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Phage Therapy an alternate disease control in Aquaculture: A review on recent advancements

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Abstract : *Nearly one-third of the world's seafood supplies come from aquaculture industry, representing the fastest growing agricultural sector. Sustainable aquaculture production is crucial to meet the future demands for seafood globally. However, one of the biggest threats it faces is infectious bacterial disease, which effect livelihoods of communities causing heavy financial and production loses and a subsequent decrease in food availability. Whilst fish vaccinology has shown remarkable developments in recent years, and major improvements have been made in good management practices, the emergence of antimicrobial resistant bacteria has become a global problem. With an increasing trend of multiple drug resistance (MDR), chemical residues, and tightening of regulations surrounding the use of chemotherapeutics, bacteriophage may provide a natural, sustainable solution to successfully address this need. Phage therapy may represent a viable alternative to antibiotics to inactivate bacteria, the main pathogenic agents in the aquaculture industry. Virulent phages are natural, sustainable antimicrobials that are nontoxic and, when correctly selected and prepared, do not pose any risk to plant, animal or the environment. Its use, however, requires the awareness of novel kinetics phenomena not applied to conventional drug treatments. This current work is a detailed review of the pros and cons of phage therapy.*

Keywords: *Aquaculture, infectious bacterial disease, multiple drug resistance, bacteriophage, phage therapy.*

I. Introduction

Aquaculture is currently one of the fastest growing food producing industries in the world with an average growth rate of 6.2% (2000-2012) [1]. In 2000, farmed food fish contributed 25.7% to global total fish production increasing to 42.2% in 2012 with a worth in excess of US$ 144 billion [1]. The aquaculture and fisheries sectors also provide significant nutritional requirements to people in developed and developing counties and a source of income and livelihood to approximately 58.3 million people, equivalent to 10-12% of the world population [1]. With a dramatic increase in population growth and an ever-increasing demand for seafood, aquaculture is an increasingly important source of sustainable food production [2]. The need for sustainable aquaculture has lead to an increase in research and development across a range of areas such as nutrition; environmental impacts, good management procedures and disease control, and consequently contributed to great improvements in these areas.

However, the greatest threats to sustainable aquaculture are biological (infectious disease) and chemical agents (agro-chemicals, chemotherapeutants, contaminants, and organic pollutants) [3]. Aquaculture industries frequently suffer heavy financial losses that threaten their growth and sustainability, mainly due to uncontrolled microbial diseases [4, 5, 6]. Several factors may contribute to disease outbreaks, such as unfavorable environmental conditions, overfeeding, high water temperature, fast bacterial growth, infrequent water renewal, and improper removal of wounded and dead fish from the farming area.

Disease outbreaks (parasitic, viral and bacterial) in the past have resulted in losses amounting to billions of dollars to the aquaculture industry [7]. Advances in the understanding of many of these pathogenic organisms cause disease and implementation of 'good management practices' within the industry have helped in the control of many pathogens. Research and development of vaccines has significantly aided in the control of many viral and bacterial pathogens and this has been extensively reviewed [8]. Vaccines developed from inactivated bacterial pathogens have been greatly successful in the control of bacterial disease caused by *Vibrio spp*., *Aeromonas spp*., *Yersinia spp*., *Edwardsiella spp*., and *Flavobacterium spp*. [9]. Yet vaccines to control viral disease are vastly fewer and no vaccines have been developed for parasites [9]. To date there have been no commercial vaccines developed for invertebrates, however a review on this subject has recently been published with some promising results [10].

Despite advancements in good management practices and vaccine production, bacterial infections still pose major problem in both hatcheries and grow-out, often resulting in mass mortalities (70-90%). These mortalities are typically associated with pathogenic *Vibrio spp.*, [11, 12, 13, 14] *Aeromonas spp*., [15, 16] *Pseudomonas spp*., [17, 16] and *Streptococcus spp.* [18, 19, 20] all of which have global significance and an increasing number of which are multidrug resistant (MDR). Out of which Vibriosis is the primary disease of marine and estuarine fish in both natural and commercial production systems throughout the world, but it may

also occur in freshwater fish [21, 22, 23, 24, 25, 26]. This bacterial infection causes significant mortality in fish, reaching up to 100% mortality in infected facilities, and is responsible for most of the current disease outbreaks in fish farming plants. Vibriosis is caused by species from the genera Vibrio (i.e., *V. anguillarum*, *V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus* and *V. salmonicida*) and Photobacteriosis is caused by Photobacterium (i.e., *P. damselae* subsp. *damselae*, formerly *Vibrio damselae*) [27, 28]. Other bacteria as *A. salmonicida*, causative agent of furunculosis, Rickettsia-like bacteria, *Cytophaga marina*, *Flavobacterium psychrophilum* and *Pseudomonas plecoglossicida* are also important groups of fish pathogens, affecting a variety of fish species from diverse geographical aquatic environments [29, 4].

A variety of antimicrobial and chemical treatments have been used to control and treat bacterial disease in human, animals and production systems. However, the WHO report on global surveillance of antimicrobial resistance states, "Existing antimicrobials are losing their effect. At the same time there is a decline in the development of new antimicrobials. Similarly, there is insufficient new research into diagnostics to detect resistant microorganisms and vaccines for preventing and controlling infections. If this trend continues, the arsenals of tools to combat resistant microorganisms will soon be depleted" [30]. To alleviate the incidences of bacterial diseases in aquaculture, different anti-microbial chemicals i.e. $KMnO_4$, $CuSO_4$, H_2O_2, Formalin, Benzolkonium chloride, stains (Crystal violet, Methylene blue and Malachite green), lime, common salt and finally antibiotics (Ofloxacin, Tetracycline, Erythromycin, Neomycin etc.) have been used. The regular use of artificial feed supplemented with antibiotics in an effort to prevent the spread of diseases and to control infections in aquaculture system, have resulted in the development of antibiotic resistance in pathogenic bacteria and posed serious problems in the treatment of infectious disease. In fact, in the marine environment, most (90%) bacterial strains are resistant to more than one antibiotic and 20% are resistant to at least five antibiotics [31, 32]. Moreover, antibiotics not only destroy the targeted bacterium but also destroy the general micro flora in intestine of fish and also disturb the ecological balance of water body. Also chemicals are toxic to fish and aquatic ecosystem and some of them (like Malachite green) gets accumulated in different organs of fish. Thus it seems that chemicals are toxic to fish and the young ones cannot tolerate their high concentration.

The emerging crisis of resistance to antibiotics has led to sporadic application of probiotics (which are beneficial microorganisms or their products) in aquaculture in order to develop immunocompetance in fish to combat with bacterial diseases and also inhibit the colonization of potential pathogens in the digestive tract through competition exclusion principle. But generally probiotics are low — immunogenic in nature, temperature and salinity sensitive and cumbersome in application.

As far as vaccination is concerned, it has proved to be an excellent method to prevent infectious disease [33, 34, 35, 36] for poultry, animal and humans due to trouble-free application in them. While in aquaculture, although commercial vaccines against vibriosis are available, fishes are under water, large scale cultured crop and it is just impossible to handle large numbers of these small sized and frail organisms and to vaccinate each and every fish. Therefore, vaccination becomes a tedious job for large-scale aquaculture systems. Also many different kinds of infectious diseases occur locally in a variety of fish species [37], thus limiting its application.

In the non availability of appropriate strategy to eradicate bacterial pathogens, alternative strategies must be developed to control fish diseases in aquaculture which should reduce the risk of developing and spreading microbial resistance, and be reasonably inexpensive and more environmentally friendly. In line with this idea, the use of phage therapy in aquaculture seems to be very promising, the most plausible and appropriate candidate to overcome the above problems. Bacteriophages (commonly phages) are bacterial viruses extremely abundant in nature and believed to be important in controlling bacterial populations in natural systems [38], even being multidrug resistant [39, 40, 41]. The use of phages to control infections in aquatic environment, such as fish diseases, seems to be particularly promising [37, 42, 43]. As the host fish organisms live in aqueous media, the therapeutic phages can have continuous and close physiological contact with the pathogens in a natural arrangement.

1.1 Bacteriophages

Bacteriophages (phages) are viruses that parasitize bacteria. Like all viruses, phages are obligate intracellular parasites, which have no intrinsic metabolism and require the metabolic machinery of the host cell to support their reproduction. Phages are the most abundant microorganisms in the ecosystem, with total numbers estimated to be more than 10^{30} [44]. Bacteriophages are ubiquitous, and are found in marine and freshwater, soils, as well as the intestinal tracts of animals, estimated to be on the order of $10^7 - 10^9$ gm^{-1} dry weight of soils and feces [44] and from 10^4 ml^{-1} to in excess of 10^8 ml^{-1} in the aquatic environment. Since their discovery 100 years ago bacteriophages have been investigated extensively and a plethora of literature reviewing these studies exists [45, 46, 47, 48, 49]. The ecological functions of bacteriophages also involves playing important roles in e.g. structuring bacterial diversity and succession in the ocean, promoting biogeochemical element cycling and as key drivers of horizontal gene transfer [50]. Phages play a crucial role in the regulation of nutrient cycling, as sources of diagnostic and genetic tools and as novel therapeutic agents. To

date, phages have been used in a number of areas of biotechnology and medical science including rapid bacterial detection and diagnosis of disease (phage typing), prevention of bacterial disease (phage vaccine), treatment (phage therapy) and biocontrol [51].

1.1.1 Bacteriophage classification

Bacteriophages are classified by the International Committee on Taxonomy of Viruses (ICTV) based on their morphology and types of nucleic acid. More than 5500 phages have been examined by electronic microscopy, ~96% are tailed [52]. These tailed phages, which belong to the order of Caudovirales, can be divided into 3 families (Myoviridae, Siphoviridae, and Podoviridae) (**Fig 1**). The differences among these three families are: a long or short contractile tail (Myoviridae), a long non - contractile tail (Siphoviridae) and a short, non - contractile tail (Podoviridae).

1.1.2 Bacteriophage life cycle

Bacteriophages are highly specific and can only infect bacterial cells that present cell surface receptors matching those of the phage (similar to a lock and key mechanism) [54, 55]. Without the matching receptors, phages are unable to multiply and can quickly be degraded in the environment. Phages can either multiply via the lytic cycle (virulent phage) or lysogenic cycle (temperate phage). While virulent phages kill the cells they infect (lytic cycle) as phage progenies are released from lysis of the bacterial host, temperate phages can establish a persistent infection of the cell without killing it (lysogenic cycle) as the temperate phage DNA is integrated into host chromosomes and replicates along with cell division.

Virulent phages are effective at controlling bacterial populations with no known side effects to human, animal or plant. The method by which virulent phages kill their specific host bacterium is called 'lysis' [55, 56]. Virulent phages attach to receptors on the surface of bacteria (host cell) and inject their nucleic acid into the cell, directing the host to produce numerous progeny. These are then released to the environment by a fatal bursting of the cell, where they can attack new bacteria (**Fig 2**).

This entire process can take as little as 25 minutes [57]. Virulent phages have been intensely investigated for their bactericidal properties and are particularly suitable for applications that require destruction of the host bacterium such as biological control and phage therapy, making them an attractive treatment alternative to antibiotics. This is evidently confirmed in in vitro test, in which lytic phages clears the bacterial lawn in Petri plate and forms clear plaques (**Fig 3**).

On the other hand, phages that replicate without immediately killing of their host bacteria are termed temperate phage. These phages can either multiply via the lytic cycle (cell death) or enter a dormant state in the cell (lysogeny). The nucleic acid of the virus becomes part of the host genome and reproduces genetic material (prophage) in the host cell. The host bacterium continues to replicate without adverse affects to the host until host conditions become unfavorable. At this point an induction event, such as a physiological stressor, can trigger this reproduction to switch to lytic cycle and ultimately the host cell is destroyed, releasing progeny phage. Temperate phages have various applications and are particularly suited to purposes that require the transport or expression of genes such as phage display, phage typing and phage vaccines [51]. Due to the largely non-lytic nature of temperate phage and their ability to exchange genes, these phages are not good candidates for therapeutic applications that require immediate destruction of the host cell, such as in the treatment or control of disease [51].

In general, the replication of phage in the bacterial cell occurs in five steps: adsorption, penetration of genetic material, replication, maturation and lysis. During adsorption, the phage gets attached to the cell in order to infect its genetic material in to the host cell. Penetration involves the actual infection of the genetic material. In replication, the viral genetic material takes over the host metabolic machinery for its own replication. While the phage becomes mature and goes into it's infectious state (maturation) and releases its progeny through lysis. Lysis occurs when the phage particle releases lytic enzyme (Lysin) that causes the cell wall to loosen, leaving it weak enough for the breakthrough of the matured phage. Lysogenic bacteriophages may incorporate into the genome of the bacterium rather than being lytic.

One of the advantages of phage therapy over antibiotics is that they are species specific. Therefore, they can destroy only the harmful bacteria without affecting the regular microflora of the environment. Antibiotics generally target both pathogenic and non-pathogenic microflora. Therefore, phage therapy is safer and there is no need of repeated administration as phages can replicate as long as the host cells are available. On the contrary, antibiotics undergo metabolic destruction and if at stable, they need in numerous molecules to act on bacteria. Additionally, phages are known to play a critical role in the evolution of pathogenic bacterial species, as it is particularly true for *V. cholerae* [58]. For example, a major virulence factor of *V. cholerae*, the cholera toxin (CT), is encoded by ctxAB in the lysogenic phage CTXΦ [59]. Likewise, cryptic prophages also were shown to help bacteria cope with adverse environments, such as cell growth, antibiotic resistance, early biofilm formation, as well as environmental stresses [60, 61].

1.2 Vibriophages

Previous seasonal and spatial studies about vibriophages were carried out mainly based on the *V. parahaemolyticus* hosts, such as vibriophages isolated from the Strait of Georgia (Vancouver, Canada) [62] and Tampa Bay (Florida, USA) [63], as well as *V. cholera* prophages such as K139 [64], and filamentous phage CTXΦ, which has been shown to be linked to the bacterial pathogenicity, CT production [65].

Among the best characterized vibriophages is bacteriophage KVP40. It was originally isolated from polluted seawater off the coast of Japan using *V. parahaemolytcius* as the host [66]. Phage KVP40 is a novel T4 - like virulent vibriophage, with a broad host-range, belonging to the Myoviridae family, and the genome size is around 244 kb. KVP40 is known to cause infection through the universal outer membrane protein K (OmpK) and has previously been shown to infect more than 8 Vibrio species, including *V. anguillarum*, *V. parahaemolyticus*, *V. harveyi*, *V. natriegens*, *V. cholerae*, as well as *Photobacterium leiognathi* [66, 67].

1.3 Bacteriophage Therapy

Phage therapy can be described as the use of bacteriophages to control specific pathogenic or problematic bacteria. In human and animal health sectors, phage therapy has been practiced in regions of Eastern Europe for more than 60 years [68]. Early phage trials often produced unreliable and inconsistent results due to a poor understanding of phage biology and quality control during the preparation of phage therapeutic formulations. Meanwhile during 1930-1940, discovery of antibiotics led towards oblivion of phage therapy in the western countries. Due to the isolation of many Eastern European countries from the advancements in antibiotic production during this time, a number of countries continued to develop and perfect phage treatments [69].

Today phage therapy is a widespread form of treatment in a number of Eastern European countries such as Russia, Poland and Georgia [69]. Due to the high degree of specificity in virulent phage, it can be considered a natural and effective way to target specific pathogenic bacteria, without affecting normal beneficial bacteria and without negatively affecting the environment. Importantly, phages are able to infect bacteria regardless of their susceptibility to antibiotics and are capable of penetrating biofilms [46, 68] described earlier, virulent phages kill their bacterial hosts and liberate large numbers of progeny, which are able to infect neighboring susceptible bacteria and start the cycle again. This replication continues until the phage can no longer find the specific targeted bacterial cells, significantly reducing bacterial biomass. It is for this reason that only virulent phages are used in phage therapies.

The use of bacteriophage preparations has advantages and challenges, the critical points being high bacterial specificity, transference of virulence or toxin genes, appropriate administration of phage preparations and the development of phage resistant bacteria (**TABLE 1**).

With advances in technologies and better understanding of bacteriophage biology, these challenges can be addressed. The use of phage products in the food industry, human medicine, agriculture and aquaculture has gathered momentum recently. A range of products have been approved by the FDA (Food and Drug Administration), US Environmental Protection Agency (EPA) and FSANZ (Food Standards Australia and New Zealand) for the control of *Listeria monocytogenes*, *Salmonella sp.*, pathogenic *E. coli* and *Pseudomonas putida*. This is primarily due to the increase in MDR bacteria, antimicrobial and chemical residues in food and the environment, and the decline in research to develop new antibiotics. An increased understanding of phage biology, a long history as therapeutics and an urgent need, as defined by the above agencies, to find alternatives to overcome antibiotic resistance in traditional medicine have also aided in the acceptance of bacteriophage products in the human food chain. This is evidence of the gathering acceptance of phage as alternative antibacterial treatments.

1.4 Bacteriophage Therapy in Aquaculture

Due to their efficient lysis, lytic phages can potentially be used against bacterial infection, and are much more specific than commonly used antibiotics. Therefore, by using phage therapy, a specific bacteriophage could theoretically be chosen to target a specific pathogen. Because of their host specificity, they would not affect beneficial bacteria (e.g. gut flora), thus reducing the chances of opportunistic infections [71]. The use of phages to prevent infection or to inactivate different fish pathogenic bacteria is well documented [72, 42, 73, 74, 75, 76, 77] Experimental results with marine animal models have demonstrated the therapeutic efficacy of phage therapy against infectious diseases caused by *Pseudomonas aeruginosa*, *Photobacterium damselae* subsp. *piscicida*, *Enterococcus seriolicida*, *Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Pseudomonas plecoglossicida*, and *Lactococcus garvieae*. Some animal models include the yellowtail (*Seriola quinqueradiata*), larval stages of shrimp (*Penaeus monodon*), Ayu (*Plecoglossus altivelis*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), seabass (*Dicentrarchus labrax*), and seabream (*Sparus aurata*) [21, 78, 37, 72, 79, 42, 80, 81]. The therapeutic potential

for the use of phage in the control of bacterial disease in aquaculture has been reported for finfish [37, 42, 79]and prawns [78, 81]with promising results.

These studies have demonstrated the potential of specific phages to significantly control pathogen density and, in some cases, reduce fish mortality. For instance, Silva's study of *V. anguillarum* and vibriophage showed that the larvae mortality in the infected and treated group was similar to normal levels and significantly lower than the infected but not treated group [82]. More recently the successful use of bacteriophage therapy in the control of pathogenic *Aeromonas hydrophila* in a Redclaw crayfish hatchery (Elliott and Valverde, 2013) has been reported in Australia. Moreover, according to Lomelí - Ortega's study, lytic phage A3S and Vpms1 were also effective to reduce larvae mortality caused by V. parahaemolyticus [83]. Similarly, in Vinod's field trail experiments, treatment with bacteriophage improved larval survival and brought about decline in luminescent *V. harveyi* counts in hatchery tanks [81].

In aquaculture, phage therapy can be applied as a preventive approach against bacterial infections during larvae production, before releasing them in the aquaculture tanks, thereby improving the overall production of adult fish and the sustainability of fish farming. During the intensive rearing of marine larvae, various forms of interactions between bacteria and biologic surfaces may occur [84], resulting in the formation of indigenous microbiota that can be beneficial or pathogenic for the animal. In aquaculture, fish larvae are maintained in incubators with hatching eggs and debris, resulting in a 1000-fold increase in bacterial counts of the culture water throughout hatching [85]. Marine fish larvae begin drinking before the yolk sac is consumed and thus bacteria enter the digestive tract before active feeding starts [84]. Older larvae may also ingest bacteria by grazing on suspended particles and egg debris [86, 87, 88].

In larval cultures, phages can be supplied in the feed, using infected bacteria as a vehicle or by direct release into the culture water [4, 72]. The use of bacteria infected with phages as carriers can be seen as a protective method to insure that phage particles are delivered directly to the organ infected without suffering any damage. However, it was demonstrated that this strategy did not enhance the protective effect [72]. When they administrated *Lactococcus garvieae* infected with phages to treat the infection, the curative effect of the phage was not influenced, but the results did not differ from those when phages were directly administrated. The later strategy is inexpensive, flexible, and requires no specific equipment, but the antimicrobial effects are assumed to depend on phage stability in the medium and their ability to arrive at the infected tissues (i.e., intestine) by passive diffusion. Consequently, to develop an effective, safe and controlled phage therapy protocol to be used in larviculture, detailed information is needed on the properties and behavior of the selected phage. The host range of the phage, the phage time of permanence in the water, its latent period, the burst size, lytic potential, its avoidance of lysogenic induction and conversion, and the potential development of host resistance are crucial factors that must be considered.

Therefore, it seems a promising strategy to apply vibriophages to gain control of vibriosis infections in fish used for aquaculture. Selection of the appropriate bacteriophage, the stage of life (eggs, larvae, juveniles, or adult fish) during which phage therapy is applied and the method of phage delivery are key factors in the success of the treatment. The success of phage therapy to control pathogenic bacteria of fish depends on virus survival in aquaculture water and their ability to inactivate a broad range of fish pathogens. The phage burst size (number of phages produce by each host cell) and the latent period (time elapsed from virus entry into the cell until the first progeny are released) are also important factors to consider when phages are selected. Phages with high burst sizes and short latent periods are more effective to inactivate bacteria; however, great burst sizes are associated with a long latent period [89] which makes the selection for phage therapy difficult. However the vast majority of publications focus on isolating and characterizing phage capable of reducing the biomass of bacterial pathogens associated with aquaculture species in vitro. There is an urgent need for studies to be undertaken in vivo to fully prove the advantages of using phage therapy as a control measure for antibiotic resistance organisms in particular. Also successful application of phage therapy in the treatment of vibriosis requires a detailed understanding of phage - host interactions in both planktonic and biofilm forms.

1.5 Bacteriophage resistance mechanisms

Predation pressure from bacteriophages is substantial, mainly because their abundance outnumbers microbial cells by an estimated 10-fold in most natural environments [90]. Accordingly, bacteriophage lytic infection imposes a strong selection for bacterial mutations/tolerance providing reduced phage susceptibilities or resistance against phage infection. Bacteria have evolved a wide range of different resistance/tolerance mechanisms including 1), preventing phage adsorption; 2), cutting phage nucleic acid; 3), abortive infection through altruistic suicide; and 4), QS-mediated receptor down-regulation, which can make the host immune to the viral infection [91]. Similarly, phages can also overcome bacterial resistance by adapting to new receptors, battling restriction-modification systems, evading CRISPR-Cas systems, and escaping abortive-infection mechanisms [92].

1.5.1 Phage receptor modification

Adsorption is a key step in recognition between phage receptor-binding protein and phage receptors on the sensitive host cells [93]. Most of phage receptors are presented on the bacterial cell walls, such as LamB for phage lambda, the prion outer membrane protein F and C (OmpF and OmpC) for phage T2 and T4, and moreover, the flagella protein for phage phi [94, 95, 96, 54].

Additionally, according to Seed's study, *V. cholerae* lipopolysaccharide O1 antigen functions as a major target of phage ICP1 [97]. By using phase variation of O antigen biosynthesis, *V. cholerae* cells can easily generate variable expression of surface components, which is generally thought to help these organisms evade the immune system and phage predation [98]. However, little is known about the requirements of these phage receptors and it is unclear if the polysaccharide was acting as a receptor or if it was facilitating reversible phage binding to a secondary receptor [99, 100].

Mutating phage receptors or producing EPS to block the interaction between phage receptor and phage receptor-binding protein to prevent phage adsorption can be the first step that bacterial cells exhibit in developing resistance/tolerance to avoiding infection [100]. Previous studies in Vibrio hosts, showed that mutations or modifications of the outer membrane protein K (OmpK), led to resistance to vibriophage 31 KVP40 [67]. Similarly, in ompK in-frame deletion also proved that OmpK acts as a phage binding receptor in *V. anguillarum* strain PF430-3 (**Fig 4**).

Furthermore, mutations in outer membrane protein A (OmpA) of E. coli K-12 appear to play a role in inhibiting phage infection [101]. Phage receptors, in addition to the phage attachment, were involved in the bacterial nutrient intakes, which may be responsible for the morphological changes of the colonies with small size, known as small-colony variants (SCVs) and fitness costs, as have been previously demonstrated experimentally, such as reduced abilities to take up specific nutrients [102] or reduced competitive abilities in general [103, 104]. For example, mutation in the LamB phage receptor caused the inability to transport long chain maltodextrin across the outer membrane [105]. Additionally, Park [79] found that bacteriophage-resistant mutants of *P. plecoglossicida* lacked virulence for ayu. In addition, in a successful phage therapy experiment of *E. coli* infection in mice and calves, the resistant mutants isolated from the treatment were the less virulent k-1 type mutants [106].

The role of spatial refuge in stabilizing bacteria-phage interactions has been observed in many ecosystems, especially, micro-colonies and biofilms, as discussed in previous studies [107, 108, 109], and even in the marine environments, such as marine snow and sediments. Because of the low dispersal rates in the heterogeneous environments, by creating ephemeral refuge may directly/indirectly block phage receptor from phage attack by non-mutation-based mechanisms [107, 108, 109, 110]; as also shown in *V. anguillarum* strain PF430-3 protected against phage KVP40 infection by increasing cell aggregation and biofilm formation, allowing coexistence rather than coevolution, finally promoting the stability of phage-host systems by reducing the risk of lytic phage attacks.

1.5.2 Cutting phage nucleic acids

Once the nucleic acid has been injected into the bacterial cell, the restriction-modification (R-M) systems protect the bacterium by cutting invading DNA into pieces [111]. When unmethylated phage DNA enters a cell harboring R-M systems, restriction enzymes, thereby, rapidly degrade the foreign genetic material functioning as a prokaryotic immune system [112]. For instance, in *Bacillus subtilis* Marburg nonB mutated strain, nonsense mutation on ydiB was found to be related to the restriction system targeting sequence of BsuMR, which was identical to XhoI (CTCGAG) [113, 114]. However, according to Krüger's study [115], R-M systems are not always perfect, for instance, phages and plasmids can acquire host modifications to avoid restriction endonuclease, which highlights an evolutionary arms race between bacterial host and phages [92].

1.5.3 CRISPR/Cas bacterial immune system cleaves bacteriophage DNA

Another mechanism recently described is CRISPR (Clustered Interspaced Short Palindromic Repeats) and the CRISPR-associated (cas) genes system, in which a CRISP-cas loci was identified composing of 21-48 bp direct repeats interspaced by non-repetitive spacers (26-72 bp) of similar length [116, 117]. By using this immunity system to acquire at least one new repeat-spacer unit at the 5' end of the repeat-spacer region of a CRISPR locus that targets foreign nucleic acids, bacteria can efficiently protect themselves from including phage DNA and plasmids [117, 118].

1.5.4 Abortive infection (ABi) system

The study of the ABi system began 50 years ago, and even now, the mechanisms underlying the infection are still not completely understood [91]. The bacterial cell can increase the chances of its own

population survival by using the abortive infection system, where phage infection leads to the death of infected bacterial cells. The system is characterized by a normal infection start (i.e., the phage adsorbs and injects its DNA into the host cell), followed by an interruption of the replication, transcription or translation, leading to the release of little or no new phage progenies [91, 119]. Recent studies in *B. subtilis* showed that the Marburg strain remained resistant to phage SP10 due to a NonA-mediated aborted infection system, acted as a second layer of protection against phage SP10 infection, specifically, the overexpression of nonA gene terminated cell growth with reduced efficiency of colony formation and respiration activity [114].

1.5.5 Regulation of phage-host interactions by extracellular signaling molecules

A recent study has demonstrated a new mechanism of phage resistance in which, bacteria can coordinate their receptor gene expression upon the environmental QS signal, to avoid the risk of infection at high cell densities [120]. Since phages require a host to replicate, it follows that the predation pressure is relatively higher at a high cell density status compared to sparsely populated environments. Hence, if bacterial hosts could regulate their anti-phage mechanisms based on cell densities, they could easily reduce their susceptibilities to infection during high cell densities, while avoiding the metabolic burden of maintaining elevated anti-phage defenses during growth at low cell densities. For instance, *E. coli* possesses the ability to use AHLs to reduce its susceptibility to at least two phages, phage λ and phage χ [120]. In the phage λ case, phage receptor LamB was shown 40% down-regulation compared to the untreated controls without AHLs [120].

1.6 Implications of phage protection mechanisms: Phage-host coexistence and co-evolution

As complexity arises within populations of bacterial resistant mutants, it may help mutants survive better or have more offspring. If so, this complexity will be favored by natural selection and spread through the bacterial population. However, most mutations with phenotypic effects are harmful, such as reducing the competitiveness of the mutant strains, as most phage receptors involve nutrient intake or pathogenicity, a receptor-deficient mutant will have a slower growth rate or reduced virulence [79, 106, 121, 122, 123]. That is, phage resistant mutants with those traits will tend to be wiped out before reproducing, taking the deleterious traits out of bacterial communities. Therefore, the non-mutation defense mechanisms among these phage hosts may suggest phage-host co-existence interactions, rather than the classical phage-host co-evolutionary arms race, known as Red-Queen theory.

The Red-Queen hypothesis was first formed by Van Valen in order to explain the “law of extinction” [124]. According to the previous phage-host interaction studies, virulent phages managing to coexist with their bacterial host leads to continuous variations and selections towards the adaptation of bacterial hosts by evolving resistance to current phages and phage evolve to counter resistance. It has been reported that the arms race has a huge impact on global nutrient cycling, on climate, on the evolution of the biosphere, and on the evolution of virulence pathogens [125]. However, this theory was also criticized because the evolution rates between phage and host are not symmetrical. Recent studies showed that, in soil, phages seem ahead of the bacterial hosts in the evolutionary arms race [126]. In the natural environment, at each stage of the arms race, one could become extinct, and without one, phage and bacteria coexistence, therefore, does not even exist. Thus, it is important to understand both evolutionary and non-evolutionary mechanisms (such as gene regulation) that can regulate phage-host interactions in the future.

Uncovering anti-phage defense mechanisms is essential for understanding phage-host dynamics and for application of phages in disease control, as they reflect the remarkable diverse interactions between bacterial hosts and viruses and play a key role as agents shaping microbial community structure. As the predation pressure from phages is a key determinant of the size and composition of bacterial populations, understanding of the potential factors that govern phage-bacterial interactions will be important in any context where the goal is to control the growth of a microbial population including, for example, the treatment of bacterial infections, development of effective probiotics, production of cultured dairy products, or manipulation of the human microbiome to prevent or treat life-style diseases.

II. Figures and Tables

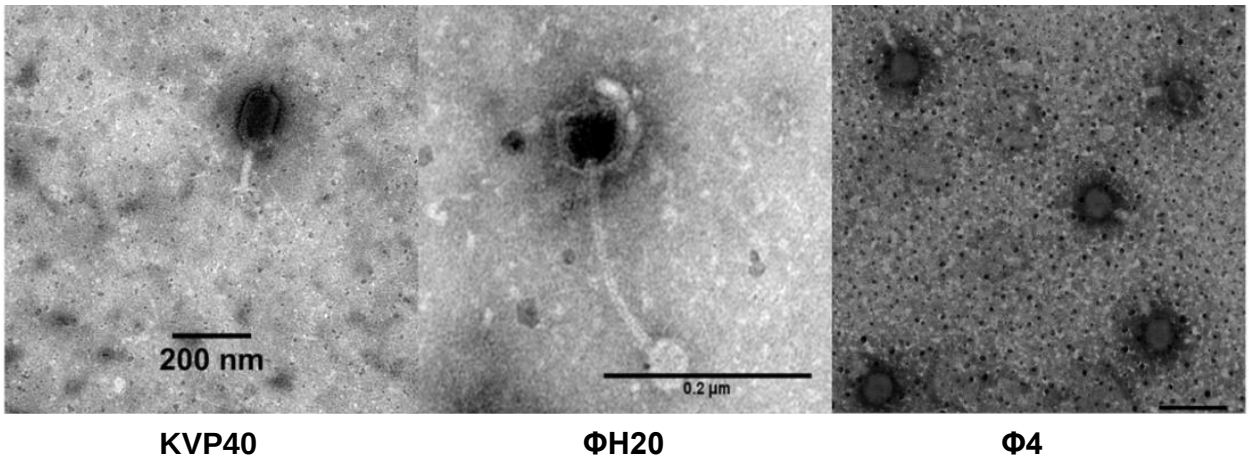

Figure 1: Transmission electron microscopy (TEM) images of selected vibriophages from three different families, scale bar indicates 200 nm. Phage KVP40, ΦH20, and Φ4-7 belong to Myoviridae, Siphoviridae, and Podoviridae, respectively [53].

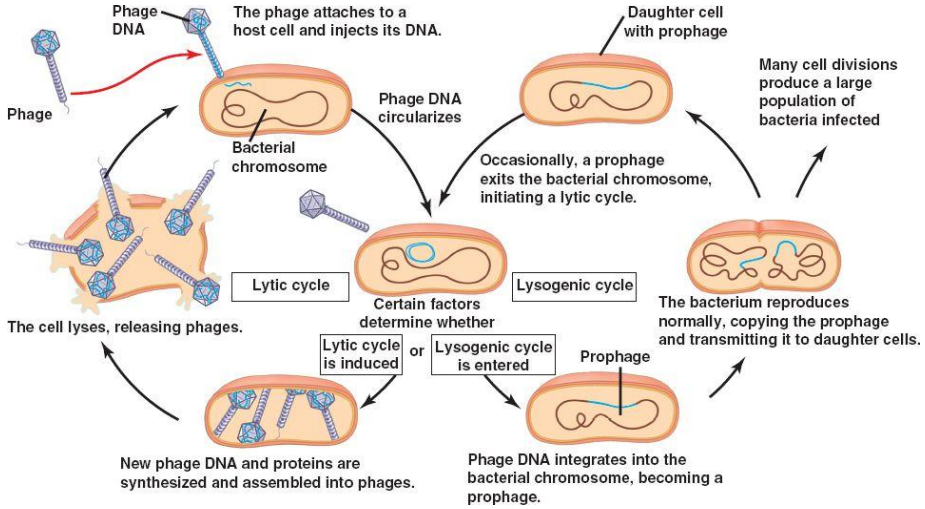

Figure 2: Schematic picture of phage lytic and lysogenic life cycles. Phage adsorption is the first key step in phage proliferation; in order to efficiently bind to the bacterial receptor, phage receptor- binding protein (RBP) is required to specifically interact with bacterial cell surface receptor to enable intracellular DNA injection. In the lytic life cycle, phage DNA replicated separately from host genome, resulting in the destruction of the infected cell and its membrane. In the lysogenic life cycle, phage DNA is integrated into the host genome and can be transferred to daughter cells, until the lytic cycle is induced. However, a portion of these induced cells could enter an abortive lytic cycle by losing prophage and becoming non-lysogens.

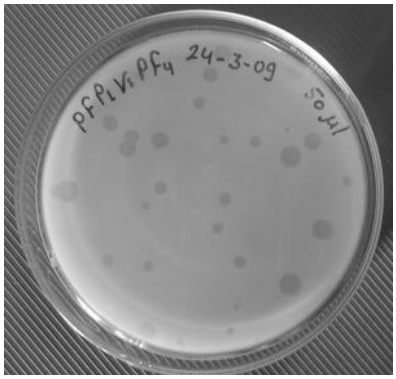

Figure 3: In vitro lytic activity of phage, showing with plaque formation in the bacterial lawn of *Pseudomonas fluorescens.*

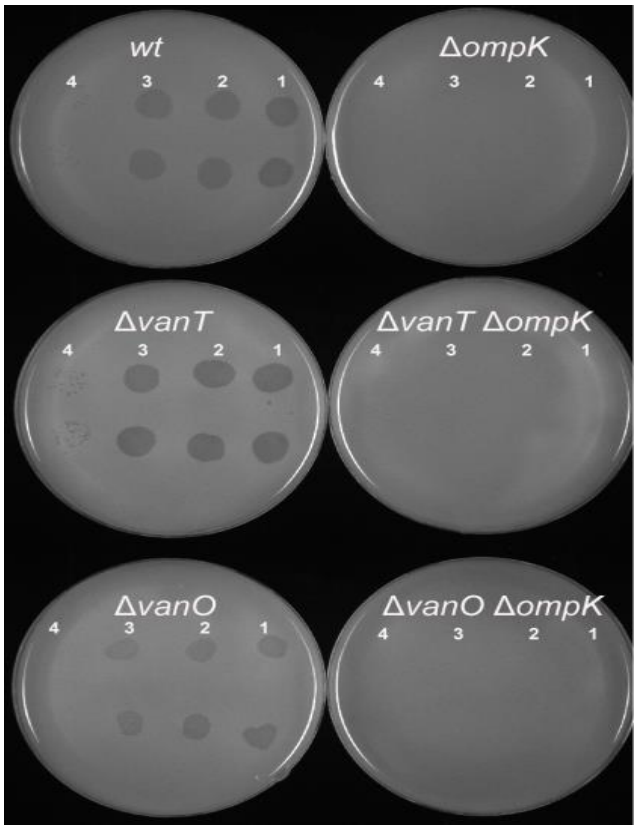

Figure 4: Spot assay for testing host specificity of phage KVP40 against wild-type, QS mutants (ΔvanT and ΔvanO) and ompK mutants (ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK) as well. Five µl serial 100-fold dilutions of phage KVP40 lysate (1, 10^9; 2, 10^7; 3,10^5; 4; 2, 10^3 PFU ml-1) were shown by spot titration onto top agar lawns of the indicated strains [53].

TABLE 1: Comparison of advantages and disadvantages of bacteriophage in phage therapy. [70]

Advantages	Impact	Disadvantages	Impact
High specificity.	Minimal disruption of normal beneficial microflora. Do not contribute to resistance in the beneficial microflora such as seen with antibiotics.	High specificity.	The disease causing bacterium must be positively identified before phage therapy can be successfully initiated. However phage can be successfully used in combination with other antimicrobials.
Virulent phages are bactericidal agents.	The target bacteria are killed and are unable to develop resistance to phage or other antimicrobials.	Temperate phages and can transfer genes between bacteria.	Phages have two life cycles, virulent (lytic) and temperate (lysogenic). For phage therapies only obligately virulent phages are used that do not possess toxin or antibiotic resistance genes or virulent factors. They kill the host bacteria.
Low inherent toxicity and low environmental impact.	Phages are protein-encapsulated nucleic acids thus are inherently nontoxic to plant, animal or environment.	May interact with the immune response.	There is little evidence of detrimental immune responses from phage themselves. However it is crucial that protocols are developed resulting in highly purified preparations to avoid contamination with bacterial components.
Administration of phages can be oral, aerosols, immersion, injection, in feed or topically.	Phage preparations can be made into tablets, liquid or powder and can be viable for many years in some preparations.	Diseased animals may not feed. Injections of large Numbers of animals (e.g. fish) may be problematic.	Phage released into the water from uneaten treated feed can also act as an immersion treatment. Advancements in vaccine delivery technologies offer relevant methods for vaccination of large numbers of animals.
Selecting new phages is a relatively rapid and cost effective process.	Evolutionary arguments support the idea that virulent phages can be selected against every antibiotic-resistant or phage resistant bacterium by the ever-ongoing process of natural selection.	Strictly virulent phages only must be selected and purified.	Advances in molecular biology and phage biology have reduced the time and cost to select for virulent phage.
Replicate at the site of infection, 'auto dosing'.	The exponential growth of phages at the site of infection may require less frequent phage administration in order to achieve the optimal		

	therapeutic effect.		
Bacteria that have become resistant to one phage continue to be susceptible to other phages.	Selecting new phages is a relatively rapid and cost effective process. The development of phage cocktails significantly reduces the appearance of phage resistant bacteria.		
Phages are active against antibiotic resistant bacteria.	Phages do not contribute to antibiotic resistance and possess different receptors to antibiotics.		

III. Conclusion

There is no doubt that sustainable aquaculture production is crucial to the future demands for seafood globally. However, one of the biggest threats to the aquaculture industry is infectious disease. Fish infection by pathogenic bacteria is a progressive problem for the development of aquaculture worldwide. Several chemotherapies, such as the utilization of antibiotics, have contributed to a rapid and effective way to treat or prevent bacterial infections. However, the increasing problem of antibiotic resistance in common pathogenic bacteria and the concern about spreading antibiotics in the environment, bring the need of finding new methods to control fish pathogenic bacteria. The consequences associated with these infections are widespread and have a significant impact on the economy, livelihood, health and welfare (human and animal) of entire communities and countries. The World Health Organization (2014) states, "Increasingly, governments around the world are beginning to pay attention to a problem so serious that it threatens the achievements of modern medicine. A post-antibiotic era—in which common infections and minor injuries can kill – far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century."

The success of phage therapy in aquaculture depends mainly on the phages selected to inactivate the fish pathogenic bacteria [127]. The selected phages must remain viable in marine waters, infecting pathogenic bacteria but not altering significantly the non-pathogenic bacteria that have an important ecological role. The results of this study showed that both phages of fish pathogenic bacteria can survive in the aquaculture water at 25 °C temperature and that after 10 hour incubation they do not alter significantly the structure of the overall bacterial community. Unlike antibiotics, phages are self-replicating as well as self-limiting and, consequently, they replicate exponentially as bacteria replicate and decline when bacterial numbers decrease [40, 128, 129]. Phage therapy is a potentially viable alternative to antibiotics, inactivating even bacteria resistant to seven different antibiotics. So it can concluded that: (1) the seasonal variation of the bacterial communities imply the need for a careful monitoring of water throughout the year in order to select suitable phages to inactivate fish pathogenic bacteria; and (2) that the spring season seems to be the critical time period when phage therapy should be applied. Consequently, the impact of the phages on the structure of the bacterial community can also vary seasonally. However, the study of the impact of the phages on the bacterial community was conducted during the warmer season which is the critical time period when phage therapy should be applied [130]. Further studies should be performed to select the most effective phage strain or effective combination of phage strains for therapeutic applications. It will be also important to characterize the capacity of phages to reduce their host fitness. Moreover, it should be emphasized that before using bacteriophages for therapy, it would be important to test whether they carry any virulence genes, that is, if there is any potential for lysogenic conversion.

Therefore, phage therapy represents a potentially viable alternative to antibiotics and to other antimicrobial compounds to inactivate indigenous and non-indigenous pathogenic bacteria in fish farming plants. Although early studies were often inconclusive, modern technology, methods and a greater understanding of phage and pathogen biology have provided an excellent basis for development of improved preparations, overcoming many of the perceived disadvantages of phage therapy. Virulent phages are natural, sustainable antimicrobials that are nontoxic and, when correctly selected and prepared, do not pose any risk to plant, animal or the environment. Future research and development of bacteriophage preparations as therapies will contribute to environmental, social and economical sustainability in global aquaculture and should be fully embraced and supported by government, researchers and farmers.

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