Virus Research 353 (2025) 199529

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Isolation, characterization and genomic analysis of bacteriophages for biocontrol of vibriosis caused by *Vibrio alginolyticus*

Nattarika Chaichana [a], Rutinan Rattanaburee [a], Komwit Surachat [c,d], Decha Sermwittayawong [b], Natthawan Sermwittayawong [a,*]

[a] Division of Biological Science, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand
[b] Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand
[c] Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Songkhla 90110, Thailand
[d] Translational Medicine Research Center, Faculty of Medicine, Prince of Songkla University, Songkhla 90110, Thailand

ARTICLE INFO

Keywords:
Bacteriophage
Bacteriophage cocktail
Biological control
Galleria mellonella
V. alginolyticus

ABSTRACT

Vibrio alginolyticus is a significant opportunistic pathogen in marine environments, affecting both marine organisms and humans. The rise of antibiotic-resistant strains has prompted the exploration of bacteriophages as alternative biological control agents. In this study, 414 lytic bacteriophages specific to *V. alginolyticus* were isolated from various seafood and environmental samples. Phages P122, P125, and P160 demonstrated the broadest host range, effectively lysing 79.01 % of fish pathogenic *V. alginolyticus* strains and 44.69 % of environmental strains. However, no activity was observed against clinical *V. alginolyticus* strains or other tested species, including *V. harveyi, Escherichia coli, Staphylococcus aureus*, and *Aeromonas hydrophila*. One-step growth curve analysis revealed latent periods of 40 to 60 min and burst sizes ranging from 140 to 367 PFU/infected cells. Transmission electron microscopy (TEM) classified these phages within the class of Caudoviricetes with an icosahedral head and a long non-contractile tail. Moreover, whole-genome sequencing (WGS) identified genome sizes of approximately 76 kb, with 272–280 open reading frames (ORFs), no tRNA and pathogenic-associated genes. Comparative genomic analysis showed over 97 % similarity with other *Vibrio* phages. Phylogenetic analysis based on the terminase subunit also confirmed phages P122, P125, and P160 belonging to the class of Caudoviricetes. The phages were non-toxic to *Galleria mellonella* larvae and showed promise in reducing mortality rates when used as a cocktail treatment. The study highlights the potential of these phages as effective biocontrol agents in aquaculture, offering a promising alternative to antibiotics for managing *Vibrio* infections.

1. Introduction

Vibrio alginolyticus is a halophilic, Gram-negative curved rod bacterium that is widely distributed in aquatic environments and marine animals. It is commonly found in bivalve shellfish, crustaceans, and other marine species, where it typically exists as a commensal organism (Kwok et al., 2024; Sampaio et al., 2022). However, *V. alginolyticus* can also act as a significant opportunistic pathogen, particularly known for causing tissue damage and gill infections in fish (Kah Sem et al., 2023). Previous studies have highlighted its high prevalence in fish, with particularly severe impacts during the early stages of life (Kalatzis et al., 2016). Pathogenic strains of *V. alginolyticus* are responsible for substantial mortality in marine animals. In humans, infection usually occurs through exposure to contaminated water, leading to skin infections (both superficial and deep) as well as infections of other organs, including the ears and eyes (Reilly et al., 2011). Moreover, gastroenteritis can result from consuming raw or undercooked seafood. Traditional methods of controlling pathogenic bacteria, such as antibiotics and chemical treatments, have led to contamination in marine environments and animal populations. Additionally, the rise of multidrug-resistant bacterial strains has created significant challenges in managing these pathogens effectively (Kang et al., 2016; Manyi-Loh et al., 2018). Recently, bacteriophages have emerged as a promising alternative for biological control, potentially reducing bacterial pathogens in aquaculture systems (Aziz et al., 2025; Garvey, 2022). Bacteriophages, which are natural bacterial viruses, contain either double-stranded or single-stranded DNA or RNA and are abundant in environments where their bacterial hosts reside, such as water, soil, and

* Corresponding author.
E-mail address: natthawan.k@psu.ac.th (N. Sermwittayawong).

https://doi.org/10.1016/j.virusres.2025.199529
Received 19 November 2024; Received in revised form 6 January 2025; Accepted 20 January 2025

Table 1
Bacterial strains used for host range determination.

Species	Strain no.	Types
	PSU 6	Reference of fish pathogenic strain
	PSU 4537, PSU 4543, PSU 4718, PSU 4794,	Clinical strains
V. alginolyticus	PSU 4912	
	JS1.2, KF2.4, MF19, MF18, MF16, MF14, GG29, TG36, TG21, TG13, TG10	Fish pathogenic strains
	W1.1, W3.5, W5.2, W4.2, W6.2, S1.3	Environmental strains
	PSU 4211, PSU 4099, PSU 3916	Pandemic strains in cockles
	PSU 4117, PSU 3909, PSU 3622	Non-pandemic strains in cockles
V. parahaemolyticus	TG56, MF36, MF37, FV01, FV09, FV14, KF1.5, KF2.1, KF2.10, CH1.1, CH2.9, JS 1.3, JS1.7, JS2.6, CH1.10	Fish pathogenic strains
	TG1, TG11, TG35, GG2, GG8, GG31, MF17,	Fish pathogenic strains
V. harveyi	MF25, MF31, KF1.4, KF2.12, CH1.4, CH2.13, JS2.4, JS2.7	
	MF13, MF24, MF32, FV05, VVA6, KF1.3,	Fish pathogenic strains
V. vulnificus	KF2.8, KF2.11, KF2.17, CH1.2, CH1.8, CH1.9, CH1.13, JS1.6, JS2.2	
E. coli	ATCC 8339	Reference
S. aureus	ATCC 13,565	Reference
A. hydrophila	PSU 37	Reference

air. The interaction between bacteriophages and their target bacteria is mediated by specific receptors on the bacterial cell surface (Van Belleghem et al., 2018).

Lytic bacteriophages are particularly effective as they replicate within the host cell by exploiting its resources, ultimately causing the lysis of the bacterial cell. This process occurs through the action of enzymes like endolysin and holin, which degrade the peptidoglycan layer of the bacterial cell wall, releasing new bacteriophages to infect additional bacterial cells (Abdelrahman et al., 2021). Bacteriophages are highly efficient at lysing host cells shortly after infection and are environmentally safe, posing no risk to other beneficial bacteria or marine animals (Hitchcock et al., 2023). However, the prolonged use of a single bacteriophage strain can result in the development of bacteriophage-resistant bacteria. Bacteria can evolve mechanisms to prevent bacteriophage adsorption, thus reducing the effectiveness of the bacteriophage (Oechslin, 2018). This limitation can be addressed by using a combination of multiple bacteriophages, known as a bacteriophage cocktail, which can delay the evolution of resistance. Bacteriophage cocktails present a sustainable and attractive alternative for controlling bacterial pathogens.

Bacteriophages specific to *V. alginolyticus* have been studied as potential biological control agents in aquaculture due to high host specificity, rapid self-proliferation, effectiveness, and environmental safety. These phages have been isolated from various environments, including seawater, sediments, and marine organisms, where their bacterial hosts reside (Le et al., 2020). The numerous studies on *V. alginolyticus* phages were focused on their ability to lyse pathogenic strains in fish and shrimp, demonstrating their potential to mitigate bacterial infections in aquaculture systems (de Souza Valente and Wan, 2021; Fu et al., 2023; Hao et al., 2023). A marine-based study revealed that the combination of two *V. alginolyticus* phages markedly improved the survival rate of sea cucumbers, with survival rates ranging from 47 % to 73 % depending on the applied multiplicity of infection (Zhang et al., 2015). Building on these findings, recent research has explored the genomic characterization and functional mechanisms of *V. alginolyticus*-specific phages to better understand their lytic capabilities and optimize their application in aquaculture systems. Genomic analysis provides insights into their structural and lytic proteins and ensures the absence of genes encoding virulence factors or antibiotic resistance, which is critical for their safe application in aquaculture settings (Wang et al., 2024; Xu et al., 2023). Moreover, the development of phage cocktails, composed of multiple phages with complementary host ranges, has been explored as a strategy to enhance efficacy and minimize the emergence of phage-resistant bacterial strains, underscoring the potential of phage therapy as a sustainable alternative to antibiotics in aquaculture systems (Abedon et al., 2021; Lin et al., 2017). Therefore, this study aims to isolate and characterize bacteriophages specific to *V. alginolyticus* for use as biological control agents, to reduce the contamination of *Vibrio* pathogens in marine aquaculture.

2. Materials and methods

2.1. Bacterial strains and growth conditions

V. alginolyticus strain PSU 6 was isolated from the wound of diseased grouper (*Epinephelus* spp.) cultured in the fish farm in Krabi province, Thailand, and was used as the host for the isolation and propagation of bacteriophages. A total of seventy-nine additional bacterial strains, including four species of *Vibrio* (*V. alginolyticus, V. parahaemolyticus, V. vulnificus*, and *V. harveyi*), as well as *Escherichia coli, Staphylococcus aureus*, and *Aeromonas hydrophila* (listed in Table 1), were used to test the host range of the isolated bacteriophages. Each bacterial strain was inoculated into Luria-Bertani (LB) broth (Difco, USA) supplemented with 1 % (w/v) NaCl, and the cultures were incubated at 30 °C with shaking at 150 rpm for 6 h. All bacterial strains used in this study were obtained from the culture collection of the Division of Biological Science, Faculty of Science, Prince of Songkla University, Thailand.

2.2. Isolation and propagation of bacteriophages

Bacteriophages were isolated from various sources, including cockles, oysters, water, soil sediments, shrimps, mussels, and green caviar. For enrichment, 25 g of the sample or 25 mL of water was added to 225 mL of double-strength LB broth supplemented with 1 % NaCl, followed by the inoculation with 100 µL of the bacterial host *V. alginolyticus* strain PSU 6. The enrichment samples were incubated overnight at 25 °C without shaking. The supernatant was harvested by centrifugation at 8000 × g for 10 min and filtered through a 0.22 µm filter membrane (Corning, Germany). Bacteriophages were then isolated using the double-layer agar method. Briefly, 100 µL of the filtered supernatant and 100 µL of *V. alginolyticus* strain PSU 6 were added to 3 mL of soft agar containing 0.7 % agar and 1 % NaCl. The mixture was overlaid on LB agar supplemented with 1 % NaCl and incubated overnight at 25 °C to examine plaque formation. A single plaque was picked and transferred into 500 µL of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, and 50 mM Tris–HCl, pH 7.5) (Tan et al., 2014). The bacteriophages were purified by re-plating three times (three passage purification) to ensure a pure isolate. For bacteriophage propagation, 100 µL of ten-fold serial dilutions and 100 µL of *V. alginolyticus* strain PSU 6 were mixed with 3 mL of soft agar containing 0.7 % agar and 1 % NaCl, and then overlaid on LB agar supplemented with 1 % NaCl. After overnight incubation at 25 °C, 2 mL of SM buffer was added. The bacteriophage suspensions were then centrifuged at 8000 × g for 10 min at 4 °C to remove intact bacteria and bacterial debris. The supernatants were filtered through a 0.22 µm filter membrane and stored at 4 °C.

2.3. Host range determination

The host range of each bacteriophage was tested using a spot assay to evaluate their ability to lyse other bacterial strains (Table 1). Briefly,

100 μL of each bacterial strain was mixed with 3 mL of soft agar supplemented with 1 % NaCl and overlaid onto LB agar containing 1 % NaCl. Subsequently, 5 μL of bacteriophage lysate was dropped onto the bacterial lawn, and the plates were incubated overnight at 25 °C. The susceptibility of the bacterial strains to each bacteriophage was assessed by the presence of a clear lysis zone (Mateus et al., 2014).

2.4. Determination of multiplicity infection (MOI)

V. alginolyticus strain PSU 6 in the early log phase was adjusted to 0.5 McFarland and then infected with five different bacteriophage lysates at ratios of 10^2, 10^1, 1, 10^{-1}, and 10^{-2} PFU/CFU (corresponding to MOIs of 100, 10, 1, 0.1, and 0.01, respectively). After incubating the mixture for 3 h at 25 °C without shaking, the bacteriophage lysates were centrifuged at 8000 × g for 10 min. The supernatants were subjected to ten-fold serial dilutions up to 10^{-8}, and the MOI was determined by a spot assay. The optimal MOI was determined as the ratio that yielded the highest titer of bacteriophages (Zhang et al., 2021).

2.5. One-step growth curve of bacteriophages

The early log phase of *V. alginolyticus* strain PSU 6 was adjusted to a 0.5 McFarland standard. One milliliter of the *V. alginolyticus* suspension was then mixed with bacteriophage lysate at the optimal MOI. The mixture was incubated at 25 °C without shaking for 10 min, followed by centrifugation at 8000 × g for 10 min to remove any free bacteriophages. The pelleted cells were resuspended in 50 mL of LB broth supplemented with 1 % NaCl and incubated continuously at 25 °C. Samples were taken at 10-min intervals for 3 h, and the phage titers were determined using the spot assay (Zhao et al., 2019).

2.6. Stability of bacteriophages

The stability of bacteriophages was tested in artificial seawater as previously described with few modifications (Stalin and Srinivasan, 2017). Bacteriophage lysate was added to artificial seawater and incubated at 25 °C without shaking. The bacteriophage titer was measured at the initial time point (zero time) and intervals of 12 h until the third day, 48 h until the seventh day, 120 h until the twelfth day, 144 h until the forty-second day, 192 h until the fiftieth day, and 240 h until the hundredth day. For each time point, 20 mL of the bacteriophage suspension was harvested, and filtered through a 0.22 μm filter membrane, and the phage titer was determined using the double-layer agar assay.

2.7. Morphology of bacteriophages

The morphology of bacteriophages was observed using transmission electron microscopy (TEM). Bacteriophage lysate was prepared, and NaCl was added to a final concentration of 1 M, followed by storage on ice for 30 min. Subsequently, 10 % polyethylene glycol 8000 was added to the mixture, which was stored at 4 °C for 12 h. After incubation, the mixture was centrifuged at 8000 × g for 20 min at 4 °C. The resulting pellets were resuspended in SM buffer and centrifuged at 5000 × g for 5 min at 4 °C. The supernatants were then subjected to further purification using a cesium chloride (CsCl) gradient centrifugation. The bacteriophage suspensions were overlaid onto a CsCl gradient of 1.0, 0.82, and 0.66 g/mL, and centrifugation was carried out at 35,000 × g in an ultracentrifuge (Beckman Coulter, USA) for 2 h at 4 °C. The purified bacteriophages were negatively stained with 2 % uranyl acetate, and their morphology was examined using transmission electron microscopy (TEM) (JEM-100CX II, JEOL, Germany) (Rodríguez-Rubio and Muniesa, 2021).

2.8. Genomic and bioinformatic analysis

DNA extraction from the *Vibrio* phages was carried out using the phenol-chloroform method as described by Higuera et al. The extracted DNA was assessed for quality by 1.5 % agarose gel electrophoresis, which was run at 80 kV for 1 h. DNA concentration was measured using a Nanodrop spectrophotometer based on the absorbance at 260 nm, and the 260/280 absorbance ratio, along with agarose gel electrophoresis, was used to evaluate the purity and integrity of the DNA. Short-read sequencing was performed using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) following the manufacturer's protocol. The quality of the sequencing reads was assessed using FASTQC, and the reads were filtered using Trimmomatic with default settings. Assembly of the reads into contigs was completed using SPAdes version 3.11.130. The bacteriophage genomes were annotated using Pharokka (Bouras et al., 2022). Open reading frames (ORFs) were identified, and the genome map for the phages was generated using Proksee (Grant et al., 2023). Furthermore, tRNAs were predicted using tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/, accessed on 30 August 2024). To identify potential virulence factors, the predicted proteins were analyzed using the Virulence Factors Database (VFDB, http://www.mgc.ac.cn/VFs/, accessed on 30 August 2024), with an E-value cutoff of 1×10^{-5}. Antimicrobial resistance genes (ARGs) were identified using ResFinder v4.1 (https://cge.cbs.dtu.dk/services/ResFinder/, accessed on 30 August 2024). The average nucleotide identity (ANI) between the phages and closely related *Vibrio* phages was calculated using FastANI v1.3.3 (Jain et al., 2018). A phylogenetic tree was constructed by comparing the terminase large subunits of the phages with their closest *Vibrio* phage relatives using the neighbor-joining method in MEGA v11, with 1000 bootstrap replications (Kumar et al., 2008). Finally, the nucleotide sequence identity between the phages and nine other *Vibrio* phages deposited in the GenBank database was calculated using VIRIDIC v1.1 (Intergenomic Distance Calculator) (https://rhea.icbm.uni-oldenburg.de/viridic/, accessed on 10 September 2024).

2.9. Ability of bacteriophages in the hemolymph of Galleria mellonella larvae

Creamy-colored *Galleria mellonella* larvae (Greater wax moth), weighing between 0.2–0.3 g, were selected for the experiment. Fifteen larvae were injected with 20 μL of each bacteriophage lysate (P122, P125, and P160). At each time interval, hemolymph was randomly collected from three larvae. The larvae were placed into a 15 mL tube and kept on ice for 5–10 min until movement ceased. A small incision was made between two segments near the tail using a scalpel to collect the hemolymph. The combined hemolymph samples were serially diluted, and the bacteriophage titer was determined using the double-layer agar method (Nale et al., 2016).

2.10. In vivo infection of bacteriophage cocktail in G. mellonella larvae infected with V. alginolyticus

To determine the lethal dose (LD_{50}) of each strain of *V. alginolyticus*, a 10-fold serial dilution of *V. alginolyticus* strains was prepared up to 10^{-4}, and 20 μL of each dilution was injected into the last proleg of fifteen *G. mellonella* larvae. To evaluate the effectiveness of bacteriophages against *V. alginolyticus* infection, the bacterial cultures were diluted to the determined LD_{50} of each strain. The bacteria were mixed with individual bacteriophages (P122, P125, and P160) or a bacteriophage cocktail (containing equal concentrations of the three bacteriophages) at three different multiplicities of infection (MOI) of 1, 10, and 100. The mixtures were incubated for 15 min to allow bacteriophage adsorption. Following incubation, 20 μL of each mixture was injected into the last proleg of fifteen *G. mellonella* larvae. Positive controls consisted of larvae injected with the LD_{50} dose of *V. alginolyticus*, while the negative controls included two groups: one injected with PBS buffer and the other with each bacteriophage or the bacteriophage cocktail. The larvae were placed in Petri dishes and incubated at room temperature in dark

Table 2
Sample collections and numbers of bacteriophages that specific for *V. alginolyticus* PSU 6 isolated from various sample sources.

Sample sources	Number of collected sample	Number of positive samples for bacteriophage	Number of bacteriophages specific to *V. alginolyticus* PSU 6
Water	19	19	104
Sediments	17	17	122
Cockles	15	15	138
Oysters	3	3	12
Shrimps	2	2	7
Mussels	2	2	27
Green caviar	1	1	4
Total	59	59	414

Table 3
Host range of isolated bacteriophages specific for *V. alginolyticus* PSU 6.

Species	Strains	Bacteriophage infected in host (%)
V. alginolyticus	Clinical	0/414 (0.0)
	Fish pathogenic	327/414 (79.0)
	Environmental	185/414 (44.7)
V. parahaemolyticus	Pandemic in cockles	82/414 (19.8)
	Non-pandemic in cockles	79/414 (19.1)
	Fish pathogenic	134/414 (32.4)
V. harveyi	Fish pathogenic	0/414 (0.0)
V. vulnificus	Fish pathogenic	21/414 (5.1)
E. coli	Reference	0/414 (0.0)
S. aureus	Reference	0/414 (0.0)
A. hydrophila	Reference	0/414 (0.0)

conditions. The survival of the larvae was monitored every 12 h to 48 h, with mortality indicated by the larvae turning black (Antoine et al., 2021).

2.11. Statistical analysis

Statistical tests were performed using SPSS version 26 (SPSS Inc., USA). The Mann-Whitney U test was employed to compare differences between groups of *G. mellonella* larvae. A p-value of < 0.05 was considered to indicate a statistically significant difference between the group treated with a single bacteriophage and the group treated with a bacteriophage cocktail. The LD_{50} of *G. mellonella* larvae was calculated using Probit analysis in Microsoft Excel.

3. Results

3.1. Isolation of bacteriophages specific to V. alginolyticus

A total of fifty-nine environmental and seafood samples were collected and enriched for bacteriophages specific to *V. alginolyticus* PSU 6. Bacteriophages were detected in the majority of the collected samples. All 59 samples were positive for 414 bacteriophages specific to *V. alginolyticus* PSU 6, as shown in Table 2.

3.2. Host range determination

A total of 414 bacteriophages isolated from *V. alginolyticus* PSU 6 were propagated and purified through a three-passage purification process. These bacteriophages were then tested for their host range by assessing their ability to lyse various *Vibrio* species, as well as other Gram-positive and Gram-negative bacteria, based on the formation of clear lysis zones. The bacteriophages specific to *V. alginolyticus* PSU 6 showed positive lysis activity against 79.0 % of fish-pathogenic *V. alginolyticus* strains and 44.7 % of environmental *V. alginolyticus* strains. Notably, none of the bacteriophages were able to infect clinical strains of *V. alginolyticus*. Moreover, 32.4 % of the bacteriophages demonstrated activity against fish-pathogenic strains of *V. parahaemolyticus*, and 5.1 % were effective against fish-pathogenic strains of *V. vulnificus*. None of the bacteriophages tested could lyse *V. harveyi, E. coli, S. aureus*, or *A. hydrophila* (Table 3). Among the bacteriophages tested, P122, P125, and P160 exhibited the broadest host range against the bacterial strains.

3.3. Optimal MOI and one-step growth curve of bacteriophages

The MOI of phages P122, P125, and P160 infecting *V. alginolyticus* PSU 6 demonstrated variations, with optimal MOI values of 100, 1, and 10, respectively. The life cycle of each bacteriophage, encompassing the latent period, burst phase, and plateau phase, was analyzed and

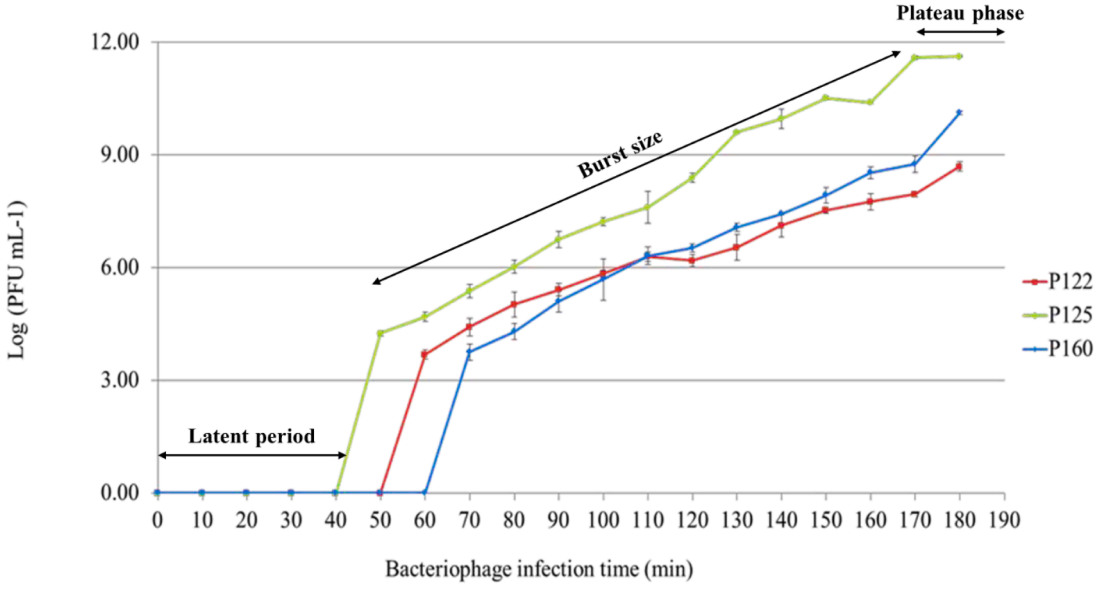

Fig. 1. One-step growth curves of bacteriophages P122, P125, and P160 in the presence of *V. alginolyticus* PSU 6. Values represent the mean of three independent experiments, with error bars indicating the standard deviation.

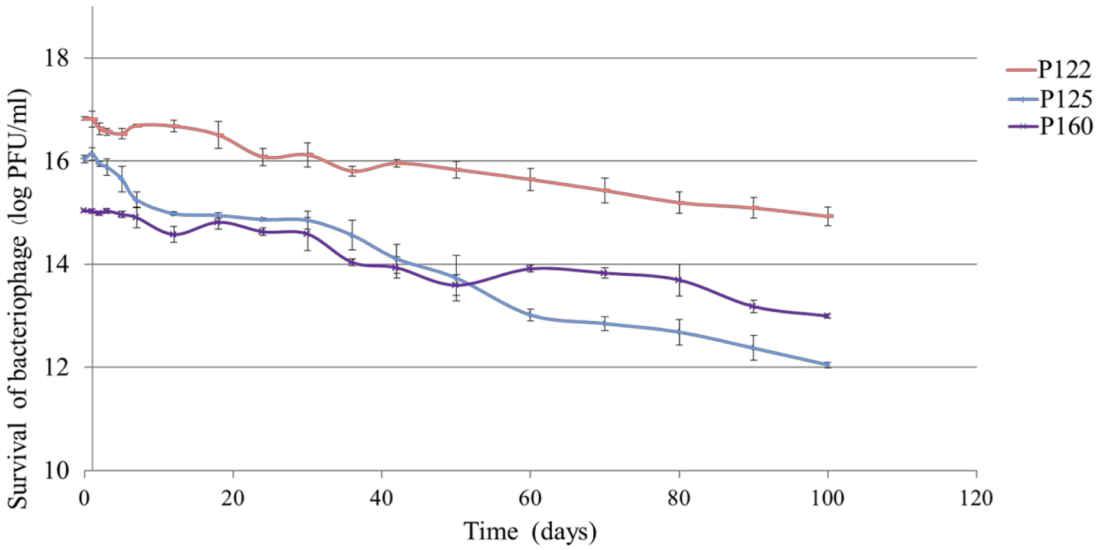

Fig. 2. Survival of three bacteriophages in artificial seawater over a 100-day period. Values represent the mean of three independent experiments, with error bars indicating the standard deviation.

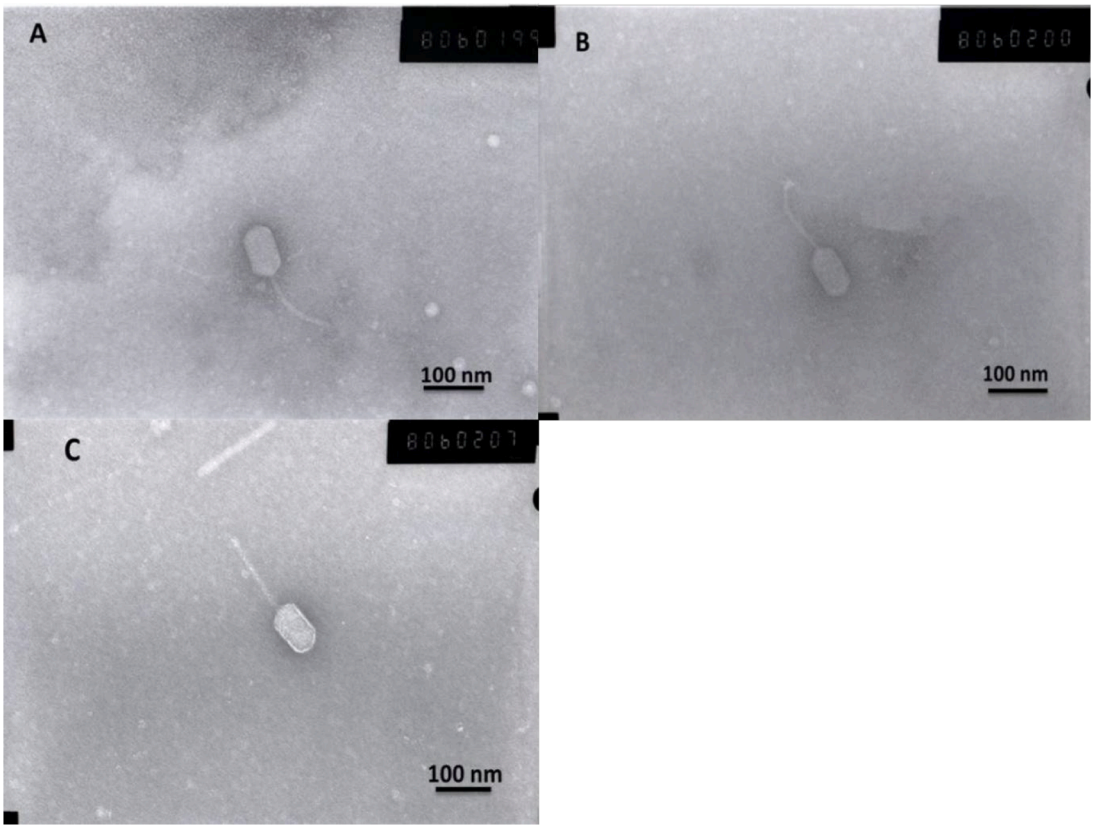

Fig. 3. Transmission electron microscopy (TEM) images showing the morphology of bacteriophages P122 (A), P125 (B), and P160 (C).

quantified using a one-step growth curve (Fig. 1). Phage P122 had a latent period of 50 min and a burst size of 140 PFU/infected cell. Phage P125 exhibited a latent period of 40 min with a burst size of 333 PFU/infected cells. Meanwhile, Phage P160 showed a latent period of 60 min and a burst size of 367 PFU/infected cells.

3.4. Stability of bacteriophages

The survival rate of three bacteriophages in artificial seawater, collected at different sampling times, showed that the titers of all three bacteriophages decreased but maintained viability over an extended period of 100 days. The titer of the P122 bacteriophage decreased by 1.89 logs during the study. It remained viable until the 40th day, after which the titer slightly decreased through to the 100th day (Fig. 2). The titer of the P125 bacteriophage decreased by 3.89 log, remaining viable until the 24th day, followed by a gradual decline until the 50th day and a significant reduction by the 100th day (Fig. 2). Similarly, the titer of the P160 bacteriophage decreased by 2.04 log, maintaining viability until

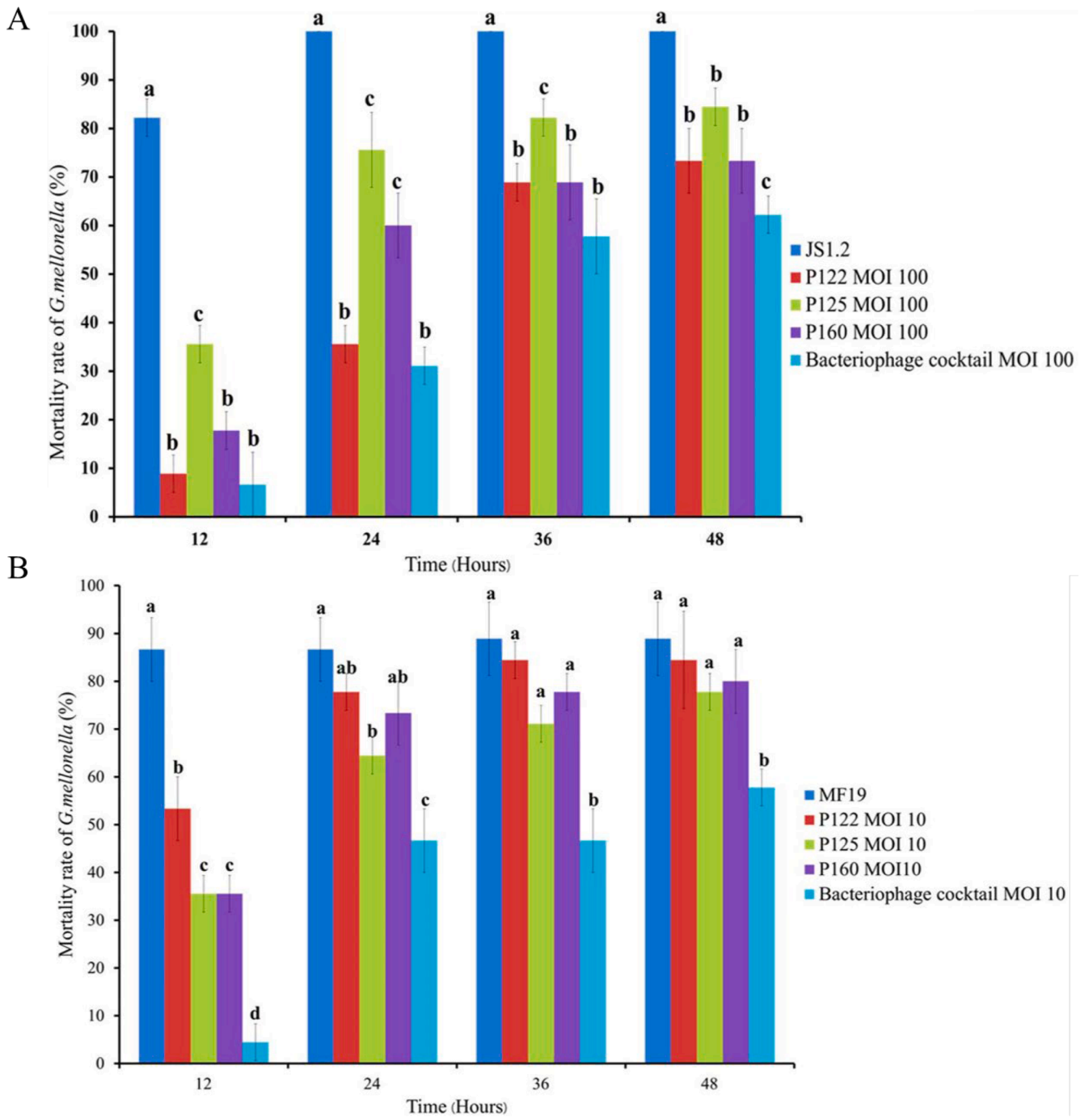

Fig. 4. The mortality rate of *G. mellonella* larvae following infection with *V. alginolyticus* and treatment with bacteriophages. (A) *V. alginolyticus* JS1.2-infected larvae treated with single bacteriophages P122, P125, P160, or a bacteriophage cocktail at an MOI of 100. (B) *V. alginolyticus* MF19-infected larvae treated with single bacteriophages P122, P125, P160, or a bacteriophage cocktail at an MOI of 10.

the 42nd day, after which it slightly declined until the 100th day (Fig. 2).

3.5. Morphology of bacteriophages

Transmission electron microscopy (TEM) revealed that the three bacteriophages have an icosahedral head and a non-contractile tail. The head of the P122 bacteriophage measured approximately 62.5 nm in width and 87.5 nm in length (Fig. 3A). Similarly, the head of the P125 bacteriophage was approximately 62.5 nm wide and 87.5 nm long (Fig. 3B). The P160 bacteriophage head was slightly smaller, measuring around 50 nm in width and 87.5 nm in length (Fig. 3C). Based on these morphological characteristics, P122, P125, and P160 bacteriophages are classified within the class Caudoviricetes and exhibit features consistent with phages previously categorized under the now-defunct family *Siphoviridae* (Ackermann, 2003).

3.6. Bacteriophage activity and in vivo efficacy in G. mellonella larvae against V. alginolyticus

In this study, the titers of bacteriophage isolates collected from the hemolymph of *G. mellonella* larvae remained stable over a 48-h period, showing no significant decrease (Fig. S1). The *V. alginolyticus* strains

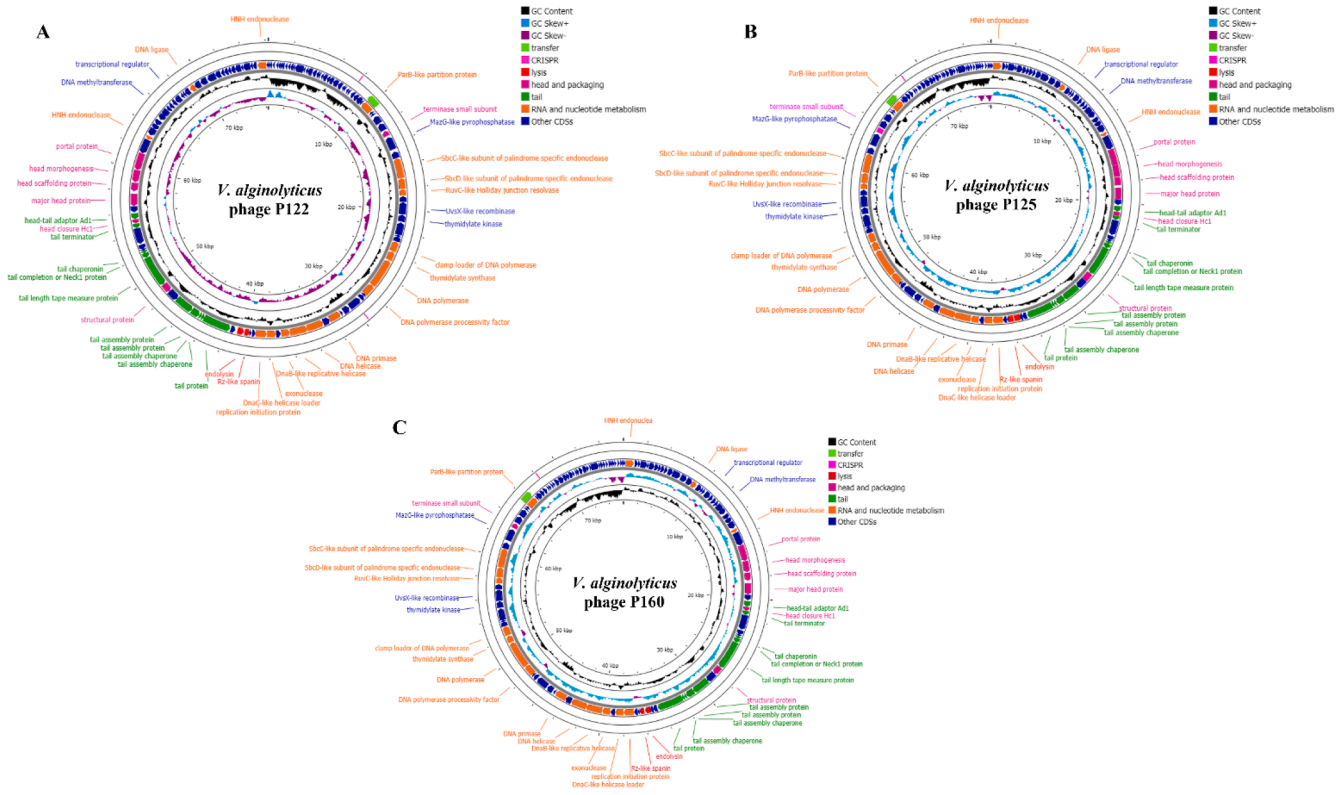

Fig. 5. The circular genome maps of *V. alginolyticus* bacteriophages P122 (A), P125 (B), and P160 (C).

MF19 and JS1.2, isolated from fish, were shown to be susceptible to all three bacteriophages (data not shown). The ability of these bacteriophages to rescue *G. mellonella* larvae infected with lethal doses (LD_{50}) of *V. alginolyticus* was evaluated. The LD_{50} for *V. alginolyticus* strains MF19 and JS1.2 was determined to be 5.2×10^4 CFU/larvae and 4.0×10^5 CFU/larvae, respectively. Bacteriophage treatments, including single bacteriophages (P122, P125, and P160) and a cocktail of all three phages at multiplicities of infection (MOI) of 1, 10, and 100, were tested for their effect on reducing the mortality of larvae infected with both strains. In larvae infected with *V. alginolyticus* strain JS1.2 and treated with single bacteriophages at an MOI of 100 for 48 h, a significant decrease in mortality was observed. The use of single phages P122, P125, and P160 reduced mortality by 27 %, 16 %, and 27 %, respectively. However, the bacteriophage cocktail, where all three phages were mixed in equal concentrations, showed a significantly higher efficiency, reducing the mortality rate by 38 % compared to untreated larvae infected with strain JS1.2 (Fig. 4A). Similarly, in larvae infected with *V. alginolyticus* strain MF19 and treated with single bacteriophages at an MOI of 10 for 48 h, mortality was reduced by 13 %, 7 %, and 9 % for P122, P125, and P160, respectively (data not shown). However, there was no significant difference in mortality between single bacteriophage treatments. The bacteriophage cocktail demonstrated a significantly higher protective effect, reducing mortality by 31 % compared to untreated larvae infected with strain MF19 (Fig. 4B). Moreover, no mortality was observed in larvae injected with PBS buffer, single bacteriophages (P122, P125, or P160), or the bacteriophage cocktail in the absence of *V. alginolyticus* infection (data not shown).

3.7. Whole-genome sequencing (WGS) and comparative genomic analysis

The whole-genome sequences (WGS) of the three bacteriophages were analyzed through Illumina sequencing technology. The final assembled genomes of *V. alginolyticus* phages P122 and P125 were 76,250 base pairs (bp), while P160 had a genome size of 76,020 bp, all composed of linear double-stranded DNA (dsDNA) with a GC content of 48.8 % (Fig. 5 and Table 4). Similar to TEM, genome analysis confirmed that all three phages belonged to the tailed complex phages from the Caudoviricetes class. Genome annotation revealed a total of 272, 276, and 280 ORFs in the genomes of phages P122, P125, and P160, respectively, all starting with the AUG codon and with no tRNA genes identified. Of these, 123 ORFs in P122, and 124 ORFs in P125 and P160 were predicted to be coding sequences (CDSs) (Table 4). Moreover, no genes related to antibiotic resistance or virulence factors were found in any of the phage genomes. The circular genome maps highlight key features, including structural proteins, regulatory elements, and enzymes involved in DNA processing and replication. Functional annotation of the ORFs for phages P122, P125, and P160 identified 82, 83, and 83 hypothetical proteins, respectively. Among the structural proteins, the major head protein was located at ORF 90 in P122 and ORF 34 in both P125 and P160, with five additional proteins associated with head and DNA packaging functions. Additionally, a portal protein was annotated at ORF 93 in P122 and ORF 31 in both P125 and P160. The genomes also encoded 11 tail proteins: ORFs 87–88, 73–77, 80–82, and 86 in P122, and ORFs 36–38, 42–44, and 47–51 in P125 and P160. Remarkably, all phages were found to encode lysis-associated proteins, including endolysin (ORF 71 in P122, and ORF 54 in both P125 and P160) and Rz-like spanin (ORF 72 in P122, and ORF 55 in both P125 and P160). Other enzymatic proteins included endonucleases (ORFs 95 and 122 in P122, and ORFs 2 and 29 in P125 and P160) and exonucleases

Table 4
Structural characteristics of phage P122, P125, and P160 genomes.

Phage	Size (bp)	GC content (%)	tRNA	ORFs	Number of Coding Sequences
P122	76,250	48.8	0	272	123
P125	76,250	48.8	0	276	124
P160	76,020	48.8	0	280	124

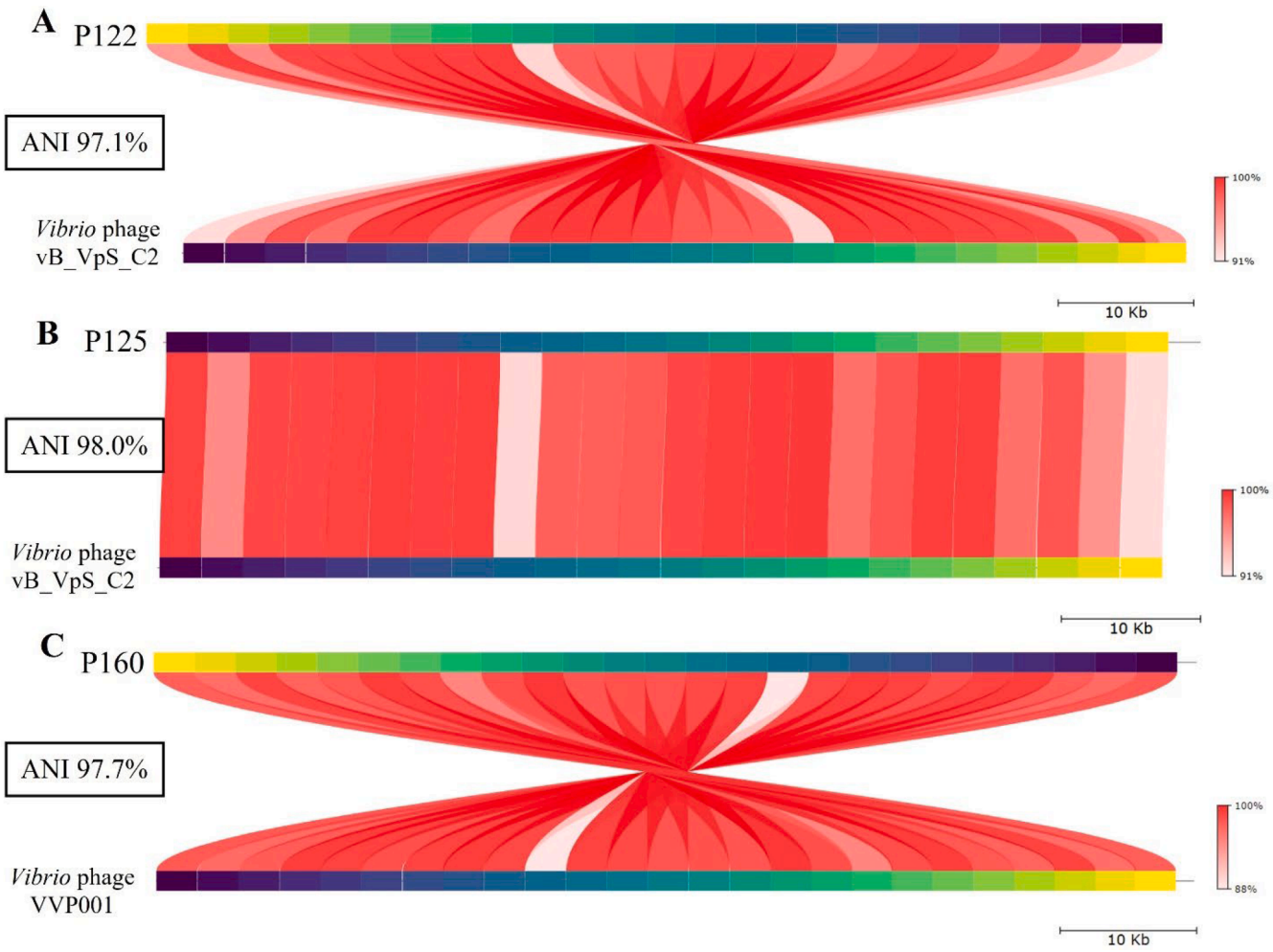

Fig. 6. Average Nucleotide Identity (ANI) comparison of *V. aliginolyticus* phages P122, P125, and P160 and their closely related *Vibrio* phage. Phages P122 (A) and P125 (B) are similar to *Vibrio* phages vB_VpS_C2 and P160 (C) is close to *Vibrio* phage VVP001. High ANI values indicate strong genetic similarity. Scale bars: 10 Kb.

(ORF 64 in P122, and ORFs 2 and 61 in both P125 and P160). A detailed list of all predicted proteins and their corresponding CDS positions is provided in Supplementary Tables S1, S2, and S3.

In addition, BLASTn similarity searches for phages P122, P125, and P160 against related phages in the GenBank database revealed over 97 % similarity to other *Vibrio* phages, including *Vibrio* phage vB_VpS_C2 (GenBank Acc. No. MZ592922.1) for P122 and P125, and *Vibrio* phage VVP001 (GenBank Acc. No. MG602476.1) for P160. Comparative genomic analysis using Average Nucleotide Identity (ANI) to assess genetic similarity is shown in Fig. 6. The synteny map displays the aligned genomic regions, with darker red areas indicating higher similarity. Phage P122 has an ANI value of 97.9 % with *Vibrio* phage vB_VpS_C2, while P125 shows a slightly higher ANI value of 98.0 % with the same phage, reflecting near-identical genomic sequences. Phage P160 has an ANI value of 97.7 % compared with *Vibrio* phage VVP001.

The phylogenetic tree presented in Fig. 7 illustrates the evolutionary relationships among various *Vibrio* phages, including our three *V. alginolyticus* phages (P122, P125, and P160), highlighted with red stars and their closest relatives. The tree was constructed based on the terminase subunit sequencing, using a neighbor-joining method with bootstrap values indicated at each node. Phages P122, P125, and P160 form a closely related clade with strong bootstrap support (99 to 100) and are closely associated with *Vibrio* phage vB ValS R12Z (GenBank Acc. No. PP094284), a phage known to specifically target *V. alginolyticus*. In contrast, other phages such as *Vibrio* phage vB VpS HCMJ (GenBank Acc. No. QEP53397) and *Vibrio* phage vB VpS CC6 (GenBank Acc. No. UTQ72760), phages known to specifically target *V. parahaemolyticus* show lower bootstrap values and clusters farther from the P122, P125, and P160 group. Also, phages that are specific to other *V. parahemolyticus* and *V. cholerae* form separate branches, reflecting their divergent evolutionary trajectories and likely different host specificities.

Genomic comparison between various phages, highlighting intergenomic similarity, aligned genome fraction, and genome length ratio among our phages P122, P125, and P160, and their close relatives, was calculated using VIRIDIC (Fig. 8). The heatmap shows that phages P122, P125, and P160 are closely related, with high intergenomic similarity (98.4 % to 100 %) and nearly complete genome alignment. Their aligned genome fractions and genome length ratios are close to 1.0, further indicating their genomic integrity and conservation. When compared to other *V. alginolyticus* phages, P122, P125, and P160 show a high degree of similarity (>91 %) with phage PVA73 and vB ValS_R12Z which are *V. alginolyticus* phages. In contrast, distant relationships are observed with phages R01, which presented lower similarity scores (~66 %) to our phages P122, P125, and P160. The genome lengths of the phages are relatively consistent, with slight variations reflecting potential structural differences.

4. Discussions

V. alginolyticus is a well-known pathogen responsible for significant economic losses in aquaculture, particularly in fish and invertebrate

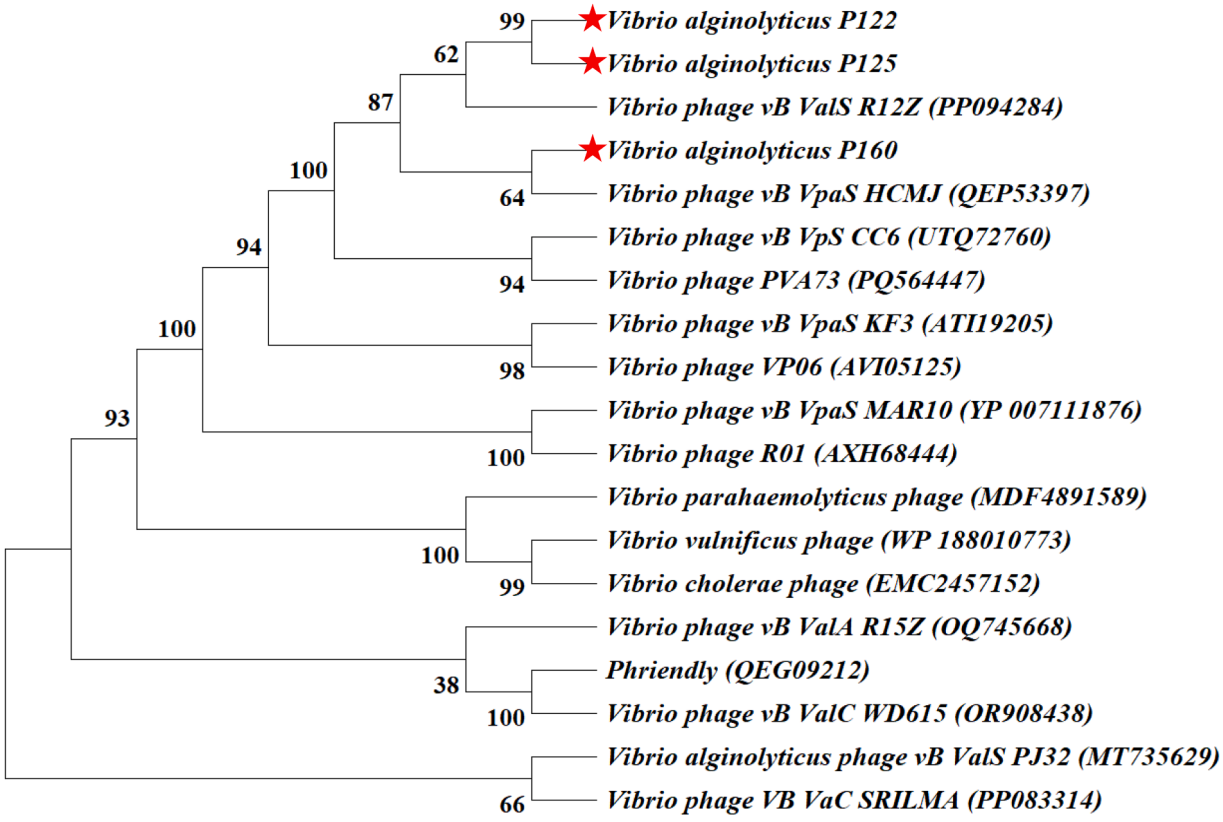

Fig. 7. Phylogenetic tree based on terminase large subunit sequences of V. *alginolyticus* phages P122, P125, and P160 (marked with red stars) and other selected phages. The tree was generated using the neighbor-joining method in MEGA version 11, with 1000 bootstrap replications.

hatcheries. It can cause severe infections in marine organisms, leading to high mortality rates and reduced production efficiency (Manchanayake et al., 2023). The contamination of marine environments and seafood with *V. alginolyticus* poses a considerable threat, as it can persist in reservoirs such as cockles, oysters, shrimp, and other marine organisms (Song et al., 2020). Traditional methods for controlling *Vibrio* outbreaks, such as antibiotics, are becoming increasingly ineffective due to the rise of antimicrobial resistance (Muthulakshmi and Mothadaka, 2023). As a result, alternative strategies, such as the use of bacteriophages, have gained interest as a targeted and environmentally friendly solution for controlling *Vibrio* infections in aquaculture (Hossain et al., 2024; Ramos-Vivas et al., 2021). In this study, 414 lytic bacteriophages specific to *V. alginolyticus* PSU 6 were isolated from seafood and marine environmental samples such as cockles, oysters, water, sediments, shrimps, mussels, and green caviar. These phages were tested for their ability to lyse *V. alginolyticus* and other bacterial strains, revealing that 79.01 % of fish pathogenic *V. alginolyticus* strains and 44.69 % of environmental strains were susceptible to these phages. Notably, none of the phages were able to infect clinical strains of *V. alginolyticus*, possibly due to structural adaptations on the bacterial cell surface that prevent phage attachment. Moreover, some bacteriophages demonstrated cross-lytic activity, lysing 32.37 % of fish pathogenic strains of *V. parahaemolyticus* and 5.11 % of *V. vulnificus* strains, suggesting that these bacteria may share common receptors with *V. alginolyticus* (Yingkajorn et al., 2014). Among these, phages P122, P125, and P160 emerged as the most promising candidates, displaying broad host ranges and high lytic activity. One-step growth experiments revealed differences in their latent periods and burst sizes. P122 had a latent period of 50 min and a burst size of 140 PFU/infected cell, P125 had a latent period of 40 min and a burst size of 333 PFU/infected cell, and P160 had a latent period of 60 min with a burst size of 367 PFU/infected cell. While the latent periods were relatively long, the high burst sizes indicate strong potential for therapeutic applications, as phages with high burst sizes are generally more effective in bacterial eradication (Kalatzis et al., 2016; Lin et al., 2012). Transmission electron microscopy (TEM) revealed that all three phages had an icosahedral head with a non-contractile tail, placing them within the class Caudoviricetes. Genomic analysis showed highly similar genomes for P122, P125, and P160, with lengths of approximately 76,250 bp for P122 and P125, and 76,020 bp for P160, and a GC content of 48.8 %. All genomes contained between 272 and 280 open reading frames (ORFs), with no tRNA genes or antibiotic resistance genes, highlighting their suitability for aquaculture applications. Key structural proteins, such as head, portal, neck, and tail proteins, were conserved across all three phage genomes. Lysis-associated proteins, such as endolysin and Rz-like spanin, were identified in the genomes, emphasizing their role in the bacteriophage lytic cycle. Endolysin, such as those identified at ORF 70 in P122 and ORF 54 in P125 and P160, degrades the bacterial cell wall, while Rz-like spanin disrupts the outer membrane, facilitating cell lysis and the release of viral progeny (Miroshnikov et al., 2021). These proteins are essential for the efficient propagation of phages and their ability to eliminate bacterial infections. Moreover, genes involved in DNA replication and repair, such as DNA polymerases, helicases, and ligases, were conserved, ensuring the stability and integrity of the phage genomes under various environmental conditions (Morcinek-Orłowska et al., 2022; Shen et al., 2012; Weitzman and Fradet-Turcotte, 2018). Notably, no genes related to antibiotic resistance or virulence were detected, emphasizing the potential of these phages for phage therapy or biocontrol applications (Li et al., 2021). Furthermore, numerous hypothetical proteins were identified, indicating the presence of novel, uncharacterized functions that warrant further investigation. Genomic similarity analysis revealed ANI values exceeding 97 % among P122, P125, and P160 with other *Vibrio* phages in the GenBank database, confirming their close evolutionary relationship (Chibani et al., 2020).

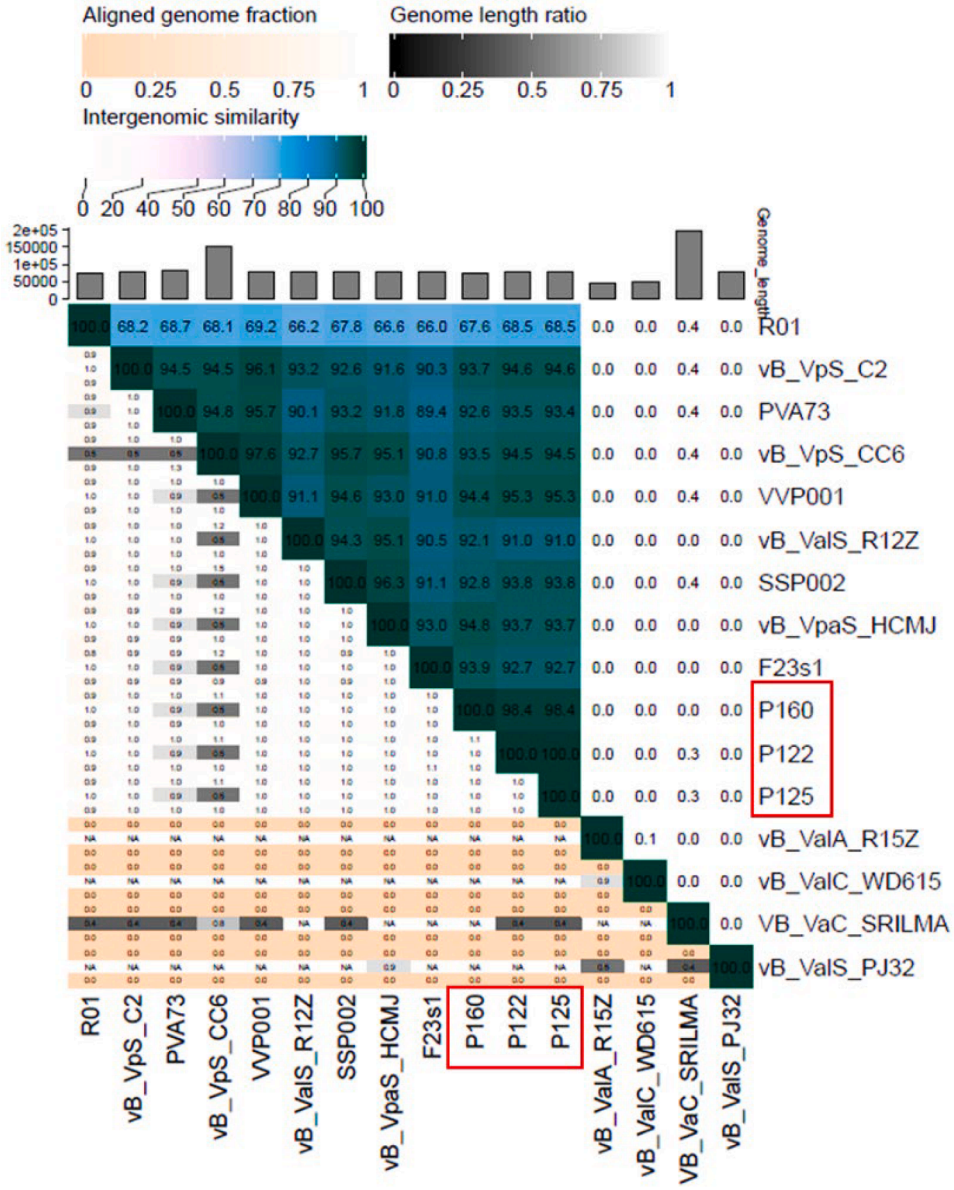

Fig. 8. Whole-genome comparison and clustering of *V. alginolyticus* phages P122, P125, and P160 (highlighted with a red frame), alongside their nine closest relatives. The analysis and clustering were conducted using VIRIDIC v1.1. The different shades of blue in the right half of the heatmap represent varying levels of genomic similarity between the phages.

The synteny map of aligned genomic regions, where darker red areas signify higher similarity, exhibits the conserved structure among these phages. This high genetic similarity, as well as the strong phylogenetic bootstrap support (99–100) for the clade containing these phages, suggests they share a recent common ancestor and have likely evolved under similar ecological conditions or host pressures (Wang et al., 2020). Moreover, they are most closely related to *Vibrio* phage vB ValS R12Z which also shares high bootstrap support, reinforcing their close genetic and functional similarities. These relationships indicate that these phages likely belong to the same genetic lineage or phage group within the *V. alginolyticus* cluster. Interestingly, phages such as *Vibrio* phage R01 and *Vibrio* phage VB Vac SRILMA are positioned further down the tree with low bootstrap values which indicates their genomic and evolutionary distance from the main *V.* alginolyticus group. This highlights the diversity of phages targeting *Vibrio* species and emphasizes the specificity of P122, P125, and P160 to their host (Hendrix et al., 1999). Furthermore, their close relationship to other *Vibrio* phages, and their suitability for targeting *Vibrio*-related diseases in marine environments, highlights their potential for managing infections in fish farming, where *Vibrio* infections are prevalent. The efficacy of these phages was further demonstrated in the *G. mellonella* model. Single-phage treatments reduced the mortality of larvae infected with *V. alginolyticus*, while a phage cocktail of P122, P125, and P160 significantly enhanced survival rates. The cocktail approach is particularly valuable for delaying the emergence of phage-resistant bacterial strains, a common challenge with single-phage therapies (Cui et al., 2021; Filippov et al., 2022; Quiroz-Guzmán et al., 2018). In this study, the bacteriophage cocktail (a mixture of P122, P125, and P160 at equal concentrations) proved to be more efficient than individual phages in reducing the mortality rate of infected *G. mellonella* larvae. This result highlights the potential of using phage cocktails to extend the effectiveness of phage therapy and mitigate the emergence of phage-resistant bacterial strains. In conclusion, this study demonstrates the potential of phages P122, P125, and P160 as effective biocontrol agents for managing *V. alginolyticus* infections in aquaculture. Their broad host range, high lytic efficiency, and genomic stability, combined with the absence

of antibiotic resistance or virulence genes, emphasize their suitability for phage therapy. The use of phage cocktails further enhances therapeutic outcomes, offering a sustainable alternative to antibiotics in combating bacterial infections in marine environments.

CRediT authorship contribution statement

Nattarika Chaichana: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Rutinan Rattanaburee:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Komwit Surachat:** Writing – review & editing, Methodology, Conceptualization. **Decha Sermwittayawong:** Writing – review & editing, Methodology, Conceptualization. **Natthawan Sermwittayawong:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by the Fiscal Fund Year 2017 and Prince of Songkla University (Grant number: SCI600266S), the Ph.D. Scholarship (Grant number: PSU_PHD2563–01) and the Ph.D. Oversea Thesis Research Scholarship (Grant number: OTR2566–006).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2025.199529.

Data availability

Data will be made available on request.

References

Abdelrahman, F., Easwaran, M., Daramola, O.I., Ragab, S., Lynch, S., Oduselu, T.J., Khan, F.M., Ayobami, A., Adnan, F., Torrents, E., Sanmukh, S., El-Shibiny, A., 2021. Phage-encoded endolysins. Antibiotics (Basel) 10 (2). https://doi.org/10.3390/antibiotics10020124.

Abedon, S.T., Danis-Wlodarczyk, K.M., Wozniak, D.J., 2021. Phage cocktail development for bacteriophage therapy: toward improving spectrum of activity breadth and depth. Pharmaceuticals (Basel) 14 (10). https://doi.org/10.3390/ph14101019.

Ackermann, H.W., 2003. Bacteriophage observations and evolution. Res. Microbiol. 154 (4), 245–251. https://doi.org/10.1016/S0923-2508(03)00067-6.

Antoine, C., Laforêt, F., Blasdel, B., Fall, A., Duprez, J.N., Mainil, J., Delcenserie, V., Thiry, D., 2021. In vitro characterization and in vivo efficacy assessment in *Galleria mellonella* larvae of newly isolated bacteriophages against *Escherichia coli* K1. Viruses 13 (10). https://doi.org/10.3390/v13102005.

Aziz, N.S., Ibrahim, S., Zaharinie, T., Tang, S.S., 2025. Bacteriophage encapsulation - trends and potential applications in aquaculture. Aquaculture 594, 741398. https://doi.org/10.1016/j.aquaculture.2024.741398.

Bouras, G., Nepal, R., Houtak, G., Psaltis, A.J., Wormald, P.-J., Vreugde, S., 2022. Pharokka: a fast scalable bacteriophage annotation tool. Bioinformatics 39 (1). https://doi.org/10.1093/bioinformatics/btac776.

Chibani, C.M., Hertel, R., Hoppert, M., Liesegang, H., Wendling, C.C., 2020. Closely related *Vibrio alginolyticus* strains encode an identical repertoire of Caudovirales-like regions and filamentous phages. Viruses 12 (12). https://doi.org/10.3390/v12121359.

Cui, H., Cong, C., Wang, L., Li, X., Li, J., Yang, H., Li, S., Xu, Y., 2021. Protective effectiveness of feeding phage cocktails in controlling *Vibrio harveyi* infection of turbot *Scophthalmus maximus*. Aquaculture 535, 736390. https://doi.org/10.1016/j.aquaculture.2021.736390.

de Souza Valente, C., Wan, A.H.L., 2021. *Vibrio* and major commercially important vibriosis diseases in decapod crustaceans. J. Invertebr. Pathol. 181, 107527. https://doi.org/10.1016/j.jip.2020.107527.

Filippov, A.A., Su, W., Sergueev, K.V., Kevorkian, R.T., Snesrud, E.C., Srijan, A., He, Y., Fouts, D.E., Lurchachaiwong, W., McGann, P.T., Ellison, D.W., Swierczewski, B.E., Nikolich, M.P., 2022. Design of a bacteriophage cocktail active against *Shigella* species and testing of its therapeutic potential in *Galleria mellonella*. Antibiotics 11 (11), 1659. https://www.mdpi.com/2079-6382/11/11/1659.

Fu, J., Li, Y., Zhao, L., Wu, C., He, Z., 2023. Characterization of vB_ValM_PVA8, a broad-host-range bacteriophage infecting *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. Front. Microbiol. 14, 1105924. https://doi.org/10.3389/fmicb.2023.1105924.

Garvey, M., 2022. Bacteriophages and food production: biocontrol and bio-preservation options for food safety. Antibiotics (Basel) 11 (10). https://doi.org/10.3390/antibiotics11101324.

Grant, J.R., Enns, E., Marinier, E., Mandal, A., Herman, E.K., Chen, C.-y., Graham, M., Van Domselaar, G., Stothard, P., 2023. Proksee: in-depth characterization and visualization of bacterial genomes. Nucleic Acids Res. 51 (W1), W484–W492. https://doi.org/10.1093/nar/gkad326.

Hao, Q., Bai, Y., Zhou, H., Bao, X., Wang, H., Zhang, L., Lyu, M., Wang, S., 2023. Isolation and characterization of bacteriophage VA5 against *Vibrio alginolyticus*. Microorganisms 11 (12). https://doi.org/10.3390/microorganisms11122822.

Hendrix, R.W., Smith, M.C., Burns, R.N., Ford, M.E., Hatfull, G.F., 1999. Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. Proc. Natl. Acad. Sci. 96 (5), 2192–2197. https://doi.org/10.1073/pnas.96.5.2192.

Higuera, G., Bastías, R., Tsertsvadze, G., Romero, J., Espejo, R.T., 2013. Recently discovered *Vibrio anguillarum* phages can protect against experimentally induced vibriosis in atlantic salmon, *Salmo salar*. Aquaculture 392, 128–133. https://doi.org/10.1016/j.aquaculture.2013.02.013.

Hitchcock, N.M., Nunes, D.D.G., Shiach, J., Hodel, K.V.S., Barbosa, J.D.V., Rodrigues, L. A.P., Coler, B.S., Soares, M.B.P., Badaró, R., 2023. Current clinical landscape and global potential of bacteriophage therapy. Viruses 15 (4). https://doi.org/10.3390/v15041020.

Hossain, M.M.M., Tanni, L.N., Rahman, M.A., Farjana, N., Moon, R.S., Tonni, N.Z., Mekat, M.R., Mojumdar, S., Rahman, N., Sen, B.K., Rojoni, S.A., Rubayea, U., Saha, P.K., 2024. Bacteriophage and non-pathogenic *Vibrio* to control diseases in shrimp aquaculture. Comp. Immunol. Rep. 6, 200126. https://doi.org/10.1016/j.cirep.2023.200126.

Jain, C., Rodriguez-R, L.M., Phillippy, A.M., Konstantinidis, K.T., Aluru, S., 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat. Commun. 9 (1), 5114. https://doi.org/10.1038/s41467-018-07641-9.

Kah Sem, N.A.D., Abd Gani, S., Chong, C.M., Natrah, I., Shamsi, S., 2023. Management and mitigation of vibriosis in aquaculture: nanoparticles as promising alternatives. Int. J. Mol. Sci. 24 (16), 12542. https://doi.org/10.3390/ijms241612542.

Kalatzis, P.G., Bastías, R., Kokkari, C., Katharios, P., 2016. 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 11 (3), e0151101. https://doi.org/10.13712/Fjournal.pone.0151101.

Kang, C.-H., Shin, Y., Jang, S., Jung, Y., So, J.-S., 2016. Antimicrobial susceptibility of *Vibrio alginolyticus* isolated from oyster in Korea. Environ. Sci. Pollut. Res. Int. 23, 21106–21112. https://doi.org/10.1007/s11356-016-7426-2.

Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinformatics 9 (4), 299–306. https://doi.org/10.1093/bib/bbn017.

Kwok, C.T., Yu, R.C., Hau, P.T., Cheung, K.Y., Ng, I.C., Fung, J., Wong, I.T., Yau, M.C., Liu, W.M., Kong, H.K., Siu, G.K., Chow, F.W., Seto, S.W., 2024. Characteristics and pathogenicity of *Vibrio alginolyticus* SWS causing high mortality in mud crab (*Scylla serrata*) aquaculture in Hong Kong. Front. Cell Infect. Microbiol. 14, 1425104. https://doi.org/10.3389/fcimb.2024.1425104.

Le, T.S., Southgate, P.C., O'Connor, W., Vu, S.V., Kurtböke, D., 2020. Application of bacteriophages to control *Vibrio alginolyticus* contamination in Oyster (*Saccostrea glomerata*) larvae. Antibiotics (Basel) 9 (7). https://doi.org/10.3390/antibiotics9070415.

Li, J., Li, Y., Ding, Y., Huang, C., Zhang, Y., Wang, J., Wang, X., 2021. Characterization of a novel *Siphoviridae Salmonella* bacteriophage T156 and its microencapsulation application in food matrix. Food Res. Int. 140, 110004. https://doi.org/10.1016/j.foodres.2020.110004.

Lin, Y.R., Chiu, C.W., Chang, F.Y., Lin, C.S., 2012. Characterization of a new phage, termed ϕA318, which is specific for *Vibrio alginolyticus*. Arch. Virol. 157 (5), 917–926. https://doi.org/10.1007/s00705-012-1244-8.

Lin, D.M., Koskella, B., Lin, H.C., 2017. Phage therapy: an alternative to antibiotics in the age of multi-drug resistance. World J. Gastrointest. Pharmacol. Ther. 8 (3), 162–173. https://doi.org/10.4292/wjgpt.v8.i3.162.

Manchanayake, T., Salleh, A., Amal, M.N.A., Yasin, I.S.M., Zamri-Saad, M., 2023. Pathology and pathogenesis of *Vibrio* infection in fish: a review. Aquac. Rep. 28, 101459. https://doi.org/10.1016/j.aqrep.2022.101459.

Manyi-Loh, C., Mamphweli, S., Meyer, E., Okoh, A., 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. Molecules 23 (4). https://doi.org/10.3390/molecules23040795.

Mateus, L., Costa, L., Silva, Y.J., Pereira, C., Cunha, A., Almeida, A., 2014. Efficiency of phage cocktails in the inactivation of *Vibrio* in aquaculture. Aquaculture 167–173. https://doi.org/10.1016/j.aquaculture.2014.01.001.

Miroshnikov, K.A., Evseev, P.V., Lukianova, A.A., Ignatov, A.N., 2021. Tailed lytic bacteriophages of soft rot *pectobacteriaceae*. Microorganisms 9 (9), 1819. https://www.mdpi.com/2076-2607/9/9/1819.

Morcinek-Orłowska, J., Zdrojewska, K., Węgrzyn, A., 2022. Bacteriophage-encoded DNA polymerases-beyond the traditional view of polymerase activities. Int. J. Mol. Sci. 23 (2). https://doi.org/10.3390/ijms23020635.

Muthulakshmi, T., Mothadaka, M.P., 2023. *Vibrio vulnificus* and its antimicrobial resistance. Springer Nature Singapore, pp. 1–18. https://doi.org/10.1007/978-981-16-9723-4_25-1.

Nale, J.Y., Chutia, M., Carr, P., Hickenbotham, P.T., Clokie, M.R., 2016. Get in early'; biofilm and wax moth (*Galleria mellonella*) models reveal new insights into the therapeutic potential of *Clostridium difficile* bacteriophages. Front. Microbiol. 7, 1383. https://doi.org/10.3389/fmicb.2016.01383.

Oechslin, F., 2018. Resistance development to bacteriophages occurring during bacteriophage therapy. Viruses 10 (7). https://doi.org/10.3390/v10070351.

Quiroz-Guzmán, E., Peña-Rodriguez, A., Vázquez-Juárez, R., Barajas-Sandoval, D.R., Balcázar, J.L., Martínez-Díaz, S.F., 2018. Bacteriophage cocktails as an environmentally-friendly approach to prevent *Vibrio parahaemolyticus* and *Vibrio harveyi* infections in brine shrimp (*Artemia franciscana*) production. Aquaculture 492, 273–279. https://doi.org/10.1016/j.aquaculture.2018.04.025.

Ramos-Vivas, J., Superio, J., Galindo-Villegas, J., Acosta, F., 2021. Phage therapy as a focused management strategy in aquaculture. Int. J. Mol. Sci. 22 (19). https://doi.org/10.3390/ijms221910436.

Reilly, G., Reilly, C., Smith, E., Baker-Austin, C., 2011. *Vibrio alginolyticus*-associated wound infection acquired in British waters, Guernsey, July 2011. Euro. Surveill. 16 (42), 19994.

Rodríguez-Rubio, L., Muniesa, M., 2021. Isolation and characterization of shiga toxin bacteriophage. Shiga Toxin-Producing E. coli: Method. Protocol. 119–144.

Sampaio, A., Silva, V., Poeta, P., Aonofriesei, F., 2022. *Vibrio* spp.: life strategies, ecology, and risks in a changing environment. Diversity (Basel) 14 (2), 97. https://www.mdpi.com/1424-2818/14/2/97.

Shen, X., Li, M., Zeng, Y., Hu, X., Tan, Y., Rao, X., Jin, X., Li, S., Zhu, J., Zhang, K., Hu, F., 2012. Functional identification of the DNA packaging terminase from *Pseudomonas aeruginosa* phage PaP3. Arch. Virol. 157 (11), 2133–2141. https://doi.org/10.1007/s00705-012-1409-5.

Song, X., Zang, J., Yu, W., Shi, X., Wu, Y., 2020. Occurrence and identification of pathogenic *Vibrio* contaminants in common seafood available in a chinese traditional market in Qingdao, Shandong province. Front. Microbiol. 11, 1488. https://doi.org/10.3389/fmicb.2020.01488.

Stalin, N., Srinivasan, P., 2017. Efficacy of potential phage cocktails against *Vibrio harveyi* and closely related *Vibrio* species isolated from shrimp aquaculture environment in the south east coast of India. Vet. Microbiol. 207, 83–96. https://doi.org/10.1016/j.vetmic.2017.06.006.

Tan, D., Gram, L., Middelboe, M., 2014. Vibriophages and their interactions with the fish pathogen *Vibrio anguillarum*. Appl. Environ. Microbiol. 80 (10), 3128–3140. https://doi.org/10.1128/aem.03544-13.

Van Belleghem, J.D., Dąbrowska, K., Vaneechoutte, M., Barr, J.J., Bollyky, P.L., 2018. Interactions between bacteriophage, bacteria, and the mammalian immune system. Viruses 11 (1). https://doi.org/10.3390/v11010010.

Wang, L., Lim, C.K., Klotz, M.G., 2020. High synteny and sequence identity between genomes of *Nitrosococcus oceani* strains isolated from different oceanic gyres reveals genome economization and autochthonous clonal evolution. Microorganisms 8 (5). https://doi.org/10.3390/microorganisms8050693.

Wang, Z., Wang, H., Chen, D., Li, Y., 2024. Genomic characterization and comparative genomic analysis of pathogenic *Vibrio* isolated from aquaculture-grown white-leg shrimp (*Penaeus vannamei*) in Guangdong and Jiangsu, China. Aquaculture 580, 740302. https://doi.org/10.1016/j.aquaculture.2023.740302.

Weitzman, M.D., Fradet-Turcotte, A., 2018. Virus DNA replication and the host DNA damage response. Annu. Rev. Virol. 5 (1), 141–164. https://doi.org/10.1146/annurev-virology-092917-043534.

Xu, Y., Sun, J., Hu, J., Bao, Z., Wang, M., 2023. Characterization and preliminary application of a novel lytic *Vibrio parahaemolyticus* bacteriophage vB_VpaP_SJSY21. Int. J. Mol. Sci. 24 (24), 17529. https://www.mdpi.com/1422-0067/24/24/17529.

Yingkajorn, M., Sermwitayawong, N., Palittapongarnpimp, P., Nishibuchi, M., Robins, W.P., Mekalanos, J.J., Vuddhakul, V., 2014. *Vibrio parahaemolyticus* and its specific bacteriophages as an indicator in cockles (*Anadara granosa*) for the risk of *V. parahaemolyticus* infection in Southern Thailand. Microb. Ecol. 67, 849–856. https://doi.org/10.1007/s00248-014-0382-9.

Zhang, J., Cao, Z., Li, Z., Wang, L., Li, H., Wu, F., Jin, L., Li, X., Li, S., Xu, Y., 2015. Effect of bacteriophages on *Vibrio alginolyticus* infection in the sea cucumber, *Apostichopus japonicus* (Selenka). J. World Aquac. Soc. 46 (2), 149–158. https://doi.org/10.1111/jwas.12177.

Zhang, Y., Ding, Y., Li, W., Zhu, W., Wang, J., Wang, X., 2021. Application of a novel lytic *Podoviridae* phage Pu20 for biological control of drug-resistant *Salmonella* in liquid eggs. Pathogens 10 (1), 34. https://www.mdpi.com/2076-0817/10/1/34.

Zhao, J., Zhang, Z., Tian, C., Chen, X., Hu, L., Wei, X., Li, H., Lin, W., Jiang, A., Feng, R., Yuan, J., Yin, Z., Zhao, X., 2019. Characterizing the biology of lytic bacteriophage vB_EaeM_φEap-3 infecting multidrug-resistant *Enterobacter aerogenes*. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.00420.