of phages to horizontal gene transfer (HGT) are needed.

Can phages ameliorate the host immune system?

The interaction between phages and the aquatic host immune system is interesting but poorly investigated. The immune system of a healthy body is constantly in dynamic balance, and proper inflammation helps to eliminate detrimental stimuli and restore health, but excessive or uncontrolled inflammation may damage healthy tissues (Kumar et al. 2004). MJG, a novel phage with positive therapeutic effects against *Aeromonas hydrophila* infection in rainbow trout, has been proven to ameliorate the immune response of rainbow trout by regulating IL-8 and IL-1β levels (Cao et al. 2020). Likewise, phage ZHF can alleviate the immune response of turbot caused by *Aeromonas salmonicida* and reduce mortality of turbot (Xu et al. 2021). Nikapitiya and colleagues' research highlights the relative mRNA expression level of CXCL-8a, IL-1β, TNF-α, IL-6, IL-10, and SOD-1 in the intestine was within the safety threshold after feeding phage ETP-1-enriched artemia compared to the control group (Nikapitiya et al. 2020b). These results are promising, but the impact of phages on the host immune system is multi-pathway and multi-faceted, and there is still a relative paucity of research to draw arbitrary conclusions.

Conclusions

Collectively, phage therapy has unparalleled advantages over other chemotherapeutics, such as high efficiency, specificity, and environmental friendliness, with great potential for application in aquaculture. This technology is expected to partially or even completely substitute antibiotics as the mainstream of biomedicine in the future. Nonetheless, most of the current research on phage therapy is limited to the laboratory stage—only a few commercially available products (Altamirano and Barr 2019; Kortright et al. 2019). Ideally, phages for therapeutic and prophylactic applications should be able to inactivate pathogenic bacteria effectively without affecting beneficial microbiota, preferably with a low resistance mutation rate and no risk of horizontal transfer of virulence genes, as well as be easy for mass production and storage, which is what we strive for.

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Declarations

Conflict of interest The authors declare no competing interests.

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