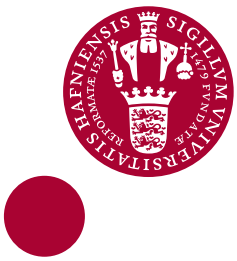

Bacteriophages in the control of pathogenic vibrios

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Review

Bacteriophages in the control of pathogenic vibrios

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ABSTRACT

Vibrios are common inhabitants of marine and estuarine environments. Some of them can be pathogenic to humans and/or marine animals using a broad repertory of virulence factors. Lately, several reports have indicated that the incidence of *Vibrio* infections in humans is rising and also in animals constitute a continuing threat for aquaculture. Moreover, the continuous use of antibiotics has been accompanied by an emergence of antibiotic resistance in *Vibrio* species, implying a necessity for efficient treatments. One promising alternative that emerges is the use of lytic bacteriophages; however, there are some drawbacks that should be overcome to make phage therapy a widely accepted method. In this work, we discuss about the major pathogenic *Vibrio* species and the progress, benefits and disadvantages that have been detected during the experimental use of bacteriophages to their control.

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1. Introduction

Vibrios are Gram-negative bacteria that can be found in marine and estuarine environments. This genus comprises several pathogenic species for humans and animals. The most clinically important pathogens for humans are *Vibrio cholerae* [1], *V. parahaemolyticus* [2] and *V. vulnificus* [3]; however, other species such as *V. fluvialis* and *V. mimicus* have been also associated with clinical cases [4,5]. *V. cholerae* is responsible for several large outbreaks of cholera, including Haiti in 2011 [6], while *V. parahaemolyticus*, although is able to cause severe mortality in aquatic animal species [7,8], in this case will be considered as human pathogen since is a major cause of severe diarrhea

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and human acute gastroenteritis worldwide [2]. *V. parahaemolyticus* is also the most common non-cholera *Vibrio* species reported to cause infection. However, the most lethal food-transmitted pathogen in USA and possibly in the world is *V. vulnificus* [3].

On the other hand, the major pathogenic vibrios for animals are *V. anguillarum*, *V. ordalii* and *Vibrio harveyi*. The first two are the ones responsible of classic vibriosis that can affect more than 50 species of marine animals [9,10], while the latter is a recurrent pathogen for aquaculture industry associated with warm waters [11]. There are also other controversial species such as *V. alginolyticus* because strains of this species, in addition to being reported as human emerging pathogen [12,13], and pathogenic for marine animals [14,15], while other have been suggested for potential use as probiotics in aquaculture [16,17]. In this case this species will be considered as marine animal pathogen.

Similarly to other animal production industries, antibiotics are used in aquaculture to control bacterial diseases, and even with prophylactic purposes. However, the use and abuse of antibiotics have led to the proliferation of multiples pathogens resistant to antibiotics. In 2014, the World Health Organization (WHO) has raised the alert against the antibiotic resistance [18], and vibrios are not the exception for this problem. Antibiotic resistance has been reported in several strains of this genus, from clinical and environmental origin [19,20,21,22]. The lack of effective treatments to control pathogenic vibrios resistant to antibiotics has led to the exploration of new alternatives. One of the most promising options is the use of lytic bacteriophages to kill pathogenic bacteria [23]. Bacteriophages are the most abundant biological entity on Earth [24,25], and they play a fundamental role in the evolution of bacteria [26,27]. Unlike antibiotics, bacteriophages are specific; therefore, their application will not disturb non-target bacterial species. Besides, they are not toxic and self-restricted, then, will remain in the environment only if the host bacteria are present [28].

This review summarizes the principal aspects of *Vibrio* as pathogens for humans and animals, as well as the principal advances, benefits and disadvantages in the use of bacteriophages to control these pathogenic bacteria. We discuss the main challenges that must be overcome in order to extend its applicability and to advance from an experimental alternative to a first choice treatment.

2. Principal pathogenic vibrios

2.1. Pathogenic vibrios in humans

There are at least twelve species of *Vibrio* which are known to be human pathogens. These species include *V. alginolyticus*, *V. cholerae*, *V. cincinnatiensis*, *V. damsela*, *V. fluvialis*, *V. furnisii*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* among others [1,2,3,5, 29,30]. They can cause three major syndromes of clinical illness, such as gastroenteritis, wound infections and septicemia, being the most common clinical manifestation a self-limiting gastroenteritis. *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* in a greater extent, and *V. alginolyticus, V. fluvialis* and *V. mimicus* in a lesser extent, are the most important in the clinical microbiology and food safety fields. These pathogens have diverse virulence factors to elicit illness in human, being *V. vulnificus* and *V. alginolyticus* primarily associated with extraintestinal infections [3,12] while *V. parahaemolyticus*, *V. mimicus* and *V. cholerae* are mainly related to gastroenteritis cases (Fig. 1) [2,31,32].

Unlike other *Vibrio spp.* which occur naturally in seafood, *V. cholerae* is primarily found in water or food sources contaminated with feces although it can also be found in the brackish river and coastal waters. At date, *V. cholerae* has been the most studied *Vibrio* due to its impact on public health and the severity of the cholera disease [1,31]. Among several virulence factors produced by this pathogen, the main ones are the cholera toxin (CT) [33], which is provided by a bacteriophage [34], the toxin co-regulated pilus (TCP) and others that facilitate its colonization in the intestine, all of them under the control of the ToxR regulon (Fig. 1) [35,36]. During infection, *V. cholerae* causes watery diarrhea, often fatal if untreated, and it is responsible for approximately between 3–5 million cases and over 100,000 deaths each year around the world according to the Center for Disease Control and Prevention (CDC) in 2017 [37].

The most common non-cholera *Vibrio* infection reported is *V. parahaemolyticus* [2,38]. Human infections caused by these bacteria are mainly produced after the consumption of raw or undercooked shellfish; only in the USA, this pathogen causes 45,000 illnesses each year. In fact, since 1996, the appearances of the pandemic clone O3:K6 caused a worldwide pandemic outbreak reaching Southeast Asia, Peru,

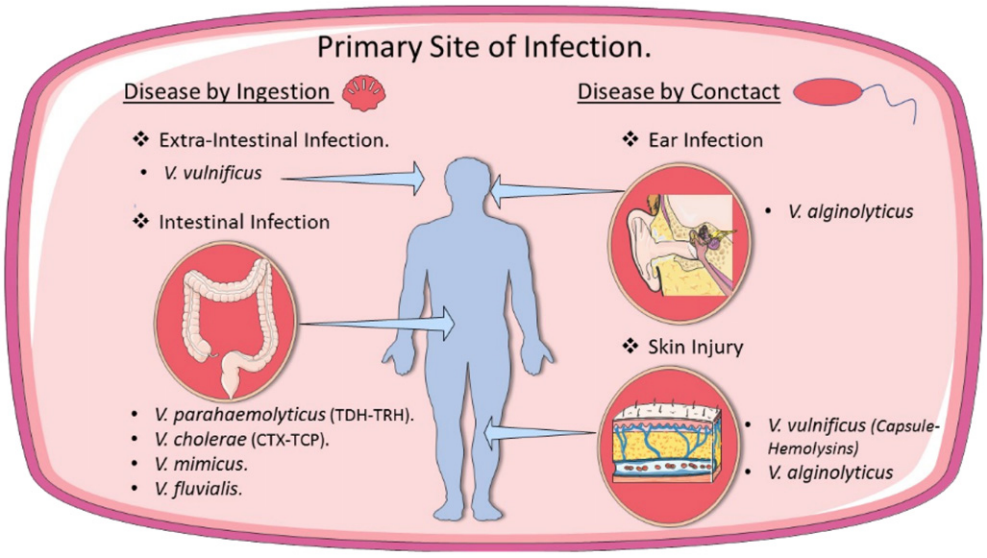

Fig. 1. Primary site of infection of different pathogenic *Vibrio* affecting humans. There are several species of pathogenic vibrios infecting humans. Some of them such as *V. cholerae* or *V. parahaemolyticus* are well characterized and their principal virulence factors have been identified while other species such as *V. mimicus* or *V. alginolyticus* are considered emergent pathogens. Infections produced by vibrios can be acquired by ingestion of contaminated food or direct contact with the bacteria, colonizing different sites in human body.

Chile, EU and USA [38,39,40,41]. Recently, others clonal complexes of Asiatic origin have also caused diarrhea outbreaks around the world [42,43]. Virulence in this species is associated to adhesins, various secretion systems, a thermostable direct hemolysin (TDH) and a TDH-related hemolysin called TRH (Fig. 1); which collaborate to produce the illness [2,38,44]; however, these genes have been found in other species [45]. The diarrhea produced by *V. parahaemolyticus* is self-limiting therefore there are several non-reported cases, even in countries with dedicated surveillance for this pathogen [46]. Rarely, *V. parahaemolyticus* can also provoke wound infections in which cases the use of antibiotics is frequently required.

Finally, *V. vulnificus* is also a relevant pathogen in clinical microbiology being the responsible of up to 94% of deaths related to infections produced by non-cholera *Vibrio* [3,47]. It possesses a repertory of virulence factors related to cytotoxicity, motility, capsule, hemolysins and expression of proteins involved in attachment and adhesion (Fig. 1). All of them are require to be expressed in a concerted manner for pathogenesis [3,47]. This bacterium is found in oysters, shellfish and warm marine waters; thus, similarly to *V. parahaemolyticus*, the risk of infection occurs when people eat raw or uncooked seafood, or when they are bathing in the sea having a cut or scratch. However, in this case the primary septicemia produced by this pathogen represents a mortality rate close to 50% in USA and therefore is considered the most lethal food-transmitted pathogen in that country, and possibly in the world [3,47].

Other less recurrent *Vibrio* pathogens are *V. mimicus* and *V. fluvialis*. The first mimics *V. cholerae* in many biochemical tests (hence its name), but do not cause epidemic cholera-like disease and less than 10% of the clinical isolates produce toxin [48]. This species carries various virulence factors that have been previously reported in other *Vibrio* species such as genes coding for ToxR, ToxS, and a type III secretion system, and it has been suggested that *V. mimicus* could be a gene reservoir for other *Vibrio* pathogens in the environment [49,50]. On the other hand, although *V. fluvialis* is an emerging foodborne pathogen over the world, generating large outbreaks in Bangladesh and India, and is occasionally reported in USA. However, its molecular epidemiological features still remain mostly unknown, and only potential virulence factors have been proposed in genetic studies [29].

Finally, *V. alginolyticus* is mainly recognized as a pathogen for fish; however, recent epidemiological data suggest an increase in the incidence of human infections. The documented cases are mainly associated to otitis and wound infections which may result from exposure of cuts or scratch to contaminated seawater; however, there are increasing reports associated to infections with this pathogen due to consumption of contaminated food [12]. The role of this species as pathogen for animals will be discussed in the next sections of this review.

Currently, several reports indicate that the incidence of human *Vibrio* infections is increasing in the United States and other countries [51]. It has been also observed a rising incidence of antimicrobial resistant pathogenic bacteria in shellfish, including *Vibrio* species [19, 21]. Many studies have reported different *Vibrio* pathogens with resistance to ampicillin, penicillin G, streptomycin, carbenicillin, kanamycin, cefalotin, sulfadiazine-trimethoprim, chloramphenicol, erythromycin, ciprofloxacin, polymyxin B, azithromycin, sulfamethoxazole, tetracycline and quinolones [19,21,52,53]. This situation has motivated the exploration of new alternatives to conventional treatments with antibiotics, especially for the multiple antibiotic resistant strains.

2.2. Pathogenic vibrios in animals

Several *Vibrio* species are also important pathogens for aquaculture industry, especially in fish farm, shellfish hatchery and wild shrimp [54,55]. The more relevant are *V. anguillarum*, *V. ordalii* and *V. harveyi*. The two first are mainly associated to fish infection while the latter is a major pathogen in shrimp (Fig. 2) [9,10,11].

Both *V. anguillarum* and *V. ordalli* are causative agents of a hemorrhagic septicemia known as classical vibriosis in marine and freshwater fish [9,10,54,56]. *V. anguillarum* is known to infect several fish species including various species of economic importance in the larviculture and aquaculture industry, including salmonids [54,57]. Although more than 20 serotypes have been identified for this species, only serotypes O1, O2 and O3 are associated with vibriosis [54,58,59]. The pathogenesis of *V. anguillarum* is multifactorial and highly complex requiring multiple crucial virulence determinants, including those involved in chemotaxis, motility, iron uptake system, hemolysins, a quorum-sensing system (QS) and sigma factor regulators RpoS and RpoN among others [9,60]. *V. ordalii* is genetically closely related to *V. anguillarum* [61,62] and vibriosis generated by them can result in 90% mortality if it is not controlled (FAO 1990). In the North Atlantic area, the impact of vibriosis in the salmonid industry has been reduced due to the development of vaccination procedures [63]; however, this remains a significant problem in farmed fish in Europe and Asia [64,65,66].

V. harveyi is widely distributed in the marine environment, either as free-living form or associated with marine animals. This pathogen is responsible of the so called luminous vibriosis infecting a great variety of aquatic animals including shrimps, finfish and mollusk, leading to severe economic losses [11]. As in many *Vibrio*, virulence in this species is QS-regulated, modulating virulence factors such as biofilm formation, motility, production of siderophore, extracellular products and type III secretion system [67,68]. There are also reports connecting virulence in this bacterium to the presence of a temperate bacteriophage [69,70]. Other two *Vibrio* species associated to luminous vibriosis are *V. campbellii* and *V. owensii*, both are closely related to *V. harveyi* and therefore frequently misidentified (Fig. 2) [71]. The virulence mechanisms of *V. owensii* are largely unknown, but it is considered virulent because it causes mortality in *Penaeus monodon* [72]. These three species trigger bioluminescent vibriosis through numerous associated virulence factors including toxic extracellular proteins such as proteases, hemolysins and cysteine proteases, siderophores, bacteriocins resistance plasmids and chitinases [67,73,74].

V. alginolyticus belongs to the so called *harveyi* clade [67]. This bacterium has been involved infections in humans [12], but it is mostly recognized as an aquaculture pathogen, causing severe mortalities in shellfish and crustaceans, particularly shrimps. Among their virulence factors repertory is possible to find lipases, proteases, siderophores and even a TDH [45,75]. Similarly, although *V. parahaemolyticus* is mainly recognized as a human pathogen, it has been also reported as the causing agent of acute hepatopancreatic necrosis disease (AHPND) which affect multiple shrimp species, such as *P. vannamei* and *P. monodon.* This diseases was first reported in 2009 in China and since then has been detected Malaysia, Thailand, Philippines and also in Mexico generating important economic losses [76,77]. The pathology of this disease is still unclear, but it has been associated to *V. parahaemolyticus* strains that harbor a specific plasmid which encode for a binary toxin $PirAB^{vp}$ [78].

Other pathogenic *Vibrio* that can cause mortality events in aquatic animals are *V. tubiashii*, which has been reported in shellfish hatcheries on USA and Chile affecting species like larval pacific oyster (*Crassostrea gigas*), Kumamoto oyster (*Crassostrea sikamea*) and Geodric clams (*Panope abrupta*) [79,80]; *V. coralliilyticus*, closely related to the previous one [81], is a well-known pathogen for different coral species, and has been recently associated with disease in variety species of fish and shellfish, including oyster larvae, bivalves larvae, great scallop (*Pecten maximus*) and rainbow trout (*Oncorhynchus mykiss*) [82,83,84]. The list of pathogenic *Vibrio* for marine species is very large and can include also *Vibrio rotiferianus* in *O. mykiss*

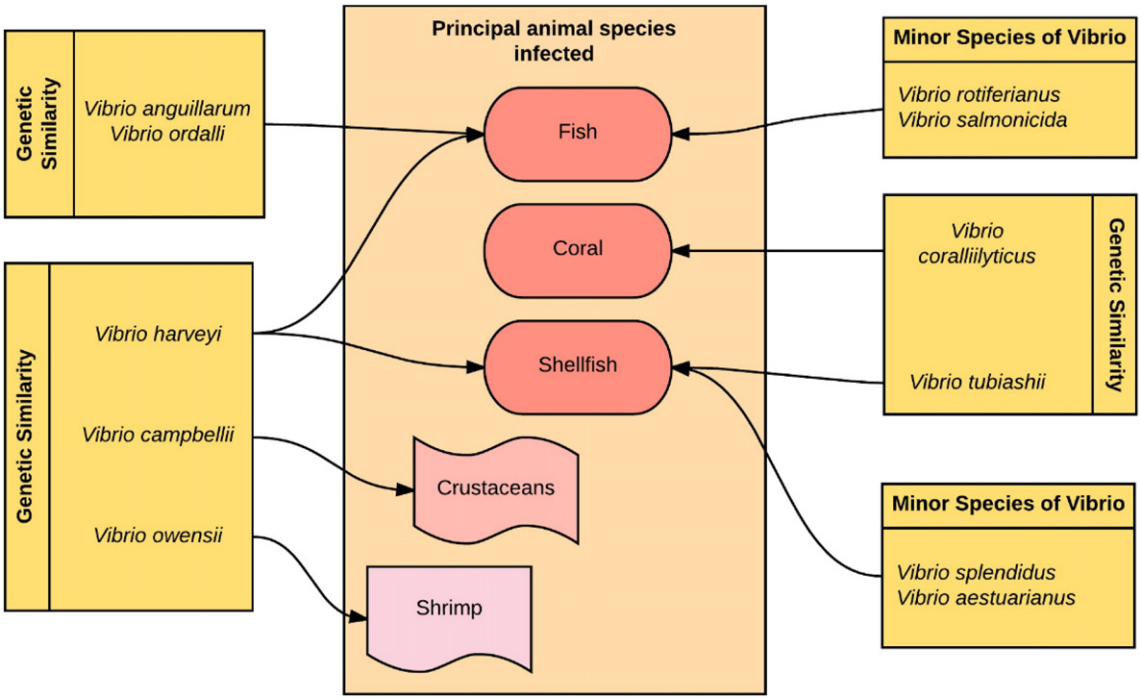

Fig. 2. Principal *Vibrio* species pathogenic for aquatic animals and principal type of host. The list of pathogenic vibrios infecting aquatic animals is very large. *V. anguillarum* and *V. harveyi* are the most important species due to their impact in Aquaculture industry, because they infect different species of fish and shellfish of economic importance. Some of these species, such as *V. anguillarum* and *V. ordalii*, or *V. coralliilyticus* and *V. tubiashii*, are very close related, making their identification difficult.

and *Artemia nauplii* [84], *Vibrio splendidus* and *Vibrio aestuarianus* in *C. gigas* [85] among others (Fig. 2).

Similarly to the situation with pathogenic vibrios for humans, in this case antibiotics have been the first line treatment to control these pathogens. The extensive use of antibiotics in the aquaculture industry has raised the concern about the occurrence of antibiotic resistant pathogens [86]. Several *Vibrio* species resistant to antibiotics have been reported [19,20,21], and some cases the lack of effectiveness of these antimicrobials has led to massive mortalities in shrimp aquaculture [87,88]. In this regard, the implementation of new alternatives, such as bacteriophages or vaccines, to control pathogenic vibrios represents an important step in the transition to a more sustainable aquaculture industry.

3. Bacteriophages for controlling pathogenic vibrios

3.1. Phage therapy to control pathogenic vibrios infecting humans

The first report about the use of phages to control a pathogenic *Vibrio* in humans was against *V. cholerae*, and it was described by Felix d'Herelle. During this work, when the cholera-patients were treated with oral doses of bacteriophage the mortality rate was 8.1%, while in the control patients treated with other medicines it was 62.9%. The mortality rate in the phage treated group was zero if treatment occurred within 6 h of appearance of the first symptoms [89]. In parallel, Asheshov performed a similar experience in different locations but with conflicting results. While in one location the treatment was successful, the phages treatment did not work in the other location. The authors mentioned that although the phage was able to arrest the progress of disease it was more effective used with prophylactic rather than a therapeutic purposes [90]. Later in the years 1958–1960, animal passaged phage preparations were successfully used in treating cholera-patients. An initial intravenous or intramuscular phage doses with saline buffer followed by oral doses for 3 d displayed positive results [91]. Despite the successful experiences, all the studies concluded that to understand better the nature of bacteriophage-host interactions *in vivo*, a good animal model of cholera phage therapy is needed.

The use of animal models has been very important to study the phage therapy to control *V. cholerae* [92,93] especially, considering that it is not ethically acceptable to experimentally infect humans with bacterial pathogens for trials purposes (Table 1). Nowadays, rabbits and mice are used like phage therapy models [92,93,94]. The first challenge using this approach inoculated rabbits with 10^9 CFU/mL of *V. cholerae* strain MAK 757 in each of the six controls and phage treated rabbits. In the phage-treated rabbits, besides the bacteria, they gave 10^8 PFU/mL cocktail of phages. Those animals developed mild to low diarrhea, and fewer pathological changes in the intestine than non-phage treated individuals. The authors concluded that this study was the first direct indication of phage multiplication in an open system such as the intestine infected by a *V. cholerae* strain. Same group later used an adult mouse model to test different oral approach to treat *V. cholerae* infection, including cocktail phages against the bacteria and antibiotics [95]. Daily application of both cocktail of phages administered at the MOI (multiplicity of infection) of 0.1 (1×10^8 PFU/mL) and ciprofloxacin antibiotic (40 mg/kg) were effective in the reduction of bacterial load, although the bacterial load reduction was greater in antibiotic treated animals. Recently, Yen et al. proved the prophylactic efficacy of a cocktail of three phages named ICP1, ICP2 and ICP3 in mice (Table 1). The results showed that oral administration of phages up to 24 h before infection with *V. cholerae* reduce the bacterial colonization in the intestinal tract and prevents cholera-like diarrhea [91]. These results suggest that phages can be effective against *V. cholerae* as a prophylactic or as a treatment.

There are several reports about phages infecting *V. parahaemolyticus* [96,97,98,99]; however, their use in phage therapy to control infections in humans have been less explored, and always used on animal models (Table 1). Recently, the therapeutic potential of a phage named pVp-1 was studied in a mouse model using a multiple-antibiotic-resistant *V. parahaemolyticus* O3:K6 pandemic clinical strain [100]. They

Table 1
Phage therapy challenges in different *Vibrio* species pathogenic for humans.

Vibrio species	Challenge model	Type of phage application (PFU/mL)	Results	Reference
V. cholerae	Human	Oral (unknown)	Protection	[86]
V. cholerae	Human	Oral (unknown)	Protection	[105]
V. cholerae	Human	Oral (unknown)	Relative protection	[106]
V. cholerae	Human	Oral, after animal passage (unknown)	Protection	[88]
V. cholerae	Human	Oral (10^{12})	Protection	[107]
V. cholerae	Human	Oral (10^{8}–10^{9})	Protection	[107]
V. cholerae	Adult rabbit	Inoculation into ileal loop (10^{11})	Unsuccessful	[108]
V. cholerae	Adult rabbit	Oral (10^{8})	Protection	[91]
V. cholerae	Adult mice	Oral (10^{8})	Protection	[92]
V. cholerae	Infant mouse Infant rabbit	Oral (10^{9})	Bacterial load reduction in mouse Protection in rabbit	[89]
V. parahaemolyticus	Adult mouse	Oral (10^{8})	Protection	[97]
V. parahaemolyticus	Oyster	Bath (10^{8})	Bacterial load reduction	[98]
V. vulnificus	Adult mice	Intravenous (10^{8})	Protection	[99]
V. vulnificus	Oyster	Bath[a]	Bacterial load reduction	[100]

[a] Phage concentration was not specified.

monitored the survivability, histopathological changes, quantified the bacterial and phage titers during phage therapy and observed the immune response induced by phage burst. The results showed that phage-treated mice presented protection from a *V. parahaemolyticus* infection and survived lethal doses of oral and intraperitoneal bacterial challenges. Despite the successful results, the authors emphasized the need to establish adequate phage preparation methodologies such as the purification and removal of endotoxins for safety in phage therapy to prevent anaphylactic responses. Same authors with different approach used the same phage pVp-1 to avoid *V. parahaemolyticus* infection due to consumption of raw contaminated seafood, especially oysters. In this case the authors designed an artificial contamination model simulating potentially contamination events during oyster processing [101]. This method showed that bacterial growth can be reduced five orders of magnitude when phages were added through bath immersion and six orders of magnitude when phages were added over the surface of the samples. In both cases, bacteria were added prior to phage treatment indicating phages could be efficient even after the pathogen started the infection.

Finally, in the case of *V. vulnificus* phage therapy has been driven using an infection model of iron-dextran-treated mice [102]. The animals were injected subcutaneously with 10^{6} CFU (10 times the lethal dose of *V. vulnificus*), while phages were administered at doses of 10^{8} PFU through intravenous injection, either simultaneously or at various times after infection. The authors showed that phage treatment has therapeutic potential for both localized and systematics infections preventing both local and systemic disease reaching the optimal protective effect when administered within 3-h post bacterial infection. Interestingly, only two out of three phages tested were effective in normal conditions. The third phage was able to lyse the bacteria only in presence of seawater, and was ineffective to protect mice during the challenges. Similarly to the case of *V. parahaemolyticus*, bacteriophages have been also used to reduce the load of *V. vulnificus* in extracts of eastern oyster (*Crassostrea virginica*) [103]. In this case, the oyster extract also have antimicrobial properties against this bacterium, and the combined effect with bacteriophages allow a bacterial load reduction from 10^{6} to 10^{1} CFU/mL after 18-h incubation at 4°C. These results add evidence that phage therapy is a viable alternative treatment for human *V. vulnificus* infections or seafood depuration. However, these also remark the importance of study the effectiveness of different phages and administration conditions for phage therapy like the proper time for phage addition.

There are other *Vibrio* species considered opportunistic pathogens in humans such as *V. fluvialis* [4] or *V. furnissii* [104] for which no phage therapy studies have been conducted yet. However, vibriophages infecting and controlling these bacteria have been isolated and characterized. For example, bacteriophages infecting *V. fluvialis*, which is considered as an emerging human pathogen [3], were characterized for bacterial typing purposes but candidates for phage therapy have remained unexplored [105]. Phages infecting *Vibrio* species in the environment have been well documented [99,106,107]. Thus, it is possible to expect new studies about phage therapy in these emerging *Vibrio* pathogens.

3.2. Phage therapy to control pathogenic vibrios infecting animals

Most of the animal species infected by vibrios reported are related to aquaculture industry. Therefore the vast majority of examples presented are associated with this productive area. Fish, mollusks and crustaceans of economic importance can be infected by *Vibrio* species such as *V. harveyi*, *V. anguillarum* and the close related species *V. ordalii*, *V. splendidus*, *V. coralliilyticus* and more recently *V. cyclitrophicus*. Among them, *V. harveyi* is the most common target. To date, there are several articles about isolation and characterization of *V. harveyi* phages or about the use of phages to control this bacterium where crustaceans are the preferred infection model [106, 112,113,114,115,116,117].

In 2000, Oakey and Owens described the phage VHML able to infect *V. harveyi* [118]. However, this phage was not suitable for phage therapy since it was shown that its presence may confer virulence to several strain of *V. harveyi* [70]. Later on, in 2006, bacteriophages isolated from shrimp farm waters demonstrated to increase the viability of *P. monodon* larvae infected with *V. harveyi* up to 80% in comparison to 25% of larvae without phages [112]. This was the first attempt to demonstrate the potential use of bacteriophages to control *Vibrio* pathogens in aquaculture. Afterwards similar approach was used with different *Vibrio* species (Table 2). By contrast to what happen with vibrios infecting humans, in this case most of the challenges have been done using the actual host of the bacteria and in conditions equal or similar to aquaculture farms [112,116,119,120,121], these include phage therapy assays against *V. splendidus* using sea cucumber (*Apostichopus japonicus*) farming [121] and assays against *V. coralliilyticus* using the non-commercial coral host (*Acropora millepora)* (Table 2) [119]. In the case of *V. splendidus*, three bacteriophages named PVS-1, PVS-2 and PVS-3 were able to inhibit the growth of the host and other 3 *Vibrio* species. A cocktail of these phages increases the survival of sea cucumber infected with *V. splendidus* from 18% to 82% in the phage-treated condition, which was indistinguishable to antibiotic-treated sea cucumber [121]. A similar approach was used by same authors to prevent infections with *V. cyclitrophicus* [122]. In this case, a single bacteriophage named vB_VcyS_Vc1 was able to increase the survival rate of juvenile

Table 2
Examples of phage therapy in *Vibrio* species pathogenic to animals.

Vibrio species	Challenge model	Type of phage application (PFU/mL)	Results	Reference
Vibrio harveyi	*Penaeus monodon*	Batch (10^9)	Increased animal survival	[109]
Vibrio harveyi	*Penaeus monodon*	Direct addition (10^6)	Increased larvae survival than antibiotic treatment	[111]
Vibrio harveyi	Shrimp post larvae	Direct addition (10^{10})	Increased shrimp survival	[113]
Vibrio harveyi	*Haliotis laevigata*	Bath (10^2)	70% increase animal survival	[114]
Vibrio splendidus	Sea cucumber *(Apostichopus japonicus)*	Injection of Single and Cocktail (10^9)	Increased animal survival	[118]
Vibrio coralliilyticus	*Acropora millepora*	Bath (10^9)	Prevent photo inactivation and coral tissue lysis	[116]
Vibrio cyclitrophicus	Sea cucumber (*Apostichopus japonicus*)	Bath (10^5)	Increased animal survival	[119]
Vibrio alginolyticus	Sea cucumber (*Apostichopus japonicus*)	Direct addition (10^5–10^7)	Increased animal survival	[124]
Vibrio alginolyticus	*Artemia salina*	Bath Cocktail phage (10^6)	Increased animal survival	[117]
Vibrio anguillarum	*Salmo salar*	Direct addition (10^6)	Increased animal survival	[120]
Vibrio anguillarum	*Danio rerio* larvae	Direct addition (10^8)	Moderate increase in larvae survival	[121]

sea cucumber from 18% to 81%, 58% and 63% when the phages were added through food, injection or bath immersion respectively, evidencing that the method for phage administration can be determining in the results obtained.

Phages have been also used to protect against *V. anguillarum* infections. In 2013, Higuera et al. [123] showed that a phage named CHOED was able to increase *Salmo salar* fish survival infected with the bacteria from 10 to even 100% rates in controlled conditions. Moreover, in aquaculture conditions, the phage was able to increase survival of fishes from 60 to 100% rates after a 20-d challenge; this was the first successful attempt to demonstrate the use of bacteriophages to control *Vibrio* pathogens in salmonids [123]. Similar results were obtained by Silva et al. but this time using zebrafish larvae as infection model [124]. In this case, the mortality rate observed in the larvae infected with the bacteria plus phage was less than 3% and was indistinguishable from the control condition (non-infected and without phages), while the infected larvae without phage addition showed a mortality rate of 17%. The lower mortality observed is probably because zebrafish is not a common host for *V. anguillarum*. However, these results suggest that phage therapy can be an alternative to protect fish against these bacteria in different developmental stages.

Besides the standard approach to phage therapy, that determines if phages can protect against bacterial infection. There are several reports about different factors that may influence and be important for the success of phages to control pathogenic bacteria. For instance, *V. harveyi* have been the subjects of studies focused on determine the effect of dissolved solids and temperature in phage therapy experiments, or to determine if phages are able to inhibit the biofilm formation in this bacterium [114,125]. Another example included experiments to test if the isolated phages against *V. harveyi* were lytic against potentially beneficial bacteria [126]. This issue is very relevant to assure the safety of phage application because in the ideal scenario the normal microbiota from the animal should not be disturbed.

A final interesting case is *V. alginolyticus*, since it has been reported as a pathogen for animals [14,15] and humans [12,13]. Zhang et al. showed in 2015 that bacteriophages against this species were able to increase the survival of sea cucumber (*A. japonicus*) from 3% in untreated-phage individuals up to 73% when phages were added at MOI of 10 [127]. More recently, it was reported that two bacteriophages ϕSt2 and ϕGrn1 against the *V. alginolyticus* strain V1 were able to significantly reduce the total *Vibrio* load in *Artemia salina* cultures [120]. However, there are reports suggesting that specific strains of this species can be used as probiotics in shrimp culture [16,17], evidencing the extreme diversity of this genus and species [128].

All these examples suggest that phage therapy can be an excellent alternative to control pathogenic vibrios, both from humans and animals. However, this approach is not widely used yet and is mainly still in a research stage. In the next section, we explore which are the main challenges ahead in order to reach efficient treatments against vibrios using bacteriophages.

3.3. Future challenges in phage therapy

To date, a search in PubMed with the words “phage”, “therapy” and “Vibrio” shows 40 results. Additionally, there are 1879 patents or patents in progress about the use of bacteriophages and its possible use in phage therapy [129]. Moreover, currently there are several commercially available products based in bacteriophages. However, unlike Eastern Europe, the use of bacteriophage as antimicrobials is still in development and subject to general discussion. There are still some areas that require more development and studies. These are related mainly to the proliferation of resistant bacteria, methods of administration, and a regulatory frame for products based on bacteriophages.

Bacteriophage resistance was reported soon after the discovery of bacteriophages, and since then, has been subject of several studies [130,131]. In the context of phage therapy, several alternatives have been proposed to overcome this problem, such as the use of bacteriophage cocktails [93,132]. The normal frequency of resistant bacteria appearance is between 10^{-6} and 10^{-8} then, if the bacteriophages used in the cocktail have different routes of infection, the probability of proliferation of resistant bacteria will be reduced to around 10^{-14} or even less depending on how many phages are used in the cocktail. In 2012, Gu et al. reported a method to generate cocktail of bacteriophages for use in phage therapy which reduce the probabilities of resistant bacteria proliferation [133]. This method consisted in sequential isolation of new bacteriophages against the bacteriophage resistant variants of the host *Klebsiella pneumoniae*. Each new phage isolated will target a variant of *K. pneumoniae* derived from the original host and resistant to the last bacteriophage isolated. In this way, they generated a cocktail composed by bacteriophages able to infect all the possible resistant variants of the original host. This method has great potential against single pathogens, however, can be very laborious if the targets are multiple pathogens or different strains of the same bacteria. Other authors have proposed the optimization of bacteriophage cocktails studying potential interactions between phages to predict synergisms or interference between phages in cocktails [134]. While these efforts try to avoid the proliferation of resistant bacteria other studies are focus in the characterization of resistant bacteria. Several reports show that bacteriophage resistant strains can have a reduction in their virulence probably as a consequence of the acquired resistance [130,135,136]; however, this situation is not common to all bacteria [137].

In recent years, numerous researches have been focused in CRISPR-cas system (For further details see [138]); this so called bacterial immune system is widely spread in bacterial species [139], including *Vibrio* [140,141,142] and therefore can be considered a big obstacle for phage therapy because bacterial pathogens can acquire resistance after multiples treatments with the same phage. Fortunately, for phage therapy enthusiast, phages have evolved different mechanisms to avoid or repress the CRISPR-cas system. Five genes with anti-CRISPR activity were described in bacteriophages against *Pseudomonas aeruginosa* and homologs to these genes were

found in other genetic mobile elements from the same species [143, 144]. Bacteria and bacteriophages have a history of co-evolution, then for each defense mechanisms generated by bacteria, bacteriophages will develop a strategy to surpass the defense.

Another challenge for phage therapy is to develop efficient mechanisms for phage administration depending on where the phages will be applied. For vibrios, the alternatives are therapy in humans and aquaculture systems. In 2006, the FDA recognize a bacteriophage preparation against *Listeria monocytogenes* as GRAS (Generally Recognized As Safe GRAS Notice 000198) authorizing its addition to ready-to-eat food. Since then, this status has been granted to five others preparations, recognizing they represent no risk for human health. Concerning actual trials of phage therapy in humans, there are reports from 1930 in India about the use of bacteriophages to control cholera outbreaks [110,145], but most of the experience comes from Eastern Europe countries, especially Georgia where the use of bacteriophages is part of the National Health System [146]. In most of the cases, bacteriophages have been applied over the skin to treat wound infections or orally for systemic diseases [147]. In the first case, phages can be applied directly over the wound or through phage soaked dressings complementarily to wound care treatments in order to get successful results. In the second case bacteriophages can reach a systemic distribution being able even to cross the blood–brain barrier [132,147]. In this situation, the main obstacle is the acidic environment of gastric fluids which can affect the viability of the phages. The encapsulation with alginate beads and other polymers can be a potential solution for this problem as has been proposed previously [148,149]. Other option explored is the utilization of bacteriophages from the same environment where they will be used [150].

The other main field of application of bacteriophages regarding *Vibrio spp.* is aquaculture. In this case, bacteriophages have been applied directly to the water [112,114,123], through intraperitoneal injection [151,152] or embedded with food [153,154]. All these three methods can have strengths and weakness depending on the aquaculture system. For example, application to the water can be the easiest way in recirculation aquaculture systems (RAS), however is not suitable for open water systems. On the other hand, the intraperitoneal injection can be a time-consuming method while application of phage-embedded food could affect bacteriophage release, viability, or even the food consumption by the cultured species. Therefore, each case should be considered particularly depending on the aquaculture system and the nature of the bacteriophages to apply.

Finally, another major obstacle for phage therapy is to deal with the lack of a specific regulatory frame designed considering the special nature of bacteriophages. Despite that bacteriophages are considered to use as antimicrobials, they have special features as self-replication, self-restriction and no toxicity [155,156], and therefore cannot be classified or regulate as antibiotics. The lack of knowledge and regulation had to lead to bacteriophages to be classified as different substances hampering clinical trials [157]. This situation has motivated a group of important researchers from Europe to claim for adequate regulations generating efficient treatments using bacteriophages [158].

4. Conclusion

Among the genus *Vibrio*, there are important bacterial species that can be pathogenic for humans and economically important animals. Worryingly, several reports indicate that the occurrence of *Vibrio* infections is increasing. Parallel, a rising incidence of antimicrobial resistant pathogenic bacteria has been observed. The evidence summarized in this work suggests that bacteriophage can be considered as a consistent alternative to control pathogenic vibrios, especially in the antibiotic resistance era. However, in spite of the information and the experience generated, there are still some drawbacks that must be overcome in order to generate safe, efficient and reproducible treatments. The achieve of these goals require joint efforts from researchers, but also from governing entities which must implement adequate regulations that allow generating reliable and efficient treatments oriented to replace or reduce the use and misuse of antibiotics.

Conflict of interest statement

This research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

[1] Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 1998;62(4):1301–14.
[2] Levin RE. *Vibrio parahaemolyticus*, a notably lethal human pathogen derived from seafood: a review of its pathogenicity, characteristics, subspecies characterization, and molecular methods of detection. Food Biotechnol 2006;20(1):93–128. https://doi.org/10.1080/08905430500524275.
[3] Oliver JD. The biology of *Vibrio vulnificus*. Microbiol Spectr 2015;3(3). https://doi.org/10.1128/microbiolspec.VE-0001-2014.
[4] Ramamurthy T, Chowdhury G, Pazhani GP, et al. *Vibrio fluvialis*: an emerging human pathogen. Front Microbiol 2014;5:1–8. https://doi.org/10.3389/fmicb.2014.00091.
[5] Chitov T, Kirikaew P, Yungyune P, et al. An incidence of large foodborne outbreak associated with *Vibrio mimicus*. Eur J Clin Microbiol Infect Dis 2009;28(4):421–4. https://doi.org/10.1007/s10096-008-0639-7.
[6] Chin C-S, Sorenson J, Harris JB, et al. The origin of the Haitian cholera outbreak strain. N Engl J Med 2011;364:33–42. https://doi.org/10.1056/NEJMoa1012928.
[7] Kumar BK, Deekshit VK, Raj JRM, et al. Diversity of *Vibrio parahaemolyticus* associated with disease outbreak among cultured *Litopenaeus vannamei* (Pacific white shrimp) in India. Aquaculture 2014;433:247–51. https://doi.org/10.1016/j.aquaculture.2014.06.016.
[8] Chonsin K, Matsuda S, Theethakaew C, et al. Genetic diversity of *Vibrio parahaemolyticus* strains isolated from farmed Pacific white shrimp and ambient pond water affected by acute hepatopancreatic necrosis disease outbreak in Thailand. FEMS Microbiol Lett 2016;363:fnv222. https://doi.org/10.1093/femsle/fnv222.
[9] Hickey ME, Lee J-L. A comprehensive review of *Vibrio* (*Listonella*) *anguillarum*: ecology, pathology and prevention. Rev Aquac 2017. https://doi.org/10.1111/raq.12188.
[10] Ruiz P, Poblete-Morales M, Irgang R, et al. Survival behaviour and virulence of the fish pathogen *Vibrio ordalii* in seawater microcosms. Dis Aquat Organ 2016;120: 27–38. https://doi.org/10.3354/dao03005.
[11] Austin B, Zhang XH. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett Appl Microbiol 2006;43:119–24. https://doi.org/10.1111/j.1472-765X.2006.01989.x.
[12] Sabir M, Ennaji Moulay M, Cohen N. *Vibrio Alginolyticus*: an emerging pathogen of foodborne diseases. Int J Sci Technol 2013;2:302–9.
[13] Schmidt U, Chmel H, Cobbs C. *Vibrio alginolyticus* infections in humans. J Clin Microbiol 1979;10:666–8.
[14] Selvin J, Lipton AP. *Vibrio alginolyticus* associated with white spot disease of *Penaeus monodon*. Dis Aquat Organ 2003;57:147–50. https://doi.org/10.3354/dao057147.
[15] Balebona MC, Andreu MJ, Bordas MA, et al. Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.). Appl Environ Microbiol 1998;64: 4269–75.
[16] Rodríguez J, Cedeño R, Bayot B, et al. Effects of the *Vibrio alginolyticus* probiotic, E-1,3/1,6-glucans and temperature on shrimp production. Dyn Biochem Process Biotechnol Mol Biol 2011;5:49–55.
[17] Thompson J, Gregory S, Plummer S, et al. An *in vitro* and *in vivo* assessment of the potential of *Vibrio spp.* as probiotics for the Pacific white shrimp, *Litopenaeus vannamei*. J Appl Microbiol 2010;109(4):1177–87. https://doi.org/10.1111/j.1365-2672.2010.04743.x.
[18] World Health Organization (WHO). Antimicrobial resistance: global report on surveillance. WHO; 2014; 8.
[19] Kuma GK, Opintan JA, Sackey S, et al. Antibiotic resistant patterns amongst clinical *Vibrio cholerae* O1 isolates from the Greater Accra Region, Ghana-2013. Int J Infect Dis 2014;21:80. https://doi.org/10.1016/j.ijid.2014.03.594.
[20] Chowdhury MA, Aziz KM, Rahim Z, et al. Antibiotic resistance patterns of *Vibrio mimicus* isolated from human and environmental sources in Bangladesh. Antimicrob Agents Chemother 1986;30(4):622–3.
[21] Elmahdi S, DaSilva LV, Parveen S. Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: a review. Food Microbiol 2016;57: 128–34. https://doi.org/10.1016/j.fm.2016.02.008.
[22] Campos LC, Zahner V, Avelar KES, et al. Genetic diversity and antibiotic resistance of clinical and environmental *Vibrio cholerae* suggests that many serogroups are

reservoirs of resistance. Epidemiol Infect 2004;132(5):985–92. https://doi.org/10.1017/S0950268804002705.
[23] Clark JR. Bacteriophage therapy: history and future prospects. Future Virol 2015; 10:449–61. https://doi.org/10.2217/fvl.15.3.
[24] Suttle CA. Marine viruses-major players in the global ecosystem. Nat Rev Microbiol 2007;5(10):801–12. https://doi.org/10.1038/nrmicro1750.
[25] Fuhrman JA. Marine viruses and their biogeochemical and ecological effects. Nature 1999;399:541–8. https://doi.org/10.1038/21119.
[26] Salmond GPC, Fineran PC. A century of the phage: past, present and future. Nat Rev Microbiol 2015;13:777–86. https://doi.org/10.1038/nrmicro3564.
[27] Koskella B, Brockhurst MA. Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS Microbiol Rev 2014;38: 916–31. https://doi.org/10.1111/1574-6976.12072.
[28] Loc-Carrillo C, Abedon S. Pros and cons of phage therapy. Bacteriophage 2011;1(2): 111–4. https://doi.org/10.4161/bact.1.2.14590.
[29] Huang Y, Du P, Zhao M, et al. Functional characterization and conditional regulation of the type VI secretion system in *Vibrio fluvialis*. Front Microbiol 2017;8:528. https://doi.org/10.3389/fmicb.2017.00528.
[30] Economopoulou A, Chochlakis D, Almpan MA, et al. Environmental investigation for the presence of *Vibrio* species following a case of severe gastroenteritis in a touristic island. Environ Sci Pollut Res 2017;24:4835–40. https://doi.org/10.1007/s11356-016-8231-7.
[31] Charles RC, Ryan ET. Cholera in the 21st century. Curr Opin Infect Dis 2011;24: 472–7. https://doi.org/10.1097/QCO.0b013e32834a88af.
[32] Zago V, Zambon M, Civettini M, et al. Virulence-associated factors in *Vibrio cholerae* non-O1/non-O139 and *V. mimicus* strains isolated in ornamental fish species. J Fish Dis 2017;00:1–12. https://doi.org/10.1111/jfd.12659.
[33] Vanden Broeck D, Horvath C, De Wolf MJS. *Vibrio cholerae*: cholera toxin. Int J Biochem Cell Biol 2007;39:1771–5. https://doi.org/10.1016/j.biocel.2007.07.005.
[34] Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 1996;272(5270):1910–4. https://doi.org/10.1126/science.272.5270.1910.
[35] Childers BM, Klose KE. Regulation of virulence in *Vibrio cholerae*: the ToxR regulon. Future Microbiol 2007;2(3):335–44. https://doi.org/10.2217/17460913.2.3.335.
[36] Krebs SJ, Taylor RK. Protection and attachment of *Vibrio cholerae* mediated by the toxin-coregulated pilus in the infant mouse model. J Bacteriol 2011;193:5260–70. https://doi.org/10.1128/JB.00378-11.
[37] Center for Disease Control and Prevention. *Vibrio cholerae* infection. https://www.cdc.gov/cholera/general/; 2017.
[38] Yeung PSM, Boor KJ. Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. Foodborne Pathog Dis 2004;1(2):74–88. https://doi.org/10.1089/153531404323143594.
[39] García K, Bastías R, Higuera G, et al. Rise and fall of pandemic *Vibrio parahaemolyticus* serotype O3: K6 in southern Chile. Environ Microbiol 2013;15: 527–34. https://doi.org/10.1111/j.1462-2920.2012.02883.x.
[40] Harth E, Matsuda L, Hernández C, et al. Epidemiology of *Vibrio parahaemolyticus* outbreaks, southern Chile. Emerg Infect Dis 2009;15:163–8. https://doi.org/10.3201/eid1502.071269.
[41] Nair GB, Ramamurthy T, Bhattacharya SK, et al. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin Microbiol Rev 2007; 20:39–48. https://doi.org/10.1128/CMR.00025-06.
[42] Gonzalez-Escalona N, Gavilan RG, Toro M, et al. Outbreak of *Vibrio parahaemolyticus* sequence type 120, Peru, 2009. Emerg Infect Dis 2016;22(7):1235–7. https://doi.org/10.3201/eid2207.151896.
[43] Han C, Tang H, Ren C, et al. Sero-prevalence and genetic diversity of pandemic *V. parahaemolyticus* strains occurring at a global scale. Front Microbiol 2016:7. https://doi.org/10.3389/fmicb.2016.00567.
[44] Wang R, Zhong Y, Gu X, et al. The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. Front Microbiol 2015;6. https://doi.org/10.3389/fmicb.2015.00144.
[45] Yáñez R, Bastías R, Higuera G, et al. Amplification of tlh gene in other *Vibrionaceae* specie by specie-specific multiplex PCR of *Vibrio parahaemolyticus*. Electron J Biotechnol 2015;18:459–63. https://doi.org/10.1016/j.ejbt.2015.09.007.
[46] Scallan E, Hoekstra RM, Angulo FJ, et al. Foodborne illness acquired in the United States — major pathogens. Emerg Infect Dis 2011;17:7–15. https://doi.org/10.3201/eid1701.P11101.
[47] Jones MK, Oliver JD. *Vibrio vulnificus*: disease and pathogenesis. Infect Immun 2009;77(5):1723–33. https://doi.org/10.1128/IAI.01046-08.
[48] Chowdhury MA, Aziz KM, Kay BA, et al. Toxin production by *Vibrio mimicus* strains isolated from human and environmental sources in Bangladesh. J Clin Microbiol 1987;25(11):2200–3.
[49] Guardiola-Avila I, Acedo-Felix E, Sifuentes-Romero I, et al. Molecular and genomic characterization of *Vibrio mimicus* isolated from a frozen shrimp processing facility in Mexico. PLoS One 2016;11(1):e0144885. https://doi.org/10.1371/journal.pone.0144885.
[50] Campos E, Bolaños H, Acuña MT, et al. *Vibrio mimicus* diarrhea following ingestion of raw turtle eggs. Appl Environ Microbiol 1996;62(4):1141–4.
[51] Martinez-Urtaza J, Bowers JC, Trinanes J, et al. Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus* illnesses. Food Res Int 2010; 43(7):1780–90. https://doi.org/10.1016/j.foodres.2010.04.001.
[52] Kitaoka M, Miyata ST, Unterweger D, et al. Antibiotic resistance mechanisms of *Vibrio cholerae*. J Med Microbiol 2011;60(4):397–407. https://doi.org/10.1099/jmm.0.023051-0.
[53] Raissy M, Moumeni M, Ansari M, et al. Antibiotic resistance pattern of some *Vibrio* strains isolated from seafood. Iran J Fish Sci 2012;11:618–20.
[54] Frans I, Michiels CW, Bossier P, et al. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. J Fish Dis 2011;34:643–61. https://doi.org/10.1111/j.1365-2761.2011.01279.x.
[55] Dubert J, Barja JL, Romalde JL. New insights into pathogenic vibrios affecting bivalves in hatcheries: present and future prospects. Front Microbiol 2017;8:762. https://doi.org/10.3389/fmicb.2017.00762.
[56] Schiewe MH, Trust TJ, Crosa JH. *Vibrio ordalii* sp. nov.: a causative agent of vibriosis in fish. Curr Microbiol 1981;6(6):343–8. https://doi.org/10.1007/BF01567009.
[57] Baudin Laurencin F, Germon E. Experimental infection of rainbow trout, *Salmo gairdneri* R., by dipping in suspensions of *Vibrio anguillarum*: ways of bacterial penetration; influence of temperature and salinity. Aquaculture 1987;67(1–2): 203–5. https://doi.org/10.1016/0044-8486(87)90028-7.
[58] Silva-Rubio A, Avendaño-Herrera R, Jaureguiberry B, et al. First description of serotype O3 in *Vibrio anguillarum* strains isolated from salmonids in Chile. J Fish Dis 2008;31:235–9. https://doi.org/10.1111/j.1365-2761.2007.00878.x.
[59] Larsen JI, Pedersen K, Dalsgaard I. *Vibrio anguillarum* serovars associated with vibriosis in fish. J Fish Dis 1994;17:259–67. https://doi.org/10.1111/j.1365-2761.1994.tb00221.x.
[60] Naka H, Crosa J. Genetic determinants of virulence in the marine fish pathogen *Vibrio anguillarum*. Fish Pathol 2011;46:1–10.
[61] Fernández J, Avendaño-Herrera R. Analysis of 16S-23S rRNA gene internal transcribed spacer of *Vibrio anguillarum* and *Vibrio ordalii* strains isolated from fish. FEMS Microbiol Lett 2009;299:184–92. https://doi.org/10.1111/j.1574-6968.2009.01755.x.
[62] Steinum TM, Karataş S, Martinussen NT, et al. Multilocus sequence analysis of close relatives *Vibrio anguillarum* and *Vibrio ordalii*. Appl Environ Microbiol 2016;82: 5496–504. https://doi.org/10.1128/AEM.00620-16.
[63] Colquhoun DJ, Lillehaug A, Gudding, Lillehaug A. Vaccination against vibriosis. In: Evensen Ø, editor. Fish vaccination. Chichester, UK: John Wiley & Sons, Ltd; 2014. p. 172–84. https://doi.org/10.1002/9781118806913.ch15.
[64] Demircan D, Candan A. Identification of *Vibrio anguillarum* by PCR (rpoN gene) associated with vibriosis in marine fish in Turkey. Turk J Vet Anim Sci 2006;30: 305–10.
[65] Geng Y, Liu D, Han S, et al. Outbreaks of vibriosis associated with *Vibrio mimicus* in freshwater catfish in China. Aquaculture 2014;433:82–4. https://doi.org/10.1016/j.aquaculture.2014.05.053.
[66] Rodger HD, Colquhoun DJ. Clinical vibriosis in farmed Atlantic cod (*Gadus morhua*) in Ireland. Vet Rec 2008;162(3):94–5. https://doi.org/10.1136/vr.162.3.94.
[67] Darshanee Ruwandeepika HA, Sanjeewa Prasad Jayaweera T, Paban Bhowmick P, et al. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the *Harveyi* clade. Rev Aquac 2012;4(2):59–74. https://doi.org/10.1111/j.1753-5131.2012.01061.x.
[68] Yang Q, Defoirdt T. Quorum sensing positively regulates flagellar motility in pathogenic *Vibrio harveyi*. Environ Microbiol 2015;17:960–8. https://doi.org/10.1111/1462-2920.12420.
[69] Austin B, Pride AC, Rhodie GA. Association of a bacteriophage with virulence in *Vibrio harveyi*. J Fish Dis 2003;26:55–8. https://doi.org/10.1046/j.1365-2761.2003.00413.x.
[70] Munro J, Oakey J, Bromage E, et al. Experimental bacteriophage-mediated virulence in strains of *Vibrio harveyi*. Dis Aquat Organ 2003;54:187–94. https://doi.org/10.3354/dao054187.
[71] Gomez-Gil B, Soto-Rodríguez S, García-Gasca A, et al. Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. Microbiology 2004;150:1769–77. https://doi.org/10.1099/mic.0.26797-0.
[72] Cano-Gómez A, Goulden EF, Owens L, et al. *Vibrio owensii* sp. nov., isolated from cultured crustaceans in Australia. FEMS Microbiol Lett 2010;302:175–81. https://doi.org/10.1111/j.1574-6968.2009.01850.x.
[73] Owens L, Austin D, Austin B. Effect of strain origin on siderophore production in *Vibrio harveyi* isolates. Dis Aquat Organ 1996;27:157–60. https://doi.org/10.3354/dao027157.
[74] Svitil AL, Chadhain S, Moore JA, et al. Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Appl Environ Microbiol 1997;63:408–13.
[75] Cai SH, Wu ZH, Jian JC, et al. Cloning and expression of gene encoding the thermo-stable direct hemolysin from *Vibrio alginolyticus* strain HY9901, the causative agent of vibriosis of crimson snapper (*Lutjanus erythopterus*). J Appl Microbiol 2007; 103(2):289–96. https://doi.org/10.1111/j.1365-2672.2006.03250.x.
[76] Hong X, Lu L, Xu D. Progress in research on acute hepatopancreatic necrosis disease (AHPND). Aquac Int 2016;24:577–93. https://doi.org/10.1007/s10499-015-9948-x.
[77] Dabu IM, Lim JJ, Arabit PMT, et al. The first record of acute hepatopancreatic necrosis disease in the Philippines. Aquacult Res 2017;48:792–9. https://doi.org/10.1111/are.12923.
[78] Lai H-C, Ng TH, Ando M, et al. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish Shellfish Immunol 2015;47:1006–14. https://doi.org/10.1016/j.fsi.2015.11.008.
[79] Elston RA, Hasegawa H, Humphrey KL, et al. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. Dis Aquat Organ 2008;82:119–34. https://doi.org/10.3354/dao01982.
[80] Rojas R, Miranda CD, Santander J, et al. First report of *Vibrio tubiashii* associated with a massive larval mortality event in a commercial hatchery of scallop *Argopecten purpuratus* in Chile. Front Microbiol 2016;7:1473. https://doi.org/10.3389/fmicb.2016.01473.
[81] Wilson B, Muirhead A, Bazanella M, et al. An improved detection and quantification method for the coral pathogen *Vibrio coralliilyticus*. PLoS One 2013;8:e81800. https://doi.org/10.1371/journal.pone.0081800.

[82] Ben-Haim Y. *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. Int J Syst Evol Microbiol 2003;53:309–15. https://doi.org/10.1099/ijs.0.02402-0.
[83] Richards GP, Watson MA, Needleman DS, et al. Mortalities of eastern and Pacific oyster larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. Appl Environ Microbiol 2015;81:292–7. https://doi.org/10.1128/AEM.02930-14.
[84] Austin B, Austin D, Sutherland R, et al. Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia nauplii*. Environ Microbiol 2005;7: 1488–95. https://doi.org/10.1111/j.1462-2920.2005.00847.x.
[85] Vezzulli L, Pezzati E, Stauder M, et al. Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. Environ Microbiol 2015;17:1065–80. https://doi.org/10.1111/1462-2920.12484.
[86] Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol 2006;8: 1137–44. https://doi.org/10.1111/j.1462-2920.2006.01054.x.
[87] Karunasagar I, Pai R, Malathi GR, et al. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 1994;128:203–9. https://doi.org/10.1016/0044-8486(94)90309-3.
[88] Scarano C, Spanu C, Ziino G, et al. Antibiotic resistance of *Vibrio* species isolated from *Sparus aurata* reared in Italian mariculture. New Microbiol 2014;37: 329–37.
[89] D'Herelle F. Studies upon Asiatic cholera. Yale J Biol Med 1929;1:195–219.
[90] Summers WC. Cholera and plague in India: the bacteriophage inquiry of 1927–1936. J Hist Med Allied Sci 1993;48:275–301. https://doi.org/10.1093/jhmas/48.3.275.
[91] Sayamov RM. Treatment and prophylaxis of cholera with bacteriophage. Bull World Health Organ 1963;28:361–7.
[92] Yen M, Cairns LS, Camilli A. A cocktail of three virulent bacteriophages prevents *Vibrio cholerae* infection in animal models. Nat Commun 2017;8:14187. https://doi.org/10.1038/ncomms14187.
[93] Jaiswal A, Koley H, Ghosh A, et al. Efficacy of cocktail phage therapy in treating *Vibrio cholerae* infection in rabbit model. Microbes Infect 2013;15:152–6. https://doi.org/10.1016/j.micinf.2012.11.002.
[94] Bhowmick TS, Koley H, Das M, et al. Pathogenic potential of vibriophages against an experimental infection with *Vibrio cholerae* O1 in the RITARD model. Int J Antimicrob Agents 2009;33:569–73. https://doi.org/10.1016/j.ijantimicag.2008.10.034.
[95] Jaiswal A, Koley H, Mitra S, et al. Comparative analysis of different oral approaches to treat *Vibrio cholerae* infection in adult mice. Int J Med Microbiol 2014;304: 422–30. https://doi.org/10.1016/j.ijmm.2014.02.007.
[96] Bastías R, Higuera G, Sierralta W, et al. A new group of cosmopolitan bacteriophages induce a carrier state in the pandemic strain of *Vibrio parahaemolyticus*. Environ Microbiol 2010;12:990–1000. https://doi.org/10.1111/j.1462-2920.2010.02143.x.
[97] Zabala B, Garcia K, Espejo RT. Enhancement of UV light sensitivity of a *Vibrio parahaemolyticus* O3:K6 pandemic strain due to natural lysogenization by a telomeric phage. Appl Environ Microbiol 2009;75:1697–702. https://doi.org/10.1128/AEM.01995-08.
[98] Zabala B, Hammerl JA, Espejo RT, et al. The linear plasmid prophage Vp58.5 of *Vibrio parahaemolyticus* is closely related to the integrating phage VHML and constitutes a new incompatibility group of telomere phages. J Virol 2009;83: 9313–20. https://doi.org/10.1128/JVI.00672-09.
[99] Comeau AM, Chan AM, Suttle CA. Genetic richness of vibriophages isolated in a coastal environment. Environ Microbiol 2006;8:1164–76. https://doi.org/10.1111/j.1462-2920.2006.01006.x.
[100] Jun JW, Shin TH, Kim JH, et al. Bacteriophage therapy of a *Vibrio parahaemolyticus* infection caused by a multiple-antibiotic-resistant O3:K6 pandemic clinical strain. J Infect Dis 2014;210:72–8. https://doi.org/10.1093/infdis/jiu059.
[101] Jun JW, Kim HJ, Yun SK, et al. Eating oysters without risk of vibriosis: application of a bacteriophage against *Vibrio parahaemolyticus* in oysters. Int J Food Microbiol 2014;188:31–5. https://doi.org/10.1016/j.ijfoodmicro.2014.07.007.
[102] Cerveny KE, DePaola A, Duckworth DH, et al. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. Infect Immun 2002;70:6251–62. https://doi.org/10.1128/IAI.70.11.6251-6262.2002.
[103] Pelon W, Luftig RB, Johnston KH. *Vibrio vulnificus* load reduction in oysters after combined exposure to *Vibrio vulnificus*–specific bacteriophage and to an oyster extract component. J Food Prot 2005;68:1188–91.
[104] Derber C, Coudron P, Tarr C, et al. *Vibrio furnissii*: an unusual cause of bacteremia and skin lesions after ingestion of seafood. J Clin Microbiol 2011;49:2348–9. https://doi.org/10.1128/JCM.00092-11.
[105] Suthienkul O. Bacteriophage typing of *Vibrio fluvialis*. Southeast Asian J Trop Med Public Health 1993;24:449–54.
[106] Payne M, Oakey J, Owens L. The ability of two different *Vibrio spp*. bacteriophages to infect *Vibrio harveyi*, *Vibrio cholerae* and *Vibrio mimicus*. J Appl Microbiol 2004;97: 663–72. https://doi.org/10.1111/j.1365-2672.2004.02362.x.
[107] Kellogg CA, Rose JB, Jiang SC, et al. Genetic diversity of related vibriophages isolated from marine environments around Florida and Hawaii, USA. Mar Ecol Prog Ser 1995;120:89–98. https://doi.org/10.3354/meps120089.
[108] Morison J. Bacteriophage in the treatment and prevention of cholera. JAMA 1933; 100:1560. https://doi.org/10.1001/jama.1933.02740190086039.
[109] Asheshov J, Asheshov I, Khan S, et al. Bacteriophage inquiry. Report on the work during the period from 1st January to 1st September, 1929. Indian J Med Res 1930;17(3):971–84.
[110] Marcuk LM, Nikiforov VN, Scerbak JF, et al. Clinical studies of the use of bacteriophage in the treatment of cholera. Bull World Health Organ 1971;45:77–83.
[111] Sarkar BL, Chakrabarti AK, Koley H, et al. Biological activity and interaction of *Vibrio cholerae* bacteriophages in rabbit ileal loop. Indian J Med Res 1996;104:139–41.
[112] Vinod MG, Shivu MM, Umesha KR, et al. Isolation of *Vibrio harveyi* bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments. Aquaculture 2006;255:117–24. https://doi.org/10.1016/j.aquaculture.2005.12.003.
[113] Crothers-Stomps C, Høj L, Bourne DG, et al. Isolation of lytic bacteriophage against *Vibrio harveyi*. J Appl Microbiol 2010;108:1744–50. https://doi.org/10.1111/j.1365-2672.2009.04578.x.
[114] Karunasagar I, Shivu MM, Girisha SK, et al. Biocontrol of pathogens in shrimp hatcheries using bacteriophages. Aquaculture 2007;268:288–92. https://doi.org/10.1016/j.aquaculture.2007.04.049.
[115] Shivu MM, Rajeeva BC, Girisha SK, et al. Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. Environ Microbiol 2007;9:322–31. https://doi.org/10.1111/j.1462-2920.2006.01140.x.
[116] Raghu Patil J, Desai S, Roy P, et al. Simulated hatchery system to assess bacteriophage efficacy against *Vibrio harveyi*. Dis Aquat Organ 2014;112(2):113–9. https://doi.org/10.3354/dao02806.
[117] Wang Y, Barton M, Elliott L, et al. Bacteriophage therapy for the control of *Vibrio harveyi* in greenlip abalone (*Haliotis laevigata*). Aquaculture 2017;473:251–8. https://doi.org/10.1016/j.aquaculture.2017.01.003.
[118] Oakey HJ, Owens L. A new bacteriophage, VHML, isolated from a toxin-producing strain of *Vibrio harveyi* in tropical Australia. J Appl Microbiol 2000;89:702–9. https://doi.org/10.1046/j.1365-2672.2000.01169.x.
[119] Cohen Y, Pollock FJ, Rosenberg E, et al. Phage therapy treatment of the coral pathogen *Vibrio coralliilyticus*. Microbiology 2013;2:64–74. https://doi.org/10.1002/mbo3.52.
[120] Kalatzis PG, Bastías R, Kokkari C, et al. Isolation and characterization of two lytic bacteriophages, φSt2 and φGrn1; phage therapy application for biological control of *Vibrio alginolyticus* in aquaculture live feeds. PLoS One 2016;11. https://doi.org/10.1371/journal.pone.0151101.
[121] Li Z, Li X, Zhang J, et al. Use of phages to control *Vibrio splendidus* infection in the juvenile sea cucumber *Apostichopus japonicus*. Fish Shellfish Immunol 2016;54: 302–11. https://doi.org/10.1016/j.fsi.2016.04.026.
[122] Li Z, Zhang J, Li X, et al. Efficiency of a bacteriophage in controlling *vibrio* infection in the juvenile sea cucumber *Apostichopus japonicus*. Aquaculture 2016;451:345–52. https://doi.org/10.1016/j.aquaculture.2015.09.024.
[123] Higuera G, Bastías R, Tsertsvadze G, et al. Recently discovered *Vibrio anguillarum* phages can protect against experimentally induced vibriosis in Atlantic salmon, *Salmo salar*. Aquaculture 2013:128–33. https://doi.org/10.1016/j.aquaculture.2013.02.013.
[124] Silva YJ, Costa L, Pereira C, et al. Phage therapy as an approach to prevent *Vibrio anguillarum* infections in fish larvae production. PLoS One 2014;9:1–23. https://doi.org/10.1371/journal.pone.0114197.
[125] Luo Z-H, Yu Y-P, Jost G, et al. Characterization of two bacteriophages for specific treatment of biofilm formed by a *Vibrio sp.* isolated from an abalone farm. Aquacult Res 2016;47:3964–72. https://doi.org/10.1111/are.12846.
[126] Surekhamol IS, Deepa GD, Somnath Pai S, et al. Isolation and characterization of broad spectrum bacteriophages lytic to *Vibrio harveyi* from shrimp farms of Kerala, India. Lett Appl Microbiol 2014;58:197–204. https://doi.org/10.1111/lam.12175.
[127] Zhang J, Cao Z, Li Z, et al. Effect of bacteriophages on *Vibrio alginolyticus* infection in the sea cucumber, *Apostichopus japonicus* (Selenka). J World Aquac Soc 2015;46: 149–58. https://doi.org/10.1111/jwas.12177.
[128] Thompson FL, Iida T, Swings J, et al. Biodiversity of vibrios. Microbiol Mol Biol Rev 2004;68:403–31. https://doi.org/10.1128/MMBR.68.3.403.
[129] EPO. European patent office. https://www.epo.org/index.html; 2017, Accessed date: 14 June 2017.
[130] León M, Bastías R. Virulence reduction in bacteriophage resistant bacteria. Front Microbiol 2015;6. https://doi.org/10.3389/fmicb.2015.00343.
[131] Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. Nat Rev Microbiol 2010;8:317–27. https://doi.org/10.1038/nrmicro2315.
[132] Chan BK, Abedon ST. Phage therapy pharmacology. Phage cocktails. Adv Appl Microbiol 2012;78:1–23. https://doi.org/10.1016/B978-0-12-394805-2.00001-4.
[133] Gu J, Liu X, Li Y, et al. A method for generation phage cocktail with great therapeutic potential. PLoS One 2012;7. https://doi.org/10.1371/journal.pone.0031698.
[134] Schmerer M, Molineux IJ, Bull JJ. Synergy as a rationale for phage therapy using phage cocktails. Peer J 2014;2:e590. https://doi.org/10.7717/peerj.590.
[135] Laanto E, Bamford JKH, Laakso J, et al. Phage-driven loss of virulence in a fish pathogenic bacterium. PLoS One 2012;7. https://doi.org/10.1371/journal.pone.0053157.
[136] Santander J, Robeson J. Phage-resistance of *Salmonella enterica* serovar Enteritidis and pathogenesis in *Caenorhabditis elegans* is mediated by the lipopolysaccharide. Electron J Biotechnol 2007;10:627–32. https://doi.org/10.2225/vol10-issue4-fulltext-14.
[137] Hosseinidoust Z, van de Ven TGM, Tufenkji N. Evolution of *Pseudomonas aeruginosa* virulence as a result of phage predation. Appl Environ Microbiol 2013;79:6110–6. https://doi.org/10.1128/AEM.01421-13.
[138] Barrangou R, Horvath P. A decade of discovery: CRISPR functions and applications. Nat Microbiol 2017;2:17092. https://doi.org/10.1038/nmicrobiol.2017.92.
[139] Marraffini LA. CRISPR-Cas immunity in prokaryotes. Nature 2015;526:55–61. https://doi.org/10.1038/nature15386.
[140] Kunin V, Sorek R, Hugenholtz P. Evolutionary conservation of sequence and secondary structures in CRISPR repeats. Genome Biol 2007;8:R61. https://doi.org/10.1186/gb-2007-8-4-r61.
[141] Box AM, McGuffie MJ, O'Hara BJ, et al. Functional analysis of bacteriophage immunity through a type I-E CRISPR-Cas system in *Vibrio cholerae* and its application in

bacteriophage genome engineering. J Bacteriol 2016;198:578–90. https://doi.org/10.1128/JB.00747-15.
[142] Sun H, Li Y, Shi X, et al. Association of CRISPR/Cas evolution with *Vibrio parahaemolyticus* virulence factors and genotypes. Foodborne Pathog Dis 2015; 12:68–73. https://doi.org/10.1089/fpd.2014.1792.
[143] Bondy-Denomy J, Pawluk A, Maxwell KL, et al. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. Nature 2013;493:429–32. https://doi.org/10.1038/nature11723.
[144] Bondy-Denomy J, Garcia B, Strum S, et al. Multiple mechanisms for CRISPR–Cas inhibition by anti-CRISPR proteins. Nature 2015;526:136–9. https://doi.org/10.1038/nature15254.
[145] Monsur KA, Rahman MA, Huq F, et al. Effect of massive doses of bacteriophage on excretion of vibrios, duration of diarrhoea and output of stools in acute cases of cholera. Bull World Health Organ 1970;42:723–32.
[146] Kutateladze M, Adamia R. Phage therapy experience at the Eliava institute. Med Mal Infect 2008;38:426–30. https://doi.org/10.1016/j.medmal.2008.06.023.
[147] Kutter E, De Vos D, Gvasalia G, et al. Phage therapy in clinical practice: treatment of human infections. Curr Pharm Biotechnol 2010;11:69–86. https://doi.org/10.2174/138920110790725401.
[148] Ma Y, Pacan JC, Wang Q, et al. Enhanced alginate microspheres as means of oral delivery of bacteriophage for reducing *Staphylococcus aureus* intestinal carriage. Food Hydrocoll 2012;26:434–40. https://doi.org/10.1016/j.foodhyd.2010.11.017.
[149] Dini C, Islan GA, de Urraza PJ, et al. Novel biopolymer matrices for microencapsulation of phages: enhanced protection against acidity and protease activity. Macromol Biosci 2012;12:1200–8. https://doi.org/10.1002/mabi.201200109.
[150] Robeson J, Turra G, Huber K, et al. A note on stability in food matrices of *Salmonella enterica* serovar enteritidis-controlling bacteriophages. Electron J Biotechnol 2014; 17:189–91. https://doi.org/10.1016/j.ejbt.2014.06.001.
[151] Madsen L, Bertelsen SK, Dalsgaard I, et al. Dispersal and survival of *Flavobacterium psychrophilum* phages *in vivo* in rainbow trout and *in vitro* under laboratory conditions: implications for their use in phage therapy. Appl Environ Microbiol 2013;79:4853–61. https://doi.org/10.1128/AEM.00509-13.
[152] Castillo D, Higuera G, Villa M, et al. Diversity of *Flavobacterium psychrophilum* and the potential use of its phages for protection against bacterial cold water disease in salmonids. J Fish Dis 2012;35:193–201. https://doi.org/10.1111/j.1365-2761.2011.01336.x.
[153] Park SC, Nakai T. Infection in ayu *Plecoglossus altivelis*. Dis Aquat Organ 2003;53: 33–9.
[154] Christiansen RH, Dalsgaard I, Middelboe M, et al. Detection and quantification of *Flavobacterium psychrophilum*-specific bacteriophages *in vivo* in rainbow trout upon oral administration: implications for disease control in aquaculture. Appl Environ Microbiol 2014;80:7683–93. https://doi.org/10.1128/AEM.02386-14.
[155] Weber-Dabrowska B, Dabrowski M, Slopek S. Studies on bacteriophage penetration in patients subjected to phage therapy. Arch Immunol Ther Exp (Warsz) 1987;35: 563–8.
[156] Bruttin A, Brüssow H, Bru H. Human volunteers receiving *Escherichia coli* phage T4 Orally: a safety test of phage therapy. Antimicrob Agents Chemother 2005;49: 2874–8. https://doi.org/10.1128/AAC.49.7.2874.
[157] Cooper CJ, Mirzaei MK, Nilsson AS. Adapting drug approval pathways for bacteriophage-based therapeutics. Front Microbiol 2016;7:1–15. https://doi.org/10.3389/fmicb.2016.01209.
[158] Verbeken G, Pirnay JP, Lavigne R, et al. Call for a dedicated European legal framework for bacteriophage therapy. Arch Immunol Ther Exp (Warsz) 2014;62: 117–29. https://doi.org/10.1007/s00005-014-0269-y.