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Mitigating acute hepatopancreatic necrosis disease in vannamei shrimp: Harnessing bacteriophages from hepatopancreas and mangrove litter for sustainable aquaculture

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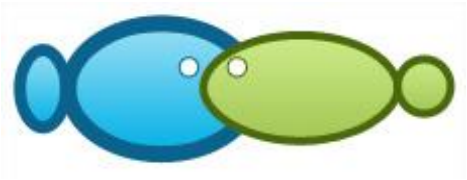

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AHPND, first identified in 2009, has rapidly become one of the most devastating diseases in shrimp aquaculture, causing high mortality rates within 30 days of pond stocking (Chandran et al 2023). This disease is primarily caused by specific strains of *Vibrio parahaemolyticus*, which secrete PirA and PirB toxins, leading to the destruction of shrimp hepatopancreatic cells (Wananda et al 2022; Beltran et al 2023; Castellanos et al 2023). The economic impact of AHPND has been catastrophic, with losses exceeding billions of dollars globally (Powers et al 2021). In Indonesia alone, the disease has contributed to major declines in shrimp production, jeopardizing the livelihoods of smallholder farmers and the broader aquaculture industry (Shinn et al 2018).

Conventional approaches to managing AHPND have predominantly relied on the use of antibiotics and other chemical treatments (Santos et al 2020; Kumar et al 2021). However, the widespread application of antibiotics has led to growing concerns about the development of antimicrobial resistance (AMR), the persistence of antibiotic residues in the environment, and the potential transfer of resistance genes to pathogenic bacteria (Hemamalini et al 2022; Andriawan et al 2023). These challenges have sparked an urgent need for sustainable and effective alternatives that can control bacterial pathogens without further exacerbating AMR or negatively impacting aquatic ecosystems.

In recent years, bacteriophages have emerged as a promising biocontrol agent in aquaculture due to their ability to specifically target and lyse bacterial cells (Ninawe et al 2020; Nachimuthu et al 2021). Bacteriophages, or phages, are viruses that infect bacteria, hijacking their cellular machinery to replicate and ultimately cause bacterial cell death (Elois et al 2023). Unlike broad-spectrum antibiotics, bacteriophages offer highly targeted action, which limits collateral damage to beneficial microbiota and reduces the risk of AMR development (Zhang et al 2022). Moreover, phages are naturally occurring and can be isolated from environments such as marine ecosystems, where host bacteria thrive (Naureen et al 2020). Their specificity and efficacy might make them an attractive alternative in combating bacterial infections in aquaculture, particularly in the context of diseases like AHPND caused by *Vibrio* species.

This study focused on the exploration of bacteriophages as potential therapeutic agents to mitigate the impacts of AHPND in vannamei shrimp. Specifically, bacteriophages sourced from shrimp hepatopancreas, and mangrove litter were targeted as local, naturally occurring phages with the potential to combat *V. parahaemolyticus*. Mangrove ecosystems, characterized by rich biodiversity and complex microbial interactions, present a unique reservoir for phage discovery (Allard et al 2020), while the shrimp hepatopancreas, being a primary site of bacterial infection in AHPND, offers a source of host-specific phages (Ghosh et al 2023).

Material and Method

Isolation of Vibrio parahaemolyticus. *Vibrio parahaemolyticus* strains were isolated from infected shrimp showing clinical symptoms of AHPND. Ten diseased shrimp samples were collected from shrimp farms located in Pasuruan, East Java, Indonesia. The hepatopancreas tissue was aseptically excised, homogenized, and diluted in sterile physiological saline (0.85% NaCl). Serial dilutions were plated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubated at 30°C for 24-48 hours. Suspected colonies of *V. parahaemolyticus* were further subcultured and confirmed by molecular identification using PCR with specific primers targeting the toxR and PirA/B genes associated with AHPND (Table 1) (Eloit & Couacy-Hymann 2023).

Table 1

First and nested primers

Primer	*Sequence (5' to 3')*
AP4-F1	5’-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3’
AP4-R1	5’-ACG-ATT-TCG-ACG-TTC-CCC-AA-3'
AP4-F2	5’-TTG-AGA-ATA-CGG-GAC-GTG-GG-3’
AP4-R2	5’- GTT-AGT-CAT-GTG-AGC-ACC-TTC-3’

Bacteriophage isolation. Bacteriophages were isolated from two different sources: (i) hepatopancreas tissue of apparently healthy shrimp and (ii) mangrove litter collected from coastal areas near shrimp farms. Samples were processed by filtering through a 0.22 µm filter to remove bacterial debris. The filtrate (5 mL) was mixed with 5 mL of an overnight culture of *V. parahaemolyticus* (10^7 CFU mL^{-1}) and incubated at 37°C with orbital shaking for 6 h. The culture was centrifuged at 8000 x g for 10 minutes, and the supernatant was filtered again to obtain phage-containing solutions. The presence of bacteriophages was confirmed using a double-layer agar method, where plaques were visualized after incubating the plates at 37°C for 24 hours (Fu et al 2023).

Bacteriophage purification and propagation. Hepatopancreas-derived bacteriophage (HB) and mangrove litter-derived bacteriophage (MLB) plaques were isolated and propagated by mixing them with an exponential phase culture of *V. parahaemolyticus*. The mixture was incubated in tryptic soy broth (TSB) supplemented with 3% NaCl at 30°C for 24 hours. A single plaque was transferred into 1 mL of sterilized PBS buffer (pH 7.2) and incubated at 40°C for 30 minutes to extract phages. The phage suspension was then centrifuged at 12000 × g for 5 minutes and filtered through a 0.22 µm membrane. The filtrate was serially diluted in 10-fold PBS, and the double-layer agar method was repeated four times to obtain uniformly sized, shaped, and clear plaques. Then, 5 mL of the lysate from a single pure plaque (in PBS) was mixed with 5 mL of the host strain's fresh culture (10^7CFU mL^{-1}) in 50 mL of sterilized 2216E medium. This mixture was incubated at 37°C with orbital shaking for 6 hours to promote phage proliferation. Cell debris was removed by centrifugation at 8000 × g for 10 minutes, followed by filtration through a 0.22 µm membrane. The bacteriophage titer of the filtrate was quantified using the double-layer agar method and reported as plaque-forming units (PFU) per mL. The proliferation solution was then stored at 4°C for further experiments (Fu et al 2023).

Stability assays for pH. Phage stability across different pH levels was assessed by incubating phage stock (10^9 PFU mL^{-1}) in 10 mL PBS buffer at varying pH values (3, 5, 7, 9, and 11, adjusted with NaOH or HCl) at 37°C for 2 hours. Phage titers were then measured using the double-layer agar technique. All experiments were performed in triplicate to ensure accuracy (Fadlilah et al 2022; Fu et al 2023).

Bacteriophage inhibition test against V. parahaemolyticus in vitro. This test was conducted to evaluate the effectiveness of phage lysates in inhibiting pathogenic bacterial growth. This process employed three groups, including one treatment group consisting of only 100 µL of *V. parahaemolyticus* ($1x10^7$ CFU mL^{-1}) in NB media as a control. Meanwhile, two treatment groups contained 100 µL of the pathogen and 100 µL of HB and MLB lysate ($1x10^9$ PFU mL^{-1}) in the given order. Bacterial growth inhibition was monitored by measuring the optical density (OD) at 600 nm every two hours over a 48-hour period. The results were compared to evaluate the bacteriophage's efficacy in suppressing *V. parahaemolyticus* growth (Choliq et al 2020).

Transmission electron microscopy. The phage lysate, with a concentration of 10^9 PFU mL^{-1}, was first filtered through a 0.22 µm membrane and then purified by centrifugation at 25000 rpm for 60 minutes. Following this, 0.1 M ammonium acetate was added. A drop of the purified phage lysate was placed on a carbon-coated copper grid, stained with phosphotungstic acid, and examined using a transmission electron microscope at 60 kV (Choliq et al 2020).

Phage treatment of shrimps infected with V. parahaemolyticus. To assess the therapeutic efficacy of the phage in controlling pathogenic bacterial infections under practical conditions, a total of 400 healthy 60-day-old *P. vannamei* (mean weight of 12±0.5 g) were cultured in 18 L glass tanks at a constant water temperature of 25±1°C. The shrimp were evenly distributed into five groups, with each group consisting of 80 shrimp. Each group was further subdivided into four parallel subgroups, with 20 shrimp per subgroup, for replication purposes. The *V. parahaemolyticus* culture was prepared by

centrifuging and washing the cells before resuspending them to a concentration of 1×10^9 CFU mL^{-1}, confirmed by OD600 measurement and TCBS plating. The phage stock (2×10^{10} PFU mL^{-1}) was then added to the glass tanks to treat the shrimp.

Five treatment groups were set up: group 1 received no treatment; group 2 was exposed to the pathogen at 1×10^6 CFU mL^{-1} for 2 days; groups 3 and 4 were treated with phage stocks at 1×10^7 PFU mL^{-1} of HB and MBL, respectively, following the same pathogen challenge; group 5 received a combined phage stock at 1×10^7 PFU mL^{-1} after the 2-day pathogen exposure. Shrimp survival rates were evaluated after 7 days, with health monitored throughout the experiment (Fu et al 2023).

Shrimp immune response and survival rate assessments. Shrimp immune responses were assessed by counting total hemocytes and phagocytes. Total hemocyte counts were performed at the of the challenge period, following the method of Ni'mah et al (2021). Hemolymph was collected from the base of the pleopod in the abdominal segment near the genital opening using a 1 mL syringe pre-treated with an anticoagulant solution (10% sodium citrate, pH 7.2, or 10% EDTA). The collected hemolymph was then transferred to a sterile microtube and kept in a coolbox. Hemocyte counts were conducted using a hemocytometer.

Hemocyte count = (amount of cells counted/volume of blood) x dilution x 10^6.

Total phagocytes were counted using a method adapted from Devitha Tri et al (2013). A suspension of *V. parahaemolyticus* in PBS was added to the hemolymph, homogenized, and incubated at room temperature for 20 minutes. Following this, a 10 µL blood sample was taken for smear preparation, allowed to air dry, and fixed with methanol for 8 minutes before drying. The smears were stained with Giemsa dye for 15 minutes, then washed and rinsed with distilled water, and air-dried. Phagocytic cells exhibiting dark purple characteristics were counted, with a total of 100 phagocytic cells observed to assess the phagocytosis process (Ni'mah et al 2021). To assess the effectiveness of bacteriophages in inhibiting AHPND and determining shrimp survival, survival rates were calculated using the following formula:

Survival rate (SR) = (Nt/No)x100

Where: SR - survival rate (%); No - number of shrimps at the beginning of treatment; Nt - number of shrimps at the end of treatment.

Statistical analysis. Data from the experiments were statistically analyzed using analysis of variance (ANOVA) with a 5% significance level, followed by Duncan's multiple range test. The analyses were performed using SPSS version 26 software.

Results and Discussion

Isolation and characterization of V. parahaemolyticus. Figure 1 illustrates the isolation of bacterial colonies from the hepatopancreas of white shrimp, which appear green and round.

This isolation was confirmed as *V. parahaemolyticus* through PCR testing, following the method outlined in the OIE 2021 Chapter 2.2.1. Figure 1 presents the positive PCR result, which verified that the isolated bacteria from the shrimp hepatopancreas were indeed *V. parahaemolyticus*.

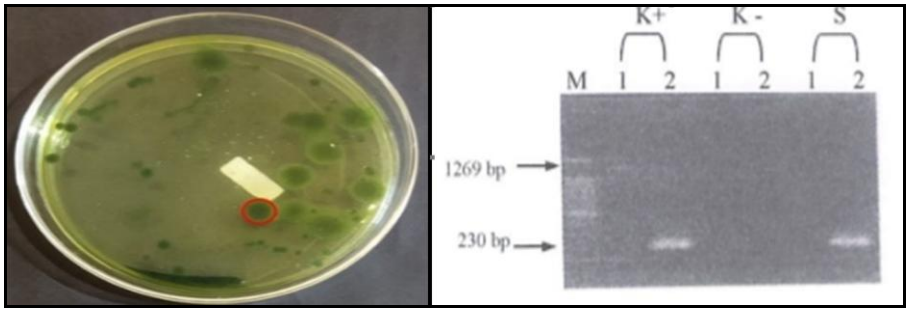


Figure 1. Results of isolation of bacterial colonies from white shrimp hepatopancreas (left) and PCR results of bacterial isolates (right); M - marker (100 bp DNA ladder); K- - negative control; K+ - positive control (AHPND); fragment size step 1: 1269 bp; fragment size step 2: 230 bp; S - isolate bacteria.

Bacteriophage isolation, purification and propagation results. Table 2 summarizes the bacteriophage isolation, purification and propagation results. It presents the plaque visualization, plaque count, and plaque titer (PFU mL^{-1}) for bacteriophages isolated from different sources.

For bacteriophages derived from shrimp hepatopancreas, two replication samples were analyzed: HB1 and HB2. Sample HB1 exhibited a plaque titer of 16×10^9 PFU mL^{-1}. In contrast, sample HB2 yielded a higher plaque titer of 20×10^9 PFU mL^{-1}. Meanwhile, sample MBL1 had a plaque titer of 24×10^9 PFU mL^{-1}, reflecting the highest concentration of phages among the samples. Sample MBL2, however, showed a plaque titer of 16×10^9 PFU mL^{-1}, which is similar to HB1 in terms of titer but lower than MBL1. However, no statistical analysis was performed on these values.

These results demonstrate that bacteriophages isolated from mangrove litter generally exhibited a higher plaque titer compared to those from shrimp hepatopancreas. The variations in plaque count and titer among the samples indicate differences in the efficiency of phage propagation and purification across the different sources.

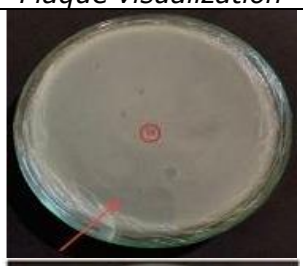
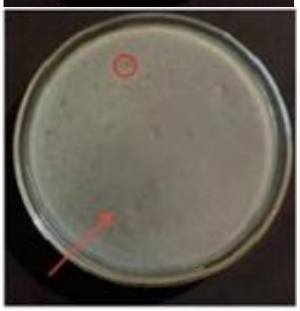
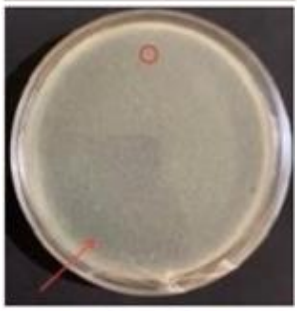
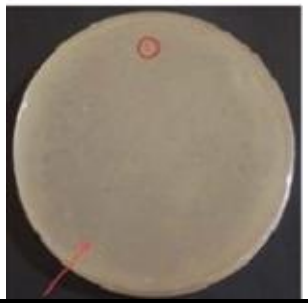
Bacteriophage growth stability test at various pH. The data on bacteriophage titers across various pH levels highlights the adaptability of bacteriophages sourced from shrimp hepatopancreas and mangrove litter, providing insight into their potential application in diverse environmental conditions (Figure 2).

At a neutral pH (pH 7), both bacteriophage sources displayed high titers, with the HP reaching 51.67×10^9 PFU mL^{-1} and MP reaching 37.33×10^9 PFU mL^{-1}. These values suggest optimal stability and replication potential for both bacteriophage types in environments close to neutrality. Interestingly, at more extreme pH levels, such as pH 3 and pH 11, HP consistently exhibited higher titers compared to their mangrove counterparts. For instance, at pH 3, the HP maintained a titer of 33×10^9 PFU mL^{-1}, while the MP only reached 17.33×10^9 PFU mL^{-1}. Similarly, at pH 11, the former registered 40.33×10^9 PFU mL^{-1}, compared to 46.33×10^9 PFU mL^{-1} for the latter.

These differences suggest that, while both bacteriophage types are effective across a range of pH environments, HP may offer greater resilience in acidic and highly alkaline conditions, making them promising candidates for therapeutic applications in varying environmental conditions.

Table 2

The result of the plaque of *Vibrio parahaemolyticus* to phages

Bacteriophage source	*Plaque visualization*	*Plaque titer (PFU mL^{-1})*
Hepatopancreas-derived bacteriophage (HB1)		16 × 10^9
Hepatopancreas-derived bacteriophage (HB2)		20 × 10^9
Mangrove litter-derived bacteriophage (MBL1)		24 × 10^9
Mangrove litter-derived bacteriophage (MBL2)		16 × 10^9

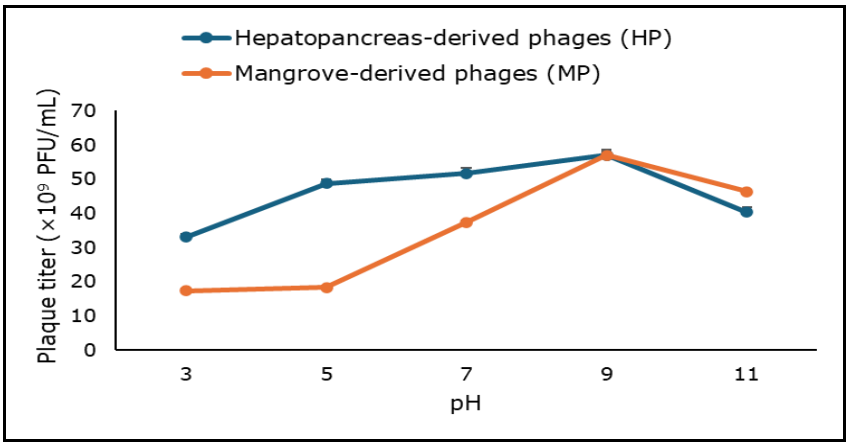


Figure 2. The pH stability test of hepatopancreas-derived phages (HP) and mangrove-derived phages (MP).

Bacteriophage inhibition test against V. parahaemolyticus in vitro. The data compares the inhibitory effects of HB and MBL on *Vibrio parahaemolyticus* by measuring optical density (OD600) over 24 hours (Figure 3). Unfortunately, at 6 and 12 hours, there were no significant differences among the values ($p>0.05$), 1.43 and 2.04, respectively. In contrast, significant inhibition ($p<0.05$) occurred at 18 hours, where HP (1.12±0.12) and MP (1.02±0.02) exhibited much lower OD600 values than the control (2.87±0.33), demonstrating strong bacterial suppression.

Although bacterial growth increased again at 24 hours, HP (2.89±0.09) and MP (3.21±0.09) remained more effective than the control (3.64±0.16), with MP showing slightly greater inhibitory capacity overall. These results suggest both phage types effectively suppress *V. parahaemolyticus*, particularly within the 18-hour window, with MP demonstrating marginally better performance based on statistical analysis ($p<0.05$).

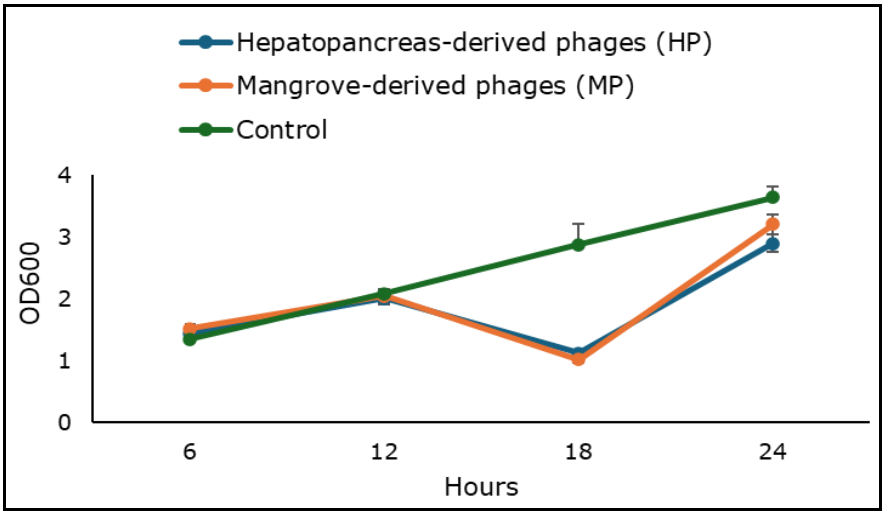


Figure 3. Growth curve for *Vibrio parahaemolyticus* in NB medium after treatments (OD readings in triplicate).

Scanning electron microscopy (SEM) observations of the morphology of *V. parahaemolyticus* cells (Figure 4) disclosed the bacteria's typical bacillus shape in the absence of bacteriophages, consistent with the known characteristics of *Vibrio* species. In contrast, bacterial cells exposed to bacteriophages exhibited signs of lysis, losing their distinct bacillus form. The irregular morphology observed is attributed to the rupture of the bacterial cell wall due to the release of newly produced bacteriophages, leading to the destruction of the bacterial cells.

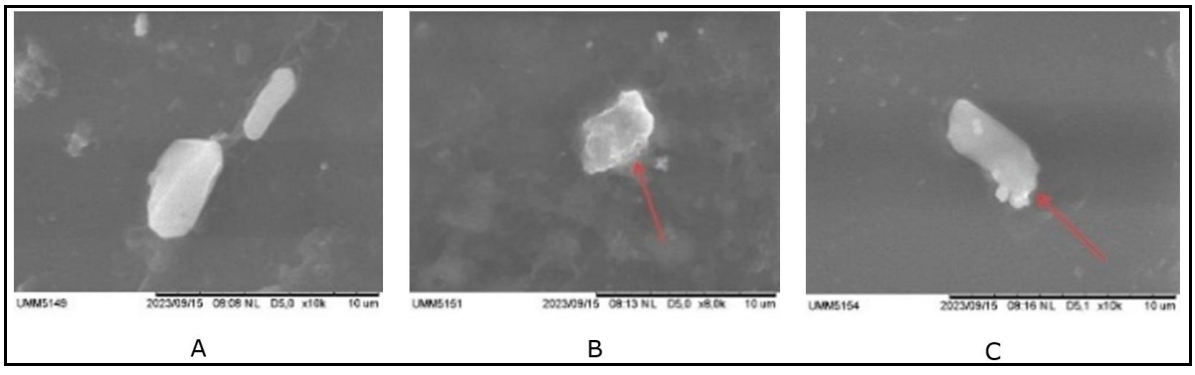


Figure 4. The SEM images of *Vibrio parahaemolyticus* show distinct cellular morphologies: A - untreated cells retain their bacillus shape; B - cells treated with HP; C - cells treated with MP display signs of lysis; arrows indicate irregularly shaped cells undergoing structural disruption from phage-induced lysis.

Shrimp immune response and survival rate assessments. The data presented reveals the effects of various treatments of phages on total hemocyte count (THC), phagocytosis activity, and survival rate (SR) in the experimental groups (Table 3).

Table 3
Immune parameters of shrimp in response to different experimental phages compared to the control group

Parameter	*Treatment*				
	Group 1 (control)	*Group 2*	*Group 3*	*Group 4*	*Group 5*
Total hemocyte count ($\times 10^5$ cells mL^{-1})	18.5±1[a]	18±0.58[a]	18.75±1.15[a]	18.25±1.53[a]	19±1.15[a]
Phagocytosis activity (%)	22.5±1.29[a]	26.5±3.11[a]	38.5±4.04[b]	39.5±3.87[b]	41.5±2.65[b]
Survival rate (%)	83.75±6.29[a]	50±4.08[a]	81.25±4.79[a]	83.75±2.5[a]	82.5±2.89[a]

Note: data are presented as mean ± SD (n=3); different letters indicate significant differences among treatments at each time point ($p<0.05$).

To begin with, the THC did not exhibit significant variation among the different treatment groups ($p>0.05$) based on similar notation. A notable variation was observed in phagocytosis activity among the groups. Group 1 exhibited a phagocytosis activity of 22.5±1.29%, which was significantly lower compared to others. Groups 3, 4, and 5, which received treatments involving HP, MP, and their combination, showed increased phagocytic activity. Statistical analysis revealed that the phagocytosis activity was significantly higher in these groups compared to the control and Group 2 ($p<0.05$), indicating an enhanced immune response due to phage treatment.

The SR across the groups varied, with group 1 and groups 4 and 5 demonstrating high survival rate. In contrast, Group 2, which was exposed to *V. parahaemolyticus* without any phage treatment, had a significantly lower ($p<0.05$) SR of 50±4.08%. The SR in group 3, which received HP phages, was 81.25±4.79%, indicating no significant difference compared to the control and phage-treated groups ($p>0.05$). This suggests that, while the phage treatments did not significantly alter the SR compared to the control, exposure to *V. parahaemolyticus* without phage intervention substantially reduced SR.

The results demonstrate that, while THC remain consistent across different treatments, phagocytosis activity is significantly enhanced with phage treatments, indicating a strengthened immune response. The SR was notably compromised only in the group exposed to *V. parahaemolyticus* without phage treatment, underscoring the protective role of phages. These findings underscore the potential efficacy of phage therapy in improving immune response and enhancing survival in the context of bacterial exposure.

The findings from Figures 1 and 2, confirming the isolation of *V. parahaemolyticus* from white shrimp hepatopancreas, align with other studies highlighting the prevalence of this pathogen in shrimp suffering from AHPND. Similar research has reported green, round colonies characteristic of *V. parahaemolyticus* and confirmed its identification through PCR-based methods, such as in studies by Raja et al (2017), Mai-Hoang et al (2021) and Tang et al (2020), which also used OIE protocols. These studies consistently observed the pathogen's role in severe disease outbreaks in shrimp aquaculture, emphasizing its pathogenicity and the critical need for accurate identification.

In corroborating these findings with other studies, it is evident that bacteriophages isolated from various environmental sources exhibit varying levels of effectiveness in propagation and plaque formation (Table 2). For instance, Pereira et al (2021) demonstrated that phages isolated from marine environments, particularly those associated with coastal sediments, tend to show higher plaque titers than phages derived from the tissues of marine organisms. Similarly, studies by Jin et al (2019), Sekar et al (2015), and Zhang et al (2023) found that bacteriophages isolated from mangrove ecosystems often exhibited stronger lytic activity and higher titers due to the high

microbial diversity in such environments, which fosters phage evolution and adaptation. These studies align with the current findings, where phages from mangrove litter (MP1 and MP2) achieved higher titers compared to those from shrimp hepatopancreas (HP1 and HP2). This may suggest that the phages derived from mangroves have a more conducive environment for propagation, similar to observations in other studies focusing on environmental phages.

Moreover, the data also indicates that bacteriophages isolated from shrimp hepatopancreas demonstrate superior stability across a wide range of pH levels compared to those from mangrove litter. Specifically, the phages from hepatopancreas maintained higher titers in both acidic (pH 3) and alkaline (pH 11) conditions. In contrast, bacteriophages from mangrove litter performed better in less extreme environments, particularly at neutral pH 7, where both sources exhibited peak titers.

Similar trends have been observed in other studies, where phages sourced from both resources tend to show enhanced resilience to environmental stressors, including extreme pH conditions. For example, research by Hao et al (2023) showed that phage VA5, isolated from aquaculture environments, exhibited stability across a pH range of 2-10, indicating resilience in acidic conditions. Similarly, phages isolated from coastal waters showed stability in a pH range of 4-10 (Gao et al 2024). Another study by Ngoc et al (2024) confirmed that phages isolated from shrimp showed significant adaptability to environmental changes, making them effective in aquatic disease management. The findings of this study, along with comparisons from existing research, suggest that hepatopancreas-derived bacteriophages could serve as potent therapeutic agents, offering high adaptability and stability under various pH conditions essential for effective treatment against bacterial infections like AHPND in aquaculture.

The findings of this study demonstrate that both HP and MP exhibit significant inhibitory effects on *V. parahaemolyticus*, especially during the 18-hour period, with MP showing slightly greater suppression than HP. These observations are in line with several previous studies investigating the antibacterial effects of phages on *Vibrio* species. For instance, a study by Ngoc et al (2024) also found that bacteriophages isolated from plenty of sources, such as pond water, healthy shrimp, and diseased shrimp, effectively reduced *Vibrio* populations over time, with substantial reductions in bacterial growth during 24 hours of exposure. Similarly, Hsu et al (2025) reported that phage BP15 exhibited a short latent period and effectively inhibited *V. parahaemolyticus* growth, even at low multiplicities of infection (MOIs), which mirrors the patterns observed in the present study. The slightly higher inhibitory capacity observed for MP in this study is consistent with research by Zhang et al (2023), which demonstrated that phages derived from mangrove ecosystems have enhanced lytic potential due to the dynamic and microbially rich environment in which they evolve. The higher microbial diversity in mangroves is believed to facilitate phage adaptation, resulting in stronger lytic capabilities compared to phages isolated from host organisms such as shrimp hepatopancreas. Therefore, the data from the current study corroborates findings from previous research, further highlighting the efficacy of phage therapy in suppressing *V. parahaemolyticus* and emphasizing the potential for mangrove-derived phages to offer slightly superior performance due to their environmental origins.

In addition, the SEM observations in this study align with previous findings regarding the morphological impact of bacteriophages on *V. parahaemolyticus*. The bacillus shape observed in the untreated *Vibrio* cells is characteristic of the species, as confirmed by studies such as those by Prosdocimi et al (2023) and Zhaolan et al (2001), which also reported similar bacillus-shaped morphology under normal conditions. The significant morphological changes seen in bacteriophage-treated *V. parahaemolyticus* — notably, the irregular forms resulting from cell lysis — are well-documented in phage studies (Figure 4). For example, Gao et al (2022) and Soonthonsrima et al (2023) observed that bacteriophages targeting *Vibrio* species cause visible damage to the bacterial cell walls, ultimately leading to the disruption of the bacillus shape. This damage is typically due to the action of phage endolysins, which degrade the bacterial cell wall, facilitating phage release and subsequent bacterial lysis.

Furthermore, the results presented align with findings from previous studies on THC, phagocytosis activity, and SR in shrimp exposed to bacteriophages and pathogenic bacteria. The lack of significant variation in total hemocyte counts across different treatment groups is consistent with studies such as those by González-Gómez et al (2023) and Alagappan et al (2016), which reported that bacteriophage treatments typically do not significantly affect overall hemocyte numbers in shrimp. This is likely due to the fact that hemocyte proliferation is often influenced more by pathogen load and immune stress rather than by bacteriophage exposure itself.

In contrast, the increase in phagocytosis activity observed in phage-treated groups (groups 3, 4, and 5) is in agreement with findings from Alagappan et al (2016) and Górski et al (2012), who demonstrated that phage therapy can enhance immune responses in shrimp by promoting phagocytic activity. These studies highlight that bacteriophage exposure can stimulate the innate immune system, leading to more robust phagocytosis of pathogens like *V. parahaemolyticus*, thereby reducing bacterial loads and improving immune defenses. The SR reported here are also comparable to previous work by Fu et al (2023), who observed that shrimp treated with phages targeting *Vibrio* species exhibited higher SR compared to those that were untreated or solely exposed to the pathogen. Similar to the current study, they found that the application of the phage vB_ValM_PVA8 resulted in a SR of 88.89% in shrimp infected with *V. parahaemolyticus*, compared to only 34.43% in the untreated control group. This underscores the protective effect of bacteriophages in mitigating the mortality associated with *Vibrio* infections, as supported by Benala et al (2023), who also noted significant reductions in mortality when shrimp were treated with bacteriophages after bacterial exposure.

Conclusions. This study demonstrates the potential of bacteriophage therapy as an effective biological tool for controlling *V. parahaemolyticus* infections in shrimp, particularly in the context of AHPND. Bacteriophages isolated from shrimp hepatopancreas and mangrove litter exhibited strong lytic activity against *V. parahaemolyticus*, significantly reducing bacterial counts and growth, as evidenced by SEM imaging that confirmed bacteriophage-induced bacterial lysis. While total hemocyte counts were unaffected by the treatments, phagocytic activity significantly increased in phage-treated groups, indicating an enhanced immune response. The survival rates of shrimp treated with bacteriophages were higher compared to those exposed to the pathogen alone, suggesting that phage therapy effectively reduces mortality. These findings suggest that bacteriophage treatments not only suppress pathogenic bacteria but also stimulate the shrimp's immune system, offering a promising, sustainable alternative to conventional antibiotics for managing AHPND in shrimp aquaculture.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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