Chapter

Phage Therapy for Control of Bacterial Diseases

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Abstract

Phage therapy is one of the most important control strategies envisaged for the management of bacterial diseases in the aquatic environment. There are no other effective alternative approaches for the natural control of bacterial diseases, while phage therapy remains the best method which has not yet been exploited. The occurrence, infectivity, lytic activities, therapeutic potentials, and efficacy of the bacteriophages of *Bacillus* spp./*Vibrio* spp. for control of pathogenic bacteria diseases such as *Vibrio vulnificus, V. damsela,* and *V. furnissii* in the cultures of crustaceans are presented. An ideal method for long-term storage and recovery of the lytic bacteriophages, agar bioassay method and one-step growth experiments, *in vivo* and *in vitro* experiments, and validation of the usefulness of phage therapy are described. The review highlights the occurrences of plagues of lytic phages of *Vibrio* sp. and *Bacillus* spp. and their control effects of vibriosis both *in vivo* and *in vitro* in the crustaceans, thus establishing the application and efficacy of the phages of *Vibrio*/*Bacillus* against the pathogenic *Vibrio* spp. Development of specific phage therapy or a cocktail of phages to a wide variety of systems is considered to represent an interesting emerging alternative to antibiotic therapy and vaccination.

Keywords: phage therapy, bacterial diseases, vibriosis, probiotics, bacteriophages, antimicrobials, antibiotic resistance, crustaceans, shrimp, lobster, crab, *Artemia*

1. Introduction

1.1 Global crustacean production and losses due to diseases

Global fish production was 171 million tons estimated at USD 362 billion in 2016, while aquaculture production was 80.37 million tons estimated at USD 232 billion [1–3] consisting of 54.1 million tons of finfish production, 17.1 million tons of molluscs, 7.9 million tons of crustaceans, and 938,500 tons of other aquatic animals such as turtles, sea cucumbers, sea urchins, frogs, and edible jellyfish [3]. Freshwater finfish represents half of the global aquaculture production (54%), molluscs being the second more produced aquaculture item in the world (24%) [2]. Crustaceans come next in production relevance, represented mostly by penaeid shrimps and grapsid crabs [2, 3]. Aquaculture is the world's fastest growing segment with a global increase of 5.7% per annum in shrimp production resulting in an increase of 18% by 2020, and the estimated world production of farmed shrimp is 3.5 million metric tons though the diseases, international market prices, and production costs

are the main challenges and constrains to the growth and productivity of the shrimp industry on a global level [4]. However, disease outbreaks have caused serious economic losses in several countries, and the estimated global losses due to shrimp diseases are around US$ 6 billion per annum [5, 6]. Such concerns confirm that the bacterial diseases are the most important contracting factors for development of the global aquaculture industry [7].

1.2 Bacterial diseases in crustaceans

Bacteria in the aquatic environment and the bacterial diseases, viz. vibriosis, shell diseases (chitinolytic bacteria), and gaffkemia of lobsters, are ubiquitous and are significant for the survival of crustaceans in confined habitats [8–22]. Diseases,

Diseases	Hosts	Causative bacterial species
Vibriosis	*Penaeus monodon*, *P. merguiensis*, and *P. indicus* (eggs, larvae, postlarvae, juveniles, and adults); *Litopenaeus vannamei*, *Macrobrachium*, lobster *Homarus americanus*, crab *Portunus trituberculatus*	*Vibrio harveyi*, *V. splendidus*; *Vibrio harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. furnissii*, *V. mimicus*, *V. damsela* [7, 13–22]
Bacterial fouling of surfaces with filamentous bacterial disease	*Penaeus monodon*, *P. merguiensis*, *P. indicus*	*Leucothrix* sp., *Thiothrix* sp., *Flexibacter* sp., *Cytophaga* sp., *Flavobacterium* sp. [7]
Shell disease, brown/black spot, black gill, black rot/ erosion, blisters, necrosis of appendages	Crabs and shrimp, white and brown shrimp *Penaeus monodon*, *P. merguiensis*, *P. indicus*	Chitonoclastic bacteria *Vibrio* spp. infections *Vibrio*, *Aeromonas*, *Pseudomonas*, *Flavobacterium* [7, 8, 11]
Chitinolytic bacterial disease, shell disease, box burnt disease, bacterial shell disease	*Cancer* spp., *Callinectes sapidus*, other crabs; lobsters, shrimps, and crayfish	Chitinolytic or chitinoclastic bacteria (Gram-negative), viz. *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp. [7, 9–11, 13, 17, 20–22]
Milky hemolymph disease (milky hemolymph syndrome [MHS])	Spiny lobster *Panulirus* spp., *Panulirus ornatus*, *P. homarus*, and *P. stimpsoni*; *Litopenaeus vannamei* (*Penaeus monodon, Carcinus maenas*)	Rickettsia-like bacterium, a-*Proteobacteria*, *Streptococcus* sp., [7, 10–12]
Gaffkemia, septicemia	Lobsters *Homarus americanus*	*Gaffkya homari* [7, 9–11]
Bacteremias	Bacterial diseases of crabs	*Vibrio*, *Aeromonas*, *Rhodobacteriales*-like organism, *Vibrio cholerae*, *Vibrio vulnificus*, chitinoclastic bacteria, *Rickettsia* intracellular organisms, chlamydia-like organism, *Spiroplasma*, chitinoclastic bacteria, *Rickettsia* intracellular organisms, chlamydia-like organism, and *Spiroplasma* [7]
Fungal diseases	*Penaeus monodon*, [larval (nauplii, zoea, and mysis) Indian tiger prawn] *Macrobrachium rosenbergii*	*Lagenidium callinectes* [16, 17]

Table 1.
Bacterial diseases, causative organisms, and their crustacean hosts.

causative organisms, and their crustacean hosts are listed in **Table 1**. Gaffkemia of lobsters is caused by *Aerococcus viridans* var. *homari* and is the root cause of mass mortalities of lobsters, while crabs, viz. *Cancer borealis* and *C. irroratus*, serve as reservoir hosts of *Aerococcus viridans* [8–13]. *Vibriosis* causes mass mortalities in several crustaceans such as penaeid shrimp *Penaeus monodon* and *P. japonicus*, fresh water prawn *Macrobrachium*, lobster *Homarus americanus*, blue crabs *Callinectes sapidus*, rock crabs *Cancer irroratus*, and shore crab *Carcinus maenas*. Shell diseases are caused by chitinolytic bacteria which were encountered in English prawn *Palaemon serratus*; American lobsters *Homarus americanus*; penaeid shrimp; king crabs, *Paralithodes camtschaticus* and *Paralithodes platypus*; and tanner crabs *Chionoecetes tanneri*, and these crustaceans are affected by rust diseases which are caused by chitin-destroying bacteria [8–22]. Significant mortalities of larval, post-larval, and adult crustaceans, viz. shrimp *Penaeus monodon*, *Litopenaeus vannamei*, and *Macrobrachium*, lobster *Homarus americanus*, and crab *Portunus trituberculatus*, are caused by common pathogens such as *Vibrio harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. furnissii*, *V. mimicus*, *V. damsela*, *Pseudomonas*, and *Aeromonas* [9–22]. Infectious diseases caused by *Vibrio* species represent the greatest challenges that cause vibriosis with considerable economic losses, and that is the most overwhelming problem in aquaculture, shrimp, and crustaceans [7–101].

2. Phage therapy

Phage therapy is a prospective ideal therapy for vibriosis in aquaculture of crustaceans. Bacteriophages are defined as bacterial viruses that can infect cells, multiply in, cytolyse, and destroy susceptible bacteria. Bacteriophages are viruses of bacteria which have a natural ability to target, infect, and destroy their host cells of a particular bacterial species or groups or even unrelated bacteria, and thus they play a major role in controlling their target bacterial population density in nature [23–60]. They are both omnipresent and copious in the aquaculture environment, especially in seawater, in which the total numbers of viruses normally surpass the bacterial cell concentration by a factor of 10 [25]. "Most phages reproduce upon entering a cell and in that process kill their host. They encode two families of proteins, holins, and lysins, which allow the phage progeny to burst through the bacterial cell wall and go off in search of new hosts. But the cell is already dead by the time that happens in terms of lethality; the lysis is just a matter of burning down the house for good measure." An estimated 10^8 strains of phage with approximately 10^{31}–10^{32} phages are known to occur in the biosphere at any given time [23]. Bacteriophages are used for the isolation and identification of specific bacteria to help in the diagnosis of the bacterial diseases, to kill antibiotic-resistant, virulent bacteria through a natural phenomenon called lysogeny, whereby one of the phage-infected bacteria in a colony kills another uninfected bacterium through phage missiles or antibacterial peptides [24, 25, 36–39, 41].

2.1 Phage therapy, an alternative to antibiotics

Due to their specific antibacterial activities and significance of the phage therapy as an alternative to antibiotics, bacteriophage therapy is re-emerging, and consequently this has become a potentially novel and useful concept to kill even intracellular pathogenic bacteria and warrant future development. Bacteriophage therapy has been extended from medical applications into the fields of agriculture, aquaculture, and the food industry [28–30, 39]. Bacteriophages specific for *Vibrio* spp. have been described [36–41]. Bacteriophages are known to infect >140 bacterial

genera, and they are the most valuable and ubiquitous (10^{31}) phage organisms in the world [26–35, 40–45]. Earliest description of phages and their antibacterial activity has been independently demonstrated [62]. D'Herelle [33] published a comprehensive account of phages, and hence the International Bacteriophage Institute was established in Tbilisi, Georgia, in 1923, now called as "the George Eliava Institute of Bacteriophages, Microbiology and Virology" [32, 34], which is still involved in researching phage therapy applications and supplies phage for the treatment of various bacterial infections. D'Herelle's first phage therapy experiments against bacterial dysentery were exceptionally successful and were very promising in the removal of the infectious organisms [32, 34]. However some of the results of the early phage therapy experiments of infectious host organisms were known to be contradictory with reports of both success and failure. Whenever failures occurred in phage therapy, they were attributed to a range of factors. They include unsatisfactory understanding of phage biology, inadequate experimental technical knowledge, poor quality of phage preparations, and a lack of understanding of the causes of illness being treated. The discovery of such specific bacteriophages were at first considered to become powerful beneficial therapeutic agents against pathogenic microorganisms but could not be put into practice because of the dawn of antibiotic era experiencing overuse/misuse of antibacterial medication that resulted in the development of untreatable antibiotic resistance [32, 36]. Commercialization of antibiotics in the Western countries in the 1940s had led to a simultaneous decline in the use of phages as human therapeutics, while in some of the Eastern European countries, exploitation of phage therapy was continued either alone or in combination with antibiotics [19, 34, 61–73]. The experimental phage therapy could be an alternative to antibiotics and replace them when they fail for the treatment of chronic infections, and such a successful eradication of drug-resistant bacteria was documented [19, 42, 61–73]. Moreover, the significantly decline costs of phage therapy constitute an important additional battle for its wider application in the current era of a worldwide circumstance in antibiotic resistance. The efficacy of phage therapy is well recognized and demonstrated in a few cases [32, 64–66]. Even a single dose of phage was reported to be much more effective than multiple doses of antibiotics such as ampicillin, tetracycline, and chloramphenicol [66]. The use of phage therapy to control fish pathogens has also been reported [23, 24, 42, 43]. Phage lysins have been used as potential therapeutics for treatment of bacterial infections [44]. Furthermore, the US Food and Drug Administration has approved commercial phage preparations to prevent bacterial contaminations. Such developments have prompted to explore the possibilities of using bacteriophages to control bacterial infections in crustacean aquaculture [36–41]. Further cautious phage collection and perfect experimental conditions for phage propagation and purification, route and timing of phage administration, and environmental monitoring of phage for use are needed. Such a focus may help in further development of probiotics, phage therapy applicable to a wide variety of systems, which is considered to signify an emerging alternative to antibiotic therapy and vaccination.

2.2 Probiotics in crustacean cultures

Probiotics are defined as applications of whole or components of microorganisms or "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" [74–93]. A probiotic is a live microbial feed supplement which benefits the host

animal by improving its intestinal microbial balance [82]. Lactic acid bacterium *Bacillus* S11 was tested as probiotics and has been proven as antagonists of shrimp pathogens [75]. *Bacillus* spores were used as biocontrol agents to reduce *Vibrio* species in shrimp culture facilities [83]. The inhibitory activity of *Bacillus subtilis* BT23, isolated from shrimp culture ponds, is against pathogenic *Vibrio harveyi* under *in vitro* and *in vivo* conditions [39, 73]. The concept of probiotics is exploited to "augment" naturally occurring pathogenic bacterial population to increase the growth rate and control diseases of organisms in aquaculture such as fish/shellfish [84, 85]. Several microorganisms have been evaluated as probiotics which have been shown to be successful in the larval stages of the aquatic organisms in preventing the diseases, improving digestion and growth [82, 84, 87]. Some of the proposed mechanisms that provide protection against pathogens involve the production of inhibitory compounds, competition for essential nutrients and adhesion sites, enhancement of disease resistance, and modulation of host immune responses [82, 87]. A probiotic containing a combination of several different bacteria has been shown to be more efficient at controlling bacterial pathogens [82, 90, 91]. Probiotics are known to act and inhibit the growth and proliferation of bacterial pathogens both *in vivo* and *in vitro* by generating antibacterial compounds such as bacteriocin, siderophores, lysozymes, proteases, hydrogen peroxide, antibiotics, and organic acids [91]. In aquaculture, some microorganisms are more beneficial to host organisms in reducing the incidence of diseases though the factors and mechanisms which mediate the benefits to the host are poorly understood. Future studies should be focused on evaluating the mechanisms by which the probiotics interact with the host and pathogens and their biology.

2.3 Proprobiotics

Proprobiotic is defined as a synbiotic comprising pre- and probiotics involving a combination of strains of nonpathogenic bacteria and yeast so as to provide supplementary nutrients and to protect against invading pathogens. Commercially available proprobiotics are known to competitively exclude the potential pathogen through the use of proprobiotics in aquaculture. Effects of Ergosan and Vibromax are used to prevent vibriosis in *Litopenaeus vannamei* [42, 43]. Probiotics (e.g., Arda-Tek, Australia, viz. DMS1000, S-1100, DMS-2001, DMS-2002, DMS-2004, etc., Wunapuo-15 of Team Aqua Corporation) are used to reduce the number of Gram-negative pathogenic bacteria including *Vibrio* in the water and to maintain stable water quality, to stabilize plankton, to reduce rate of sludge buildup on the pond bottom, and to digest microbial slime [73, 82, 84–91]. To assess the actual impact of these probiotic products in the field, it is fundamental to determine the efficacy of the probiotics and the continuance of their application. The potency of the probiotic products against selected pathogenic bacteria (and viruses) also needs to be critically evaluated. The application of some beneficial bacteria has been an option in aquaculture to achieve a number of benefits, viz. to reduce mortality in shrimp, to enhance production and increase harvest yield and eliminate antibiotic use, and to enhance immunity and disease resistance in black tiger shrimp *Penaeus monodon* by a probiont bacterium (*Bacillus* S11) [84–91]. Immunity enhancement occurs in black tiger shrimp *Penaeus monodon* by a probiont bacterium (*Bacillus* S11), and the immune stimulator capacity of probiotics may be affected by factors such as source, type, dose, and duration of supplementation [80, 85]. The use of probiotic mixtures consisting of *Bacillus tequilensis* and *B. endophyticus* along with commercial probiotics was shown to be beneficial in altering the bacterial community of larval shrimp *Litopenaeus vannamei* when the hosts were challenged with *Vibrio parahaemolyticus*. Similarly application of *Bacillus* sp. promoted cellular and

humoral immune resistances and provided disease protection in tiger shrimp *P. monodon*. The effects of probiotics such as *Bacillus cereus*, *Paenibacillus polymyxa*, and *Pseudomonas* sp. PS-102 as biocontrol agents against pathogens of various *Vibrio* species in shrimp were evaluated. Growth promoter probiotics have been used in aquaculture to enhance the growth of cultivated species, whereas their side effects if any on the host need to be investigated. The probiotics was shown to increase the survival and growth of white shrimp (*Litopenaeus vannamei*), and the production increased by 35%, whereas with the use of antimicrobials, it decreased by 94% and thus demonstrated the significance of endemic *Bacillus* probiotics on the improvement of the health of larval shrimp [71, 73]. The efficacy of the non-hemolytic probiotic *Bacillus* strains against pathogenic *Vibrio* species, viz. *Vibrio campbellii*, *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* on the growth of the larval shrimp was tested by using a daily concentration of 1×10^5 cfu ml^{-1} with initial bioassay density of 225 nauplii L^{-1}, and the treatments promoted a considerable increase in survival and growth of the larval shrimp compared to the control, and thus the study has established a significant antagonistic activity of the probiotic *Bacillus* strains against the *Vibrio* species [48]. Various strains of *Bacillus*, or a commercial product made from *Bacillus* sp., *Saccharomyces cerevisiae*, *Nitrosomonas* sp., and *Nitrobacter* sp., used as probiotics show prevention of infection and promotion of growth rate of all the stages of the white shrimp *Litopenaeus vannamei* Boone and *Fenneropenaeus indicus* when the diet was supplemented with 50 g of probiotic kg^{-1} of food. The use of probiotic *Shewanella* algae in shrimp farms is found to be safe for the consumer of shrimp [92]. A scientific rational approach for the evaluation of aquaculture probiotics and guidelines is needed for their use and safety.

2.4 *Bacillus* for the control of vibriosis

Pathogenic *Vibrio* spp. were controlled by cell-free extracts of *Bacillus* under *in vitro* and *in vivo* conditions indicating that probiotic treatment offers a promising alternative to the use of antibiotics in shrimp aquaculture [41, 63, 70, 85]. Though probiotic bacteria are currently used to improve the health of the shrimp ponds and increase productivity and reduce mortality of prawns, little attention has been paid to bacteriophages preying on the probiotic bacteria of crustacean culture such as *Vibrio*/*Bacillus* spp. from aquaculture systems. A special focus needs to be given to bacteriophages infecting probiotic bacteria to explore the antibacterial potential of bacteriophages of *Bacillus* spp., *Vibrio* spp., and other bacteria. The existence of bacteriophages in the probiotic *Bacillus* sp. and *Vibrio* spp. controlling the infectivity of the *Vibrio* spp. with a significant reduction in the mortality of infected shrimp was demonstrated [37, 41]. Anti-*Vibrio* activities of *Bacillus* spp. mainly due to the production of bacteriocin or bacteriocin-like substances have been reported from *B. subtilis*, *B. megaterium*, *B. stearothermophilus*, *B. licheniformis*, *B. thuringiensis*, *B. thermovorans*, and *B. cereus* [16, 22, 45, 47, 68, 69, 83]. *P. monodon* larvae fed with *Bacillus* S11 showed 100% survival after challenge with pathogenic *V. harveyi*, whereas only 26% control animals survived.

2.5 Antimicrobials

Vibrio species are Gram-negative curved rod-shaped bacteria that belong to the *Vibrionaceae* family, and they naturally inhabit the estuarine, coastal, and marine environment worldwide. *Vibrio* species occur as the dominant flora in all developmental stages of *Penaeus monodon*, and they have been described as the causal pathogens. *Vibriosis* is a severe bacterial disease in penaeid shrimp and is responsible

for large-scale losses in the aquaculture industry, leading to prophylactic as well as therapeutic use of antimicrobials [18–22, 70–73]. Antibiotics are used in shrimp farming to control or treat bacterial disease outbreaks with an expected 100,000–200,000 tons of antibiotics being consumed in the world every year [57]. Oxytetracycline, tetracycline, quinolones, sulfonamides, and trimethoprim are among the antimicrobials utilized to control bacterial infections in the aquaculture industry. Indiscriminate prophylactic uses of diverse antibiotics in the shrimp hatcheries/farms have resulted in antibiotic resistance of the bacteria causing mass mortalities of the hosts [73–75]. The potential negative consequences are the development of drug-resistant bacteria and reduced efficacy of antibiotic treatment. Shrimp farmers are exceedingly dependent on various antibiotics as a preventive measure against shrimp bacterial infections with 14% of farmers using antibiotics on a daily basis in their farms [22].

2.6 Antibiotic-resistant bacterial infections in crustaceans

All of the 121 isolates of *Vibrio* spp. were found to be 100% resistant to ampicillin, cloxacillin, oxacillin, erythromycin, vancomycin, penicillin G, and furazolidone and partially resistant to cefaclor, streptomycin, rifampicin, oxytetracycline, nalidixic acid, cefotaxime, and chlorotetracycline [14, 16]. Molitoris et al. [14] reported a high degree of resistance to ampicillin and furazolidone, and incidence of resistance to chloramphenicol, neomycin, and gentamycin has also been detected [16, 18–20, 22, 46, 69]. Multidrug-resistant *Vibrio harveyi* isolated from black gill-diseased *Fenneropenaeus indicus* and antibacterial activity against pathogenic *Vibrio harveyi* and its protective efficacy on juvenile *F. indicus* were reported. In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds and the effect of probiotics, antibiotics, and pathogenicity of *Listonella anguillarum*-like bacteria isolated from *Penaeus monodon* culture systems and antibiotic-resistant *Vibrio* spp. were commonly isolated from hatchery-reared postlarvae compared to farm-reared *P. monodon* [61, 62, 72, 73, 79, 85]. The unrelenting use of antibiotics against diseases in human beings and other life forms may pollute the aquatic system, and their impact on developing antibiotic-resistant *Vibrio* sp. may be a serious threat in addition to the use of antibiotics in aquaculture farms [63, 70]. Control of the bacterial diseases depends on improvement of management practices to minimize the risk of introduction of infectious agents into aquaculture systems and to reduce predisposing factors such as overcrowding and overfeeding.

2.7 Antibiotic-resistant genes (ARGs)

A study assessed a variety of antibiotic-resistant bacteria and detected the presence of their resistance genes from mariculture environments. A variety of antibiotics that were used in aquaculture have led to the occurrence of antibiotic-resistant genes in bacteria, and in these bacteria many different ARGs can be found, and they are β-lactam- and penicillin-resistant genes *penA* and *blaTEM-1*; chloramphenicol-resistant genes *cat*I, *cat*II, *cat*III, *cat*IV, and *floR*; tetracycline-resistant genes *tatA*, *tatB*, *tatC*, *tatD*, *tatE*, *tatG*, *tatH*, *tatJ*, *tatY*, and *tatZ*; and many more [65, 68]. ARGs can be transferred among bacteria via conjugation, transduction, or transformation. The widespread emergence of antimicrobial resistant bacteria worldwide has become a major therapeutic challenge. Therefore there is a need for development of novel non-antibiotic approach such as phage therapy biocontrol agents to fight against resistant bacterial infections due to the shortage of new antibiotics in developmental pipeline [19, 61–73]. Thiel [25] stated that phage therapy is "a nearly

forgotten therapy that may yet re-emerge as a savior to this accelerating crisis of antibiotic resistance. For every bacterium known on this planet, there are legions of bacteriophages—tiny viruses that seek out bacteria and use them as a breeding ground, almost invariably destroying their prokaryotic host in the process. That makes them harmless to even to nontarget bacteria—distinguishing them from broadspectrum antibiotics, which, when they work, can wipe out beneficial flora along with a troublesome infection." The phage specificity has its drawbacks as there is a requirement of the right match between phage and bacteria which needs to be determined for the phage therapy to work. There has been renewed concern in the application of bacteriophage as a non-antibiotic approach to control bacterial infections in various fields including human infections, food safety, agriculture, and veterinary applications. However the guideline data on the use of phage therapy applied to invertebrates, like shrimp and other crustaceans, are nonexistent [64, 68, 92, 93].

2.8 Bacteriophage therapy for biocontrol of vibriosis in crustaceans

A bacteriophage isolated from a shrimp hatchery was shown to infect *V. harveyi*, signifying its potential as a biocontrol agent of luminous vibriosis. In vitro treatments of bacteriophages were shown to exhibit a significant reduction (2–3 log units) in the number of *V. harveyi* host cells [41, 46, 48, 49]. These studies showed that bacteriophages could be used for biocontrol of *V. harveyi* and that bacteriophage therapy could be effective as an alternative to antibiotics in the control of luminous vibriosis in shrimp hatchery systems [23–69]. In vitro experiments confirmed that bacteriophages could be effectively used *in vivo* as biological agents to control *Vibrio* sp. in aquaculture systems [36–39, 41, 46, 48, 49]. Investigation on the occurrences of luminescent *V. harveyi* and their bacteriophages in shrimp showed that the presence of low concentrations of bacteriophages in the larval rearing tank waters could not prevent the development of luminous vibriosis [46–49], and this study showed that the presence of optimal concentration of phages is required for effective reduction of the pathogenic bacteria. A lytic spectrum of bacteriophages (Viha1, Viha2, Viha3, Viha4, Viha6, Viha7) occurring in nature exhibited a wide spectrum of activity against *V. harveyi*, suggesting their potential as agents for biocontrol of vibriosis in aquaculture environments [51]. A lytic phage (PW2) was isolated and characterized under controlled conditions in the laboratory from the host bacterium *V. harveyi* CS101 [49], and the useful bacteriophages for the biocontrol of vibriosis have been explored. A probiotic strain *V. alginolyticus* reduced the diseases and mortality of infected *P. monodon* with *A. salmonicida*, *V. anguillarum*, and *V. ordalli* [49]. A soil bacterial strain, PM-4, was shown to exhibit *in vitro* inhibitory effect against *V. anguillarum* and to promote the growth of *P. monodon* nauplius [53]. Inoculation of *Bacillus* S11, a saprophytic strain, resulted in greater survival of the post-larval *P. monodon* that were challenged with pathogenic luminescent bacterial culture [51]. These works strongly suggest an effective control of microflora in crustaceans can be achieved in aquaculture environments by bacteriophage-producing bacteria. The crustacean host, causative agent, diseases, and source of bacteriophages/bacteria are listed in **Table 2**.

2.9 In vitro and *in vivo* effects of *Bacillus* against vibriosis in shrimp

In vitro and *in vivo* antagonistic effects of *Bacillus* against the pathogenic *Vibrio* species were evaluated [40]. Cell-free extracts of *Bacillus subtilis* BT23 were shown to exhibit inhibitory effects against the growth and proliferation of *Vibrio harveyi* isolated from black gill-diseased *Penaeus monodon*. The probiotic effect of *Bacillus*

Crustacean host	Bacterial agent	Infection/ disease	Bacteriophage	Source of the bacteriophage	Efficacy of bacteriophage	Outcome of the phage therapy	References
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	*Myoviridae* (VHLM)	Extracted from a toxin-producing strain of *V. harveyi* isolated from moribund prawn	Vibriolysis	VHML showed a narrow host range with a preference for *V. harveyi* rather than 63 other *Vibrio* isolates and 10 other genera	[100]
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	*Siphoviridae*	Shrimp farm waters from the West coast of India	18-day-old PL shrimp were challenged with the bacteria (10^5 cells ml^{-1}, laboratory trial: (1) bacteriophage suspension (10^9 pfu ml^{-1}) was added initially; after 24 h (another 0.1 ml), (2) only once initially with 0.1 ml of the phage suspension; (3) no addition. Hatchery trial (1) treatment with bacteriophage (10^9 pfu ml^{-1}) at the rate of 200 ppm daily so that phage concentration in the water was 2^9–10^5 pfu ml^{-1}; (2) treatment with antibiotics (oxytetracycline 5 ppm, kanamycin 10 ppm daily); (3) no treatment	Enhanced survival (80%) of *P. monodon* larvae on treatment with two doses of bacteriophage when compared with the control (25%). Hatchery trial: survival in the control tank was only 17%, while in antibiotic-treated tanks, survival was 40%; in the bacteriophage-treated tank, survival was 86%. Bacteriophage therapy has an excellent potential in management of luminous vibriosis in aquaculture systems	[48]
Larval shrimp *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	*Siphoviridae*	Three from oyster tissue and one from shrimp hatchery water	Hatchery tanks, with post-larval five-stage larvae, presenting luminescence and mortality, were used. Bacteriophage treatment (two tanks): one suspension (2^9 10^6 pfu ml^{-1}) was added by day following the order: Viha10, Viha8, Viha10, and Viha8 chemotherapy (two	Bacteriophage treatment resulted in over 85% survival of *Penaeus monodon* larvae. The normal hatchery practice of antibiotic treatment resulted in a survival range from 65 to 68%. This study shows that bacteriophages could be used for biocontrol of *V. harveyi*	[46]

Crustacean host	Bacterial agent	Infection/ disease	Bacteriophage	Source of the bacteriophage	Efficacy of bacteriophage	Outcome of the phage therapy	References
					tanks): oxytetracycline (5 mg L^{-1}), kanamycin (10 mg L^{-1})		
Penaeid shrimp *P. monodon*	*Vibrio harveyi*	Luminous vibriosis	Seven bacteriophages specific to *Vibrio harveyi* (Viha1 to Viha7), six from *Siphoviridae* and one *Myoviridae* (Viha4)	Coastal aquaculture systems like shrimp farms, hatcheries, and tidal creeks along the East and West coast of India		All the phages were found to be highly lytic for *V. harveyi*. The phages exhibited a different lytic spectrum for a large number of bacterial isolates tested. Three of the phages (Viha1, Viha3, and Viha7) caused 65% of the strains to lyse, while Viha2, Viha4, and Viha6 caused 40% of the host strains to lyse. Only Viha5 had a narrow spectrum (14%). Six of the seven phages isolated had a broad lytic spectrum and could be potential candidates for biocontrol of *V. harveyi* in aquaculture	[51]
Shrimp *P. monodon*	*Vibrio harveyi*	Luminous vibriosis	*Siphoviridae* (VH1 to VH8)	Shrimp farm	*In vitro* experiment	All the isolates of bacteriophage (VH1–VH8) caused lysis of the host bacterial cells within 2 h. The propagation curve for each phage showed a burst time from 1 to 10 h. Bacteriophages of *Vibrio* sp. shall be effectively used *in vivo* as biological agents to control these pathogenic bacteria in aquaculture systems	[39]
Shrimp *P. monodon*	*Vibrio harveyi* CS101	Luminous vibriosis	*Siphoviridae* (phage PW2)	Shrimp pond water		Phage adsorption rate increased rapidly in the first 15 min of	[52]

Crustacean host	Bacterial agent	Infection/ disease	Bacteriophage	Source of the bacteriophage	Efficacy of bacteriophage	Outcome of the phage therapy	References
						infection to 80% and continued to increase to 90% within 30 min of infection. The stability of phage PW2 was dependent on temperature and pH. It was inactivated by heating at 90°C for 30 min and by treating at pH 2, 3, 11, and 12. One-step growth curve and latent and burst periods were 30 and 120 min, respectively, with a burst size of 78 pfu per infected center. Six structural proteins were detected	
Larval shrimp *P. monodon*	*V. harveyi*	Luminous vibriosis	ϕH17-5c, ϕH17-7b, ϕH17-8b, and ϕH17-9b	Secluded from shrimp farm water from the West coast of India and demonstrated to exhibit a broad lytic activity against *V. harveyi* isolates	In a set of laboratory experiments, post-larval *Penaeus monodon* was exposed to 10^6 cfu ml^{-1} cells of *Vibrio harveyi* and was treated with 100 ppm phage which has led to a drastic reduction of *Vibrio harveyi* counts with 86% survival of the infected larvae, while the survival of the phage-untreated larvae was 25%	In the antibiotic (oxytetracycline 5 ppm, kanamycin 100 ppm/ day)-treated hatchery tanks, an initial reduction of luminous bacterial counts were shown, and after 48 h the bacterial count increased to 106 ml^{-1} showing the luminous vibriosis and mortality of the nauplii of *Penaeus monodon* with 40% larval survival. In contrast, in the bacteriophage-treated tank, the larval survival was 86%, and the survival rate in the control tank was only 17%	[48, 49]
Shrimp, *P. monodon*	*V. vulnificus*	Vibriosis	Phages VV1, VV2, VV3, and VV4 from *V. vulnificus*	VV1, VV2, VV3, and VV4 phages were detected from	In vitro experiments show successful potential phage therapy; lytic *V. vulnificus* phages	*V. vulnificus* phages exhibited a broad lytic spectrum and potential biocontrol of luminous	[36–39]

Crustacean host	Bacterial agent	Infection/ disease	Bacteriophage	Source of the bacteriophage	Efficacy of bacteriophage	Outcome of the phage therapy	References
				shrimp aquaculture system	infect a wide variety of other *Vibrio* spp./isolates	vibriosis in the shrimp aquaculture system	
Phyllosoma larvae of the tropical rock lobster *Panulirus ornatus*	*V. harveyi*	Luminous vibriosis	Six bacteriophages from *Siphoviridae* (VhCCS-01, VhCCS-02, VhCCS-04, VhCCS-06, VhCCS-17, and VhCCS-20) and two from *Myoviridae* (VhCCS-19 and VhCCS-21)	Isolated from an epizootic in aquaculture-reared larval phyllosomas of the ornate spiny lobster *Panulirus ornatus* water samples from discharge channels and grow-out ponds of a prawn farm	Exhibited a clear lytic activity against *V. harveyi* (1) Addition of phage VhCCS-06 (1 ml) 2 h after inoculation; (2) addition of phage VhCCS-06 (1 ml) 6 h after Bacteria-free supernatants were obtained by centrifugation at 10,000 *g* for 15 min and by filtration of the aliquots of the enriched water samples; supernatants (10 ll) were inoculated onto NAMS plates, grown at 28°C for 24 h, and the bacteria-free supernatants were stored at 4°C	Exhibited a clear lytic activity against *V. harveyi* with no apparent transducing properties. Phages of VhCCS-19 and VhCCS-21 are ***Myoviridae*** bacteriophages and lysogenic and induce **bacteriocin production** in the host bacteria (*V. harveyi* strain 12); ***Siphoviridae*** phage (VhCCS-06) delayed the entry of a broth culture of *V. harveyi* strain 12 into exponential growth, though it could not prevent the overall growth of the bacterial strain. This effect was due to the multiplication of **phage-resistant cells** of *V. harveyi*. Phage resistance is an obstacle to the use of phage as therapeutic agents. The isolated phages exhibited lytic activity against strains of *V. harveyi*, a primary pathogen of phyllosoma of the tropical rock lobster, *P. ornatus*. These phages can be used as a biocontrol agent to combat vibriosis in the rearing	[55]

Crustacean host	Bacterial agent	Infection/ disease	Bacteriophage	Source of the bacteriophage	Efficacy of bacteriophage	Outcome of the phage therapy	References
						system of phyllosoma of the tropical rock lobster, *P. ornatus*	
Live prey *Artemia salina*	*V. alginolyticus* strain V1	Vibriosis	Two novel bacteriophages φSt2 and φGrn1	*Vibrio alginolyticus* strain V1, isolated during a vibriosis outbreak in cultured seabream	In vitro cell lysis experiments against the bacterial host *V. alginolyticus* strain V1 and also against 12 *Vibrio* strains originating from live prey *Artemia salina* cultures, viz. *V. anguillarum*, *V. harveyi*, *V. alginolyticus*, *V. ordalii*, *V. parahaemolyticus*, *V. splendidus*, and *V. owensii).* It indicated a strong lytic efficacy of the 2 phages	In vivo administration of the phage cocktail consisting of φSt2 and φGrn1, directly on live prey *A. salina* cultures, has led to a 93% decrease of *Vibrio* population, viz. *V. anguillarum*, *V. harveyi*, *V. alginolyticus*, *V. ordalii*, *V. parahaemolyticus*, *V. splendidus*, and *V. owensii*, after 4 h of treatment	[98, 99]
Brine shrimp nauplii *Artemia franciscana*	*V. parahaemolyticus*	Vibriosis of *Artemia franciscana*	VPMS1 phage	VPMS1 is a lytic phage of *Vibrio parahaemolyticus*, isolated from a marine clam	*V. parahaemolyticus*-infected brine shrimp nauplii were treated with a single dosage of VPMS1 phage, which was effective enough to eliminate the adverse effects of *V. parahaemolyticus* in brine shrimp. Efficacy was not affected by the reduction in the dosage	The phage therapy was successful in preventing vibriosis; a single dosage of VPMS1 phage was effective enough to get rid of the adverse effects of *V. parahaemolyticus* in brine shrimp; the beneficial effects of the therapy were compromised if the application of phages was delayed	[97]
Penaeus monodon rearing waters in shrimp ponds in Palk Strait, South East coast of India	*Vibrio parahaemolyticus*	*Vibrio parahaemolyticus* and its potential lytic phage from *Penaeus monodon*	Lytic phage (VVP1) belongs to the *Myoviridae* family	Lytic phage (VVP1) able to infect strains of N1A and N7A, *V. parahaemolyticus*, and strains of N3B and N13B *Vibrio alginolyticus*	One-step growth experiments, multiplication and host range, and pH and temperature stability of the lytic phage (VVP1) were shown; the phage showed protective biocontrol effects in reducing the pathogenic *V.*	*P. monodon* larvae infected with *V. parahaemolyticus* showed enhanced survival of the larvae in the presence of phage treatment at lytic phage (VVP1), 2.3×10^{10} PFU ml^{-1}, when compared with the control and	[101]

Crustacean host	Bacterial agent	Infection/ disease	Bacteriophage	Source of the bacteriophage	Efficacy of bacteriophage	Outcome of the phage therapy	References
					parahaemolyticus in infected shrimp larvae	showed that the application of phage therapy is a useful strategy to prevent and eliminate or reduce shrimp pathogenic *V. parahaemolyticus* in the aquaculture system	
V. alginolyticus phages were isolated from seawater samples after enrichment	*V. alginolyticus*, *Vibrio alginolyticus*, a zoonotic pathogen that causes mass mortality in aquatic animals and infects humans	Vibriosis, a zoonotic pathogen causing mass mortality in aquatic animals and infecting humans	Phage pVa-21, *Myoviridae*	Phage pVa-21 infects bacteria belonging to the family *Vibrionaceae*, *viz. V. alginolyticus* and *V. harveyi*, and could not infect *V. parahaemolyticus*, *V. anguillarum*, *V. campbellii*, and *V. vulnificus*	Bacteriophage pVa-21 belongs to *Myoviridae*, characterized as a candidate biocontrol agent against *V. alginolyticus*. It exhibits planktonic or biofilm lytic activity and showed stability under various conditions. It has latent period and burst size approximately 70 min and 58 plaque-forming units/cell, respectively. Phage pVa-21 can inhibit bacterial growth in both the planktonic and biofilm states. The phage is related to the giant phiKZ-like phages, classified as a new member of the phiKZ-like bacteriophages that infect bacteria belonging to the family *Vibrionaceae*	Infect bacteria, viz. *V. alginolyticus* and *V. harveyi* in both the planktonic and biofilm states	[101]

Table 2.
Crustacean host, bacteria, disease, and source of bacteriophages/bacteria for bacteriotherapy.

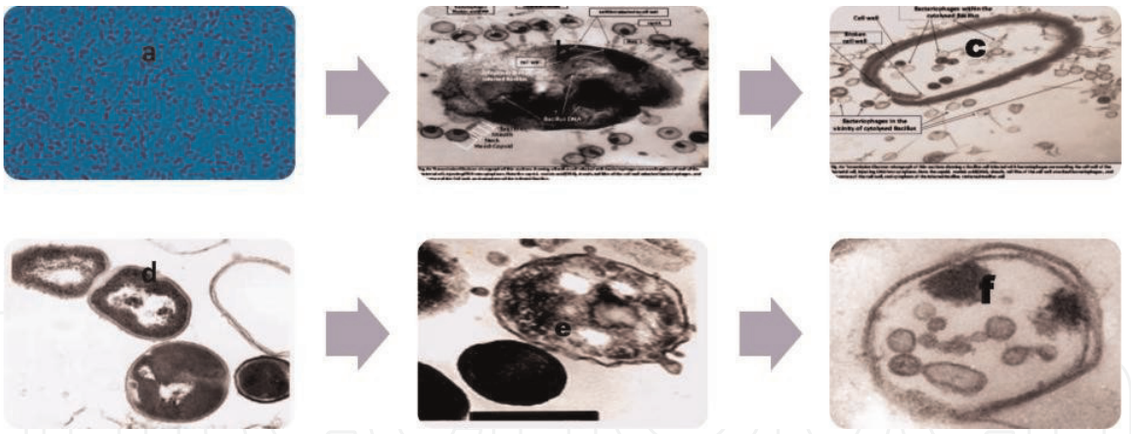

Figure 1.
(a) Light micrograph of uninfected Bacillus sp.; (b) TEM micrograph showing phages on the surface of the infected Bacillus. (c) TEM micrograph of phage-lysed bacterial cell. (d) TEM micrograph of uninfected Vibrio cells. (e) TEM micrograph of phage-infected Vibrio cell. (f) TEM micrograph of phage-lysed Vibrio cell.

was tested by exposing shrimp to *B. subtilis* BT23 at a density of $10^6/10^8$ cfu ml^{-1} for 6 days before a challenge with *V. harveyi* at $10^3/10^4$ cfu ml^{-1} for 1 h infection (**Figure 1a-f**). Probiotic treatment of *B. subtilis* BT23 showed a 90% reduction in accumulated mortality of *P. monodon*. Pathogenic *Vibrio* species were controlled by *Bacillus* under *in vitro* and *in vivo* conditions, and the results indicated that probiotic treatment offers a promising alternative to the use of antibiotics in shrimp aquaculture.

The occurrence of phages in infected cells of *Bacillus* sp. is illustrated (**Figure 1a-c**). Each bacteriophage particle of the bacterium *Bacillus* sp. presented as electron-dense objects comprising a distinctive head with a flexible noncontractile tail. The preparations consisted of 30 min–2 h exposures of *Bacillus* cells to *in vitro* phage infection, and the TEM of such experimentally phage-infected cells showed actively adsorbing and infecting phages onto the surface of bacterial cell membrane (**Figure 1b**), while phage particles occurred within the completely lysed cells in 8–24 h (**Figure 1c**).

TEM analysis of the phages of *Vibrio* host cells showed the occurrence of tailless phages with a double-layer membrane covering the icosahedral head. Phage-uninfected, phage-infected, and completely lysed *Vibrio* cells are shown (**Figure 1d–f**).

2.10 Antagonism of *Bacillus* against *Vibrio* spp.

An antagonism assay consisting of cell-free extract of *Bacillus* BT21, *Bacillus* BT22, and *B. subtilis* BT23 showed inhibitory activity against several pathogenic species of *Vibrio* [40]. They reported that *B. subtilis* BT23 exhibits a relatively higher inhibitory activity than the other two *Bacillus* BT21 and *Bacillus* BT22. Moreover *B. subtilis* BT23 was shown to inhibit the growth and proliferation of 112 isolates of *Vibrio* spp. consisting of *V. harveyi* (39 isolates), *V. anguillarum* (24 isolates), *V. vulnificus* (30 isolates), and *V. damsela* (19 isolates) which were obtained from *P. monodon* culture hatcheries and ponds. The growth and proliferation of pathogenic *V. harveyi* was inhibited by *B. subtilis* BT23 culture inoculated at a preliminary level of 10^5–10^9 cfu ml^{-1}, whereas lower concentrations of *B. subtilis* BT23 (10^5 and 10^7 cfu ml^{-1}) allowed early growth and proliferation followed by a decrease in the total viable counts of *V. harveyi*. Co-culture experiments demonstrated that the growth and proliferation of *V. harveyi* was controlled under *in vitro* conditions when the concentrations of *B. subtilis* BT23 were increased. Experiments of cell-free

extracts of *Bacillus subtilis* BT23 established the inhibitory effects on the growth and proliferation of *V. harveyi* in liquid culture under aerobic conditions. The inhibitory efficacy was higher in *B. subtilis* BT23 cell-free extracts of 10^8 cfu ml^{-1} and low in 10^4 cfu ml^{-1}. Cell-free extracts of *Bacillus subtilis* BT23 initially could not limit the growth and proliferation of *V. harveyi* for 2 days, and afterward the growth and proliferation of *V. harveyi* was extremely inhibited when compared with the growth of *V. harveyi* without *B. subtilis* BT23. The studies on the probiotic treatment *V. harveyi*-infected *P. monodon* revealed a substantial reduction in the mortality of shrimp which were treated with *B. subtilis* BT23 strains under *in vivo* conditions. The cumulative mortality of *V. harveyi* post-infection and probiotic *B. subtilis* BT23-untreated *P. monodon* was 50% on the ninth day and 100% on the seventeenth day of post-infection. In contrast, in the probiotic treatment groups, the cumulative mortality of shrimp was 10% in the combined treatment after 5 days post-infection. Long-term and short-term treatments of *V. harveyi*-infected *P. monodon* with *B. subtilis* BT23 showed a decrease in cumulative mortality of 32 and 60%, respectively. In control groups of *V. harveyi*-uninfected *P. monodon*, there was no mortality. The growth of pathogenic *V. harveyi* was inhibited by nonpathogenic *B. subtilis* BT23 under *in vivo* and *in vitro* conditions. Co-culture experiments showed that the inhibitory activity of *B. subtilis* BT23 increased with increasing density of the antagonist. A high concentration of *B. subtilis* BT23 (antagonist) was essential to obstruct the growth and multiplication of *V. harveyi* in the co-culture experiments. The antagonist required being present at significantly higher levels than the pathogen, and the degree of inhibition increased with the level of antagonist. During the co-culture, $10^7/10^8$ cfu ml^{-1} was required to inhibit the growth of the pathogen *V. harveyi*. Therefore, a potential probiotic co-culture must either be supplied on a regular basis or be able to colonize and multiply on or in the host. A similar control of pathogenic *Vibrio* in fish and shellfish, by the use of nonpathogenic bacterial strains and disease prevention, has received much attention during the last decade [42, 43, 56, 81]. Purification and characterization of the antibacterial substances from the host bacteria and the phages could help to understand the mechanism of antibacterial activity of *Bacillus* and other strains. Probiotic treatment offers a very promising alternative to the use of antibiotics in fish and shrimp aquaculture. Further study is needed to elucidate the exact mode of action of the observed beneficial effects of the probiotics and to understand the possibilities and limitations of microbial control in aquaculture.

2.10.1 Long-term storage of phages

The usefulness of a phage lysate preparation and treatment method adopted for long-term storage of phages was elucidated in a study that demonstrated the infectivity of the phages of *Vibrio* spp. that remained unaffected with chloroform and DMSO treatments and storage at −40°C for 30 days [37–39, 41]. Similarly, phages were shown to be highly stable under normal storage conditions and also stable in NaCl and $MgSO_4$ due to its stabilizing effect. Substantial amounts of viable phages were reported to occur even after storage even in distilled water. Phage isolates were found to be stable upon storage at 4°C, and a rapid loss of phage infectivity was encountered with repeated freezing and thawing at −70°C. *Bacillus* phage was stable to a 1-hour exposure to chloroform, indicating that it probably does not contain chloroform-soluble lipids and lipoproteins. *V. vulnificus* phage infectivity could not be inhibited with trypsin, protease, and ribonuclease treatments, while the infectivity of the *Vibrio* phages was inhibited with lysozyme and SDS treatments, perhaps demonstrating that the enzyme has interfered with the adsorbing of the phages [38, 39, 41]. The enzymatic treatments and inhibition of phage

infectivity of several phages were reported. Proteinase K treatment could not alter the adsorption ability of phage particles. These studies show that binding of the phages to its host cell membrane is the first step of lytic cycle and this can be an irreversible mechanism, and a similar mechanism may exist in the infection of vibriophages as the infectivity of the phages were inhibited by lysozyme and SDS treatments. Treatment with 1% SDS did not affect the adsorption ability of phage particles. Similarly *Mycoplasma arthritidis* virulent 1 (MAV1) phage infectivity was reported to be unaffected by treatment with Triton X-100 and was resistant to nonionic detergents. *Vibrio* phages were found to be fairly resistant to chloroform [46, 101]. Further studies on the proteins and lipids of *V. vulnificus* phages may help us to understand their role in the life cycle and infectivity of phages. *V. vulnificus* phages survived 100% at pH 7 and exhibited infectivity, while none of the phages survived at extreme pH conditions (pH 3 and pH 12) [41]. *V. harveyi* phages were inactivated at pH 3 or less and at pH 12 or greater. In contrast, VPP97 phage was totally inactivated at pH below 5 or over 10. All *V. vulnificus* phages from the shrimp *Penaeus monodon* exhibited optimal survival at 37°C, but infectivity occurred, and plaques were observed up to a maximum temperature of 50°C [41]. JSF9 phage was shown to be stable at a temperature below 37°C, and the phages were rapidly inactivated above 50°C temperature. *V. parahaemolyticus* phage (VPP97) has been shown to be stable up to a temperature of 65°C and was totally inactivated at 70°C [41, 54, 58, 77, 101]. Phages were reported to survive extremes of temperature up to 95°C [54]. Phages, viz. T-ϕD0, T-ϕD2S, T-ϕHSIC, and T-ϕD1B, exhibited a latent period ranging from 90 to 180 min [77]. The results have clearly shown the physicochemical parameters are very important for the survival and infectivity of phages [37, 38, 46, 49]. Additional studies related to infectivity, stage specific expression of proteins, and specific effects of the purified phage proteins are needed to better understand their functions and applications in the control and process of infectivity of *V. vulnificus* phages.

2.10.2 Phage therapy for vibriosis in shrimp

Phage therapy is a re-emerging field, and the bacteriophages represent potential biocontrol agents for the control of virulent and drug-resistant bacteria [19, 23–73]. However the use of phage therapy in shrimp is still in its early years, and these are highlighted especially the need for using more than one kind of bacteriophage in aquaculture to evade development of bacterial resistance [47–49, 58–60]. Phage therapy has been effectively used to protect against *Vibrio* diseases in a shrimp and prawn hatchery. Inhibitory effects of bacteriophages against shrimp pathogenic *Vibrio* spp., efficacy of potential phage cocktails against *Vibrio harveyi* and closely related *Vibrio* species isolated from shrimp, morphological characterization and biocontrol effects of *Vibrio vulnificus* phages against vibriosis in the shrimp, and a phage therapy in aquaculture system have been described [37–39, 41]. Four new *V. vulnificus* phages were detected from shrimp aquaculture system, named VV1, VV2, VV3, and VV4 [39]. All lytic *V. vulnificus* phages belonged to *Tectiviridae* family with typical double-layered elongated icosahedral head and tailless morphology [39, 101]. Lytic *V. vulnificus* phages which infect other *Vibrio* isolates were further characterized for long-term storage by enzyme treatment, organic solvent treatment, detergent treatment, pH stability, temperature stability, and agar bioassay method and one-step growth experiment. The infectivity, growth, and multiplication of VV1, VV2, VV3, and VV4 phages were unaffected by the treatment effects of chloroform, acetone, ethyl alcohol, methyl alcohol, ribonuclease (RNase), trypsin, protease, and Triton-X100. The phages (VV1–VV4) were inactivated completely with temperature (over 60°C), pH (below 3 and above 12), and

lysozyme and sodium dodecyl sulfate (SDS) treatment. One-step growth experiments indicated a latent period of 3 h and a burst size at 37°C. Agar bioassay method indicated that the percentage inhibition of the bacteria *Vibrio* was 75 (VV1) and 70 (VV2, VV3, and VV4), respectively. *V. vulnificus* phages had a broad lytic spectrum and potential biocontrol of luminous vibriosis in the shrimp aquaculture system [39, 41, 101]. The lytic *Vibrio vulnificus* phages may provide a better understanding of phage-host interactions and development of phage therapy in the aquaculture system. Besides, 12 *V. harveyi* phages showing broad host ranges were recovered from seawater samples. Further, some of the phages of ϕH17-5c, ϕH17-7b, ϕH17-8b, and ϕH17-9b were reported to show inhibitory activity against *V. harveyi* based on various tests, viz. heat stability, chloroform stability, adsorption rate, and one-step growth experiment [58]. A bacteriophage of *Vibrio harveyi* was secluded from shrimp farm water from the West coast of India and demonstrated to exhibit a broad lytic activity against *V. harveyi* isolates [48, 49]. *V. harveyi*-infected larval shrimp exhibited a higher rate of survival in the presence of the bacteriophage than the uninfected control larval shrimp. Bacteriophage treatment of the vibriosis of shrimp in hatchery tanks enhanced the survival of the *V. harveyi*-infected shrimp larvae and reduced the bacterial counts [50–55, 57, 58]. Bacteriophages secluded from a shrimp hatchery and farmed *P. monodon* samples exhibited lytic activity against *V. harveyi* and also controlled the population of *V. harveyi* and improved the survival of *Penaeus monodon* larvae [41, 48–52, 58]. Purified bacteriophages exhibited lytic activity, indicating they are appropriate for phage therapy application [29, 53]. A few bacteriophages that infected *V. harveyi* in a shrimp hatchery were unable to control the outbreak of luminescent vibriosis disease in a shrimp culture system [75]. The potential of the bacteriophages of *Vibrio harveyi* was reported to control population of pathogenic *Vibrio harveyi* in a hatchery setting [48]. In a set of laboratory experiments, post-larval *Penaeus monodon* was exposed to 10^6 cfu ml^{-1} *cells of Vibrio harveyi* and was treated with 100 ppm phage which has led to a drastic reduction of *Vibrio harveyi* counts with 86% survival of the infected larvae, while the survival of the phage-untreated larvae was 25%. In the antibiotic (oxytetracycline 5 ppm, kanamycin 100 ppm/day)-treated hatchery tanks, an initial reduction of luminous bacterial counts was shown, and after 48 h the bacterial count increased to 10^6 ml^{-1}, showing the luminous vibriosis and mortality of the nauplii of *Penaeus monodon* with 40% larval survival. In contrast, in the bacteriophage-treated tank, the larval survival was 86%, and the survival rate in the control tank was only 17% [48]. The presence of *V. mimicus* (15 isolates), exhibiting a typica profile of *Vibrio* [49] (two isolates), was obtained from diseased tissues of penaeid shrimp. A prominent occurrence of *V. harveyi*, *V. furnissii*, *V. mimicus*, *V. damsela*, and *V. anguillarum* in the hatchery tank water besides MBV- and WSSV-uninfected *P. monodon*, sea sediment, seawater, and shrimp culture pond sediment and shrimp culture pond water has been recorded [30, 53]. Vibriosis (*V. alginolyticus*)-associated mortality has been recorded in several invertebrates such as *Penaeus monodon* and *Macrobrachium rosenbergii*. Phage therapy has been shown to inhibit the growth and multiplication of *V. harveyi*, *V. parahaemolyticus*, and *V. anguillarum*. Bacteriophages belonging to *Siphoviridae* family are positive to control *Vibrio* species as the *Siphoviridae* phages are considered to have a specific host range consisting of species of *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio campbellii* [41, 44, 46, 48–53, 58, 60, 69]. A lytic phage PW2 was obtained from shrimp pond water in Songkhla Province, Thailand, and the morphological characteristics of the phage consisted of an icosahedral head and a long noncontractile tail categorized under the order *Caudovirales* and family of *Siphoviridae*. The phage PW2 showed lytic properties against *Vibrio harveyi* [52]. Most of the *Vibrio harveyi* phages were found to be siphophages [25, 26, 29, 41, 51]. However, *Vibrio harveyi*

phages which were from other families such as *Myoviridae* and *Podoviridae* were also reported [41, 51]. Selection of a suitable bacteriophage or a cocktail of phages is the key issue in the success of phage therapy of *Vibrio* species. Moreover phage cocktails have been demonstrated to be more effective than individual phages in treatment of *Vibrio* infection. By making a phage cocktail, it would become easier to treat a wide range of drug-resistant bacterial infections [58, 68]. Moreover phage-resistant mutants are exceptional, and hence the use of a multiphage therapy might decrease the resistance mechanism [61–65, 81, 82]. The limitations of the use of bacteriophages have been recognized, and the phages cannot be used if they (1) have toxin gene insertion; (2) have endotoxin release due to their lytic effect on the host bacterial cells; (3) have a risk of genetic material exchange, i.e., transduction or phage conversion; (4) have propagation and continuous maintenance; (5) have development of anti-bacteriophage bodies against them; (6) have the cost temperate phages contributing to bacterial virulence; (7) have emergence of phageresistant bacteria; (8) have disease outbreak of unknown bacteria when phage specificity is a problem; and (9) are continuously removed by the immune system [80–82]. Vibriophages themselves may have some protective effects though they can be candidates as therapeutic agents in bacterial infections in the aquaculture system, and therefore there is a need to be further investigating them in their natural environment. Future work may involve obtaining consistent credible results which can substantiate the application of bacteriophages to control vibriosis in shrimp and extending such research to other organisms. The usefulness of phage therapy to control microbial infections that occur in dissimilar organisms at various stages of from eggs to brood stock, as well as in laboratory, tanks, or field applications, was shown in experiments made with shrimp larvae, showing a promising potential for phage therapy [46]. The life-saving antibiotics and the new-generation antibiotics cannot be used in aquaculture, and therefore the bacteriophages have the natural advantages and potential to be used in management of the vibriosis in aquaculture.

2.10.3 Phage therapy in lobsters

Significant (71%) mortalities of larval, post-larval, and adult lobsters were caused by shell diseases where *Vibrio* spp., besides *Pseudomonas*, and *Aeromonas* have also been isolated. A strain of *Vibrio owensii* (DY05) was isolated from an epizootic in aquaculture-reared larval phyllosomas of the ornate spiny lobster *Panulirus ornatus* [10–12, 55]. Bacteriophage therapy was one of the techniques that controlled and removed the pathogenic *Vibrio* spp. from the larval cultures of the tropical rock lobster, *Panulirus ornatus*. *V. harveyi* has been found to be associated with diseases in spiny lobster [55].

Crothers-Stomps and colleagues [55] demonstrated that from eight bacteriophages (six phages belonged to the family *Siphoviridae*, and two belonged to the family *Myoviridae*), only one bacteriophage from the family *Siphoviridae* was shown to exhibit a clear lytic activity against *V. harveyi* with no apparent transducing properties. They have identified the occurrence of phage resistance as a major constraint to the use of phage therapy in aquacultures as the pathogenic bacteria were not completely eliminated [29, 41], and a similar approach on other phages is essential for understanding of the mechanism of development of phage resistance.

2.10.4 Diseases of Macrobrachium rosenbergii (DeMan)

Bacterial and fungal diseases are accountable for the high mortality and profound economic loss encountered in the giant freshwater prawn *M. rosenbergii* aquaculture industry [19–21]. Vibriosis (*V. alginolyticus*)-associated mortality was

recorded in *M. rosenbergii* [19–21]. They have shown the occurrence of a very high load of total heterotrophic bacterial count and a total presumptive *Vibrio* count in the 11 different larval stages of *M. rosenbergii*, whereas the total heterotrophic bacterial count was higher in the larval tank water throughout the cycle of the *M. rosenbergii* larval culture. The control treatment methods for the *vibriosis* consisted of chlorination, application of antibiotics, UV radiation alone, and a combination of sequential treatments starting from chlorination (2 ppm) followed by sand filtration, dechlorination, UV radiation (10 s) and microfiltration (5 μm size), and the sequential treatments in *M. rosenbergii*, and these had resulted in the gradual reduction of *V. alginolyticus* counts, and these were shown to be the best methods to control the pathogenic bacterial population in hatcheries of freshwater prawn *M. rosenbergii*. Antimicrobial resistance profile of *Vibrio* species isolated from the hatchery system of *M. rosenbergii* has been reported [19–21]. Vibriosis of *M. rosenbergii* can be controlled through phages *φ*St2 and *φ*Grn1 of *V. alginolyticus* from live feed *A. salina* [97–99].

2.10.5 Diseases of crabs

Shell diseases are caused by chitinolytic bacteria which were encountered in English prawn *Palaemon serratus*; American lobsters *Homarus americanus*; penaeid shrimp; and king crabs, *Paralithodes camtschaticus* and *Paralithodes platypus*; and the tanner crabs *Chionoecetes tanneri* are affected by rust diseases caused by chitin-destroying bacteria [7]. Biocontrol method against the infection of *V. anguillarum* for rearing the swimming crab larvae *Portunus trituberculatus* in the aquaculture water has been described [94–96]. A bacterial strain PM-4 *Thalassobacter utilis* isolated from a crustacean culturing pond was shown to inhibit the growth of pathogenic *Vibrio anguillarum* in seawater and to improve the growth of larval crab. The cells of the bacterial strain PM-4 *T. utilis* were cultured in a large quantity and were added daily for 6^3 of seawater used for culturing larval crab *Portunus trituberculatus*. Initial *V. anguillarum* bacterial density in the crustacean culture water was 10^6 cells ml^{-1}, while at the crab larval growth stage zoea II, the bacterial density was found to increase to more than 10^7 cells ml^{-1} and reduced the pathogenic *V. anguillarum* bacteria to 10^6 cells ml^{-1}. When the bacterial strain PM-4 *Thalassobacter utilis* dominated the bacterial populations, the numbers of pathogenic bacteria *Vibrio* spp. were reduced or could not be detectable in seawater. The growth and production of the larval crab *P. trituberculatus* were found to be increased by the addition of the bacterial strain PM-4 to the culture water. Investigations on the bacteriophages and phage therapy specific for aquaculture of crabs are desirable.

2.10.6 Phage therapy for vibriosis of Artemia salina

The occurrences of *V. harveyi*, *V. furnissii*, *V. mimicus*, *V. damsela*, and *V. anguillarum* in the nauplii of *Artemia* and the presence of *V. harveyi* and *V. mimicus* in the *Artemia*-reared water have been recorded [12, 18, 22, 39]. A prominent occurrence of *Vibrio* in the *Artemia* nauplii, *Artemia*-reared water, egg samples, hatchery tank water besides MBV- and WSSV-uninfected *P. monodon*, sea sediment, seawater, and shrimp culture pond sediment and shrimp culture pond water was recorded. Vibriosis (*V. alginolyticus*)-associated mortality has been recorded in several invertebrates such as *Penaeus monodon* and *Macrobrachium rosenbergii* [18–21, 34, 97–99]. *Vibrio* species normally reside in the live feed organism such as *Artemia salina* which serves as means of carrying and establishing the bacteria into the hatchery and aquaculture system where *V. alginolyticus* has been reported as the

prevailing member of the cultivable bacterial community of *Artemia*. There are a number of studies indicative that *Artemia* nauplii are vectors of potentially harmful bacteria such as *Vibrio* spp. Goulden et al. [12] reported that *Vibrio owensii* (DY05)-infected *Artemia* (brine shrimp) was responsible for 84–89% mortality of the aquacultured larval phyllosomas of the ornate spiny lobster *Panulirus ornatus*, and an understanding of the infection processes can help to improve targeted biocontrol strategies. The existing disinfection techniques such as filters, chlorination, ozone, UV, etc. could not prevent the occurrence of bacterial pathogens in the hatcheries and may perhaps help in the growth, proliferation, and multiplication of the opportunistic pathogen application of broad-spectrum antibiotics in the feed and hatchery water which has been the most natural strategy to control bacterial infections. The practice of applying antibiotics in aquaculture has become unattractive as many important bacterial pathogens belonging to the genera *Aeromonas* and *Vibrio* evolve antibiotic resistance. The excessive and indiscriminate use of antibiotics has resulted in the development of multidrug-resistant bacterial strains and public health problems. In shrimp hatcheries, the use of bacteriophages that infect bacteria is an alternative method to selectively eliminate their bacterial hosts while leaving normal microbiome unaltered. Phage therapy has been shown to inhibit the growth and multiplication of *V. harveyi*, *V. parahaemolyticus*, and *V. anguillarum* [39, 97–99].

2.10.7 In vitro lytic effect of phages φ*St2 and* φ*Grn1 on Vibrio strains of Artemia salina*

In vitro cell lysis experiment of phage (a) *φ*St2 and (b) *φ*Grn1 was carried by infecting the fresh cultures of the host bacteria *V. alginolyticus* strain V1 which was collected from live feed *A. salina* culture [97–99]. The lysis of the host bacteria *V. alginolyticus* strain V1 grown in TCBS was proportional to the multiplicity of infection (MOI) (which is defined as the ratio between the number of viruses in an infection and the number of the host cells which can be resolved by correcting the relative concentration of virus and host) used. The lowest (MOI = 1) showed no effect, while the highest (MOI = 100) showed a complete inhibition of bacterial growth. The phages were also tested *in vitro* against 12 different bacterial isolates grown in TCBS which originated from live feed *A. salina* culture. A phage mixture of *φ*St2 and *φ*Grn1 at MOI = 100 was shown to affect the growth of all 12 bacterial strains tested, and the study showed that in all cases, there was a delay in the exponential phase, and even when the cultures reached a plateau of growth, the density of phage-treated bacteria was lower than their corresponding controls.

2.10.8 In vivo efficacy of phages on the vibriosis of A. salina culture

The effect of the phage mixture (*φ*St2 and *φ*Grn1 at MOI = 100) was examined by administrating the phages *in vivo* in the live prey cultures of *A. salina*. After 4 h of administration of the phage mixture, *Vibrio* count was not altered in the phage-untreated control cultures, while the *Vibrio* count drastically reduced in the phage-treated cultures of *A. salina* [97]. The total *Vibrio* counts in the phage-treated cultures of *A. salina* was $5.3 \times 10^3 \pm 3.1 \times 10^3$ cfu ml^{-1}, which was 93% lesser than that of the initial total *Vibrio* counts. The *φ*St2 and *φ*Grn1 phages affected the growth of a range of *Vibrio* spp. and exhibited lytic activity in eight different *V. alginolyticus* strains [97]. The phages also exhibited infection in 10 out of the 25 *Vibrio* strains (40%) tested and lysis in bacterial strains such as *V. harveyi* and *V. parahaemolyticus* species and were known to affect the growth of several strains of bacteria *V. harveyi* strains isolated from an *Artemia* live feed organism. A broad host range of *φ*St2 and *φ*Grn1 phages indicated the occurrences of a very similar host

structures as receptors, viz. LPS phage receptors on the outer membrane of many of Gram-negative bacteria and the genetic similarity contributing to this broad lytic spectrum of the phages φSt2 and φGrn1 phages which exhibited a strong lytic effect against *V. alginolyticus*-type strain DSM 2171 [98]. The bacteriophages, φSt2 and φGrn1, are having a broad host range, and such biological attributes make them the potential candidates for phage therapy application, and these advocate that phage therapy can be an alternative to antibiotics in aquaculture. The phages, viz. φSt2 and φGrn1 and a giant bacteriophage pVa-21, can effectively be used in the biological control of pathogenic *Vibrio* species in marine hatcheries [97–99]. These studies indicate the potential applications of the phages for various purposes outside aquaculture, and further research on the specificity, phage-host interactions, proteomics, and genomics of phages are needed to establish their usefulness and utilization. Efficacy of phage therapy was shown to prevent mortality of the *vibriosis*-infected brine shrimp *Artemia franciscana* [97–99]. Application of single-type/cocktails of phages against *V. parahaemolyticus*- and *V. harveyi*-infected cysts and nauplii *Artemia franciscana* showed a drastic improvement in the survival rate (from 85 to 89%) and hatching success (100% in both cases) in groups treated with phages, whereas the control groups exhibited a survival rate of 40–50% and hatching success (50%). These studies indicate that the phage cocktails offer an alternative to chemotherapeutic agents and can be used in brine shrimp production.

3. Conclusions

The callous use of antibiotics against bacterial diseases in the culture of crustaceans in the aquatic system has led to the development of antibiotic-resistant bacterial infections which can be a serious threat to all life forms and public health, and the phage therapy may help to overcome such complex problems. Control of bacterial diseases in the future may depend on development of novel drugs, innovative approaches, and management practices to minimize the risk of introduction of infectious agents into aquaculture systems and to reduce predisposing factors. The occurrences of lytic bacteriophages of *Bacillus* spp./*Vibrio* spp. as well as their efficacy were established. Phage therapy has been shown to inhibit the growth and multiplication of many different pathogenic bacteria, viz. *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. furnissii*, *V. mimicus*, *V. damsel*, and *V. anguillarum*, and promote better survival and production of the crustaceans in aquaculture. Infected live feed *Artemia* is responsible for the establishment of the bacterial infections in the hatchery and aquaculture system causing mass mortality of the larval crustaceans in culture, and such bacterial infections can be controlled through lytic phages, viz. ϕH17-5c, ϕH17-7b, ϕH17-8b, and ϕH17-9b from *V. harveyi*; phages VV1, VV2, VV3, and VV4 from *V. vulnificus*; and φSt2 and φGrn1 of *V. alginolyticus* of *A. salina*. However development of specific cocktails of phage therapy for aquaculture of crabs, prawns, lobsters, and crabs is desirable. The phage therapy is an ideal method for the control of microbial infections that occur in dissimilar organisms at various stages from eggs to brood stock as well as in laboratory, tanks, or field applications, and extending such research to other organisms could be one of the valuable strategies, to provide evidences and validate the usefulness and therapeutic potential of the phage therapy. An understanding of the infection processes, phage resistance, the efficacy of phage therapy on the targeted pathogens, and their impact on the normal microbiome can help to improve biocontrol strategies. The potential applications of the phages for various purposes outside aquaculture and further research on the specificity and phage-host interactions are needed to establish their usefulness and exploitation. Future work may be

carried out on the intricacies of phage lifestyles and their dynamics in natural systems, genome and viriomes, proteome analysis, genes coding for their proteins, and DNA polymerase phylogeny, which can help us in identifying novel methods of phage-host interaction and in understanding the way in which phages control their hosts. The use of probiotic organisms and phage therapy in crustacean aquaculture is found to be safe for the consumer of shrimp, and therefore a scientific rational approach is needed to evolve guidelines for phage therapy/probiotic applications in aquaculture and for their use and safety.

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Conflict of interest

The author declares that there is no financial or conflict of interests.

Author's contribution

The author (Dr P. Ramasamy) has made a significant contribution to the conception, design, and execution of the reported study and drafted the manuscript writing and discussion.

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