

Faculty of Resource Science and Technology

Prevalence of *Vibrio* spp. Infection in Shrimp and Bio-control Using Bacteriophages Isolated from Shrimp Farms in Kuching

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Prevalence of *Vibrio* spp. Infection in Shrimp and Bio-control Using Bacteriophages Isolated from Shrimp Farms in Kuching

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



……

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ABSTRACT

Shrimp farming, a highly profitable sector in global aquaculture, has seen remarkable growth in recent years, with global shrimp consumption projected to reach US$ 74 billion by 2032. This increasing demand and the expansion of farming operations, including in Sarawak, Malaysia, highlight the sector's potential. However, the industry faces significant challenges, particularly the prevalence of vibriosis, a bacterial infection caused by *Vibrio* species. Contamination of food products has also increased the risk of vibriosis in humans. The widespread use of antibiotics to combat this disease has led to the rapid emergence of antimicrobial resistance (AMR) bacteria. This study presents a comprehensive investigation into the surveillance profiles and bio-control of *Vibrio* spp. isolated from a shrimp farm in Sarawak, Malaysia. A total of 48 (n=48) samples, including water, sediment, shrimp, and effluent, were collected from two ponds throughout the production cycle. The prevalence and quantification of *Vibrio* species were assessed by implementing the MPN-multiplex polymerase chain reaction (PCR) method. The findings revealed high prevalence rates, with *Vibrio parahaemolyticus* being the most prevalent species (97.92%), followed by *Vibrio cholerae* (47.92%) and *Vibrio alginolyticus* (25.0%). The MPN values of *V. parahaemolyticus* were as high as >1,100 MPN/mL or MPN/g in the water, sediment, and shrimp samples of both ponds, which are the highest among the three *Vibrio* species. The MPN values of *V. cholerae* and *V. alginolyticus* remained less than 1,100 MPN/mL or MPN/g in water, sediment, and shrimp samples towards the end sampling period despite starting high initially. Notably, *V. parahaemolyticus* exhibited an increasing trend from stocking to harvesting periods, whereas *V. cholerae* and *V. alginolyticus* showed a decreasing trend. Further analysis involved antibiotic susceptibility testing of 30 (n=30) *Vibrio* spp. isolates by using 18 antibiotics, revealing resistance to at least two antibiotics.

Antibiotics Ceftazidime, Meropenem, Gentamicin, Tetracycline, Nalidixic acid, Norfloxacin, Ciprofloxacin, and Chloramphenicol were 100% effective against all isolates of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus.* Meanwhile, 100% of *V. parahaemolyticus* and *V. alginolyticus* isolates were completely resistant to Penicillin G and Bacitracin, whereas 100% of *V. cholerae* isolates exhibited resistance to Penicillin G. The MAR indices of the isolates ranged from 0.11 to 0.39. In response to the escalating antibiotic resistance, bacteriophages emerged as promising alternatives. Two novel myophages, EniLVP01 and EniLVP02, targeting *V. parahaemolyticus* were isolated, characterised, and found to exhibit narrow host ranges and large burst sizes of 110 and 144 phages per infected cells, respectively. Notably, they effectively prevented and reduced bacterial biofilms. In the biofilm prevention, the absorbances were reduced from 0.592 ± 0.055 to 0.218 ± 0.039 for EniLVP01 and to 0.204 ± 0.016 for EniLVP02. Meanwhile, in the biofilm destruction assay, the mixture treated with the phage lysate of EniLVP01 and EniLVP02 showed an absorbance of 0.139 ± 0.009 and 0.174 ± 0.026, respectively, compared to the untreated samples with an absorbance of 0.843 ± 0.0029. Both phages also demonstrated promising efficacy in reducing *V. parahaemolyticus* counts on retail shrimp matrices, with a bacterial reduction of 98% achieved using a cocktail both phages. Phage EniLVP01 and EniLVP02 exhibited stability across a wide range of pH (pH 4.0 – 9.0) and temperature (28 ℃ - 65 ℃) conditions. Genomic sequencing revealed high similarity between EniLVP01 and EniLVP02, suggesting they may belong to the same species in the *Caudovirales* order or are very closely related despite originating from different sources. Importantly, the absence of lysogeny-related, antibiotic, and virulence genes in their genomes supports their safety for therapeutic use. These findings represent a significant advancement in understanding the potential of

phage therapy in the battle against bacterial infections and antibiotic resistance issues within Malaysia generally, and within Sarawak specifically.

Keywords: Vibriosis, antibiotic resistance, phage therapy, bacteriophages, shrimp aquaculture

Kawalan Biologi Terhadap Jangkitan Vibrio Spp. dalam Udang dengan Menggunakan Bakteriofaj yang Diasingkan dari Ladang Udang di Kuching.

ABSTRAK

Akuakultur udang, salah satu sektor yang paling menguntungkan dalam akuakultur global, telah menyaksikan pertumbuhan yang ketara dalam beberapa tahun kebelakangan ini, dengan penggunaan udang global dijangka mencecah AS$ 74 bilion menjelang tahun 2032. Permintaan yang semakin meningkat dan pengembangan operasi penternakan, termasuk di Sarawak, Malaysia, mengembangkan potensi sektor ini. Namun, industri ini menghadapi cabaran yang besar, khususnya dalam menangani wabak vibriosis, sejenis jangkitan bakteria yang disebabkan oleh spesies Vibrio. *Pencemaran pada produk makanan juga telah meningkatkan risiko kejadian vibriosis dalam kalangan manusia. Penggunaan antibiotik yang meluas untuk mengatasi penyakit ini telah menyebabkan kemunculan bakteria rintang antimikrob (AMR) dengan cepat. Kajian ini berkaitan penyelidikan menyeluruh mengenai profil pengawasan dan bio-kawalan* Vibrio *spp. yang dipencilkan dari ladang udang di Sarawak, Malaysia. Sejumlah 48 (n=48) sampel, termasuk air, endapan, udang, dan sisa kolam, dikumpulkan dari dua kolam sepanjang kitaran pengeluaran udang. Dengan menggunakan kaedah Most Probable Number-reaksi berantai polimerase multiplex (MPN-mPCR), kelaziman dan kuantiti spesies* Vibrio *dinilai. Penemuan menunjukkan kadar kelaziman yang tinggi, dengan* Vibrio parahaemolyticus *menjadi spesies yang paling berleluasa (97.92%), diikuti oleh* Vibrio cholerae *(47.92%) dan* Vibrio alginolyticus *(25.0%). Nilai MPN* V. parahaemolyticus *adalah >1,100 MPN/mL atau MPN/g dalam sampel air, endapan, dan udang di kedua-dua kolam, yang merupakan tertinggi di antara ketiga spesies* Vibrio. *Nilai MPN* V. cholerae *dan* V. alginolyticus *kekal kurang daripada 1,100 MPN/mL atau MPN/g dalam sampel air, endapan, dan udang menjelang akhir tempoh*

pensampelan walaupun bermula tinggi pada awalnya. Selain itu, V. parahaemolyticus *menunjukkan tren peningkatan dari tempoh penyimpanan stok hingga tempoh menuai, manakala* V. cholerae *dan* V. alginolyticus *menunjukkan tren penurunan. Analisis lanjut melibatkan ujian kepekaan antibiotik terhadap 30 (n=30) isolat* Vibrio *spp. mendedahkan ketahanan terhadap sekurang-kurangnya dua antibiotik. Antibiotik Ceftazidime, Meropenem, Gentamicin, Tetracycline, Nalidixic acid, Norfloxacin, Ciprofloxacin, dan Chloramphenicol adalah 100% berkesan terhadap semua isolat* V. parahaemolyticus, V. cholerae, *dan* V. alginolyticus. *Sementara itu, 100% isolat* V. parahaemolyticus *dan* V. alginolyticus *adalah tahan sepenuhnya kepada Penicillin G dan Bacitracin, manakala 100% isolat* V. cholerae *menunjukkan ketahanan kepada Penicillin G. Indeks MAR isolat berjulat dari 0.11 hingga 0.39. Sebagai penyelesaian terhadap peningkatan ketahanan antibiotik di kalangan backteria, bakteriofaj menunjukkan pontesinya sebagai alternatif untuk antibiotik. Dua myofaj yang baru, EniLVP01 dan EniLVP02, yang berupaya menmusnahkan* V. parahaemolyticus *diasingkan, dicirikan, dan didapati menunjukkan julat hos yang kecil dan saiz pemecahan yang besar masing-masingnya 110 dan 144 faj per sel terinfeksi. Faj-faj ini mengurangkan dan mencegah biofilem bakteria secara berkesan. Dalam pencegahan biofilm, penyerapan berkurang dari 0.592 ± 0.055 kepada 0.218 ± 0.039 untuk EniLVP01 ($P < 0.05$) dan kepada 0.204 ± 0.016 untuk EniLVP02. Sementara itu, dalam ujian pemusnahan biofilem, sampel yang dirawat dengan lisat faj EniLVP01 dan EniLVP02 menunjukkan penyerapan 0.139 ± 0.009 ($P < 0.05$) dan 0.174 ± 0.026 ($P < 0.05$) berbanding dengan sampel yang tidak dirawat yang mempunyai penyerapan 0.843 ± 0.0029. Selain itu, kedua-dua faj juga menunjukkan keberkesanan yang memberangsangkan dalam mengurangkan bilangan* V. parahaemolyticus *pada matriks udang, dengan pengurangan bakteria sehingga 98% menggunakan koktel faj. Faj EniLVP01 dan EniLVP02,*

menunjukkan kestabilan melangkaui julat pH (pH 4.0 - 9.0) dan suhu (28 °C - 65 °C) yang tinggi. Analisa genomik mendedahkan kesamaan tinggi antara EniLVP01 dan EniLVP02, mencadangkan kedua-dua faj ini mungkin spesies yang sama dalam order Caudovirales atau sangat berkaitan meskipun berasal dari sumber yang berbeza. Ketiadaan gen berkaitan lizogeni, antibiotik, dan virulensi pada genom faj EniLVP01 dan EniLVP02 menyokong keselamatan dalam penggunaan terapeutik. Penemuan ini menunjukkan kemajuan penting dalam memahami potensi terapi faj bagi melawan jangkitan bakteria dan isu ketahanan antibiotik di Malaysia umumnya dan di Sarawak khususnya.

Kata kunci: *Vibriosis, ketahanan antibiotik, terapi faj, bakteriofaj, akuakultur udang*

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistant
ARG	Antibiotic resistance gene
MGE	Mobile genetic element
MDR	Multi drug resistant
APW	Alkaline peptone water
TCBS	Thiosulfate-citrate-bile salts-sucrose
MPN	Most Probable Number
mPCR	Multiplex Polymerase Chain Reaction
PFU	Plaque forming unit
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ORF	Open reading frame
CDS	Coding sequence
NGS	Next generation sequencing
WGS	Whole genome sequencing

CHAPTER 1

INTRODUCTION

1.1 Study Background

Aquaculture has emerged as an important sector in global food production, catering to the escalating demand for high-quality protein sources for human consumption (Brye, 2023). Traditionally, freshwater and seafood procurement relied heavily on fishing, a practice susceptible to overexploitation, food chains disruption, and subsequently, ecological imbalance. The advent of aquaculture revolutionized this practice, offering a controlled and sustainable approach to aquatic organism cultivation and harvesting for human consumption (NOAA, 2016; Brye, 2023). This transition has not only mitigated the strain on natural fish stocks but also fostered economic growth and stability, as evidenced by the exponential rise in global aquaculture production reaching up to 185 million metric tons in 2023 with 96 million metric tons came from the aquaculture-raised fish (FAO, 2023). Global aquaculture food production encompasses a diverse array of finfish, molluscs, crustaceans, and other aquatic animals (Chatterjee and Haldar, 2012).

Among the most profitable ventures within aquaculture, shrimp farming stands out prominently. The escalating demand for shrimp, evident in its substantial global consumption valued at US$ 18.30 billion in 2020, surged significantly to US$ 69 billion by 2023, emphasizing its economic significance (Renub Research, 2021; IMARC, 2024). Leveraging this demand surge, countries in Southeast Asia, notably Malaysia, have capitalised on commercial shrimp aquaculture as a mean to bolster their economic industry (Ismail and Abdullah, 2013). Malaysia, in particular, benefits from natural regional advantages that are conducive to shrimp farming, such as favourable climate conditions and

abundant water resources. These factors create an optimal environment for implementing sustainable aquaculture practices (Hashim and Subramaniam, 2005).

The commercial success of Malaysia's shrimp aquaculture industry is exemplified by its substantial contributions to the global market. In 2018 alone, Malaysia contributed approximately 36,007 tonnes of Whiteleg shrimp (*Penaeus vannamei*) and 9,906 tonnes of giant tiger prawn (*Penaues monodon*), valued at USD 203.82 million and USD 70.19 million respectively, accentuating its pivotal role in meeting the increasing global demand (FAO, 2020). The industry's profitability and resilience have fuelled its rapid expansion, positioning Malaysia as a key player in the international shrimp market (Chowdhury et al., 2013). As such, the trajectory of Malaysia's shrimp aquaculture industry is set to continue growing as long as the demand for shrimp remains strong and the wild shrimp catch continues to diminish.

However, with growing industries comes a myriad of challenges. One such challenge is the prevalence of disease outbreaks. According to a 2017 report by Future Market Insights, the growth trajectory of the shrimp industry is significantly impacted by disease outbreaks, resulting in annual losses amounting to as much as 40% of the total global income which translated into billions of US dollars (Shinn et al., 2018). In Malaysia, for instance, the shrimp aquaculture industry suffered losses of approximately US$ 0.1 billion due to outbreaks of Acute Hepatopancreatic Necrosis Disease (formerly known as Early Mortality Syndrome) caused by bacteria *V. parahaemolyticus* across multiple locations in 2011 (NACA, 2012).

One of the most commonly reported ailments in shrimp is vibriosis, diseases caused by Gram-negative bacteria belonging to the *Vibrionaceae* family (Mastan and Begum,

2016). Although *Vibrio* species are ubiquitous in the environment, infections remain manageable when occurring at tolerable levels. However, environmental fluctuations, such as temperature and pH shifts, can trigger exponential bacterial proliferation. This uncontrolled upsurge in *Vibrio* spp. populations causes vibriosis outbreaks capable of decimating entire shrimp populations within infected ponds (Mastan and Begum, 2016). Furthermore, the consumption of *Vibrio*-infected shrimp presents health hazards to consumers, potentially resulting in acute gastroenteritis and other related symptoms (Sharihan et al., 2018).

To combat these persistent disease challenges, shrimp farmers and aquaculture companies often resort to the administration of antibiotics such as chloramphenicol, furazolidone, and oxytetracycline as a primary treatment strategy (Okocha et al., 2018). Antibiotic therapy is favoured for its expediency and cost-effectiveness, with their ready over-the-counter availability facilitating their widespread and unregulated use (Thornber et al., 2020). However, the indiscriminate use of antibiotics has given rise to the emergence of antibiotic-resistant bacterial strains (CDC, 2013). These strains harbour antimicrobial resistance (AMR) genes, which enable them to withstand the effects of drugs or chemicals that are typically efficacious against non-resistant strains (Thornber et al., 2020). Moreover, aside from fostering bacterial resistance, the consumption of antibiotics through contaminated foodstuffs can lead to a spectrum of health issues in human. Toxicity, allergic reactions, and an elevated risk of cancer development are among the documented effects associated with the ingestion of antibiotics-contaminated food products (Okocha et al., 2018). Hence, while antibiotics offer a swift, short-term remedy for disease prevention or control in shrimp farms, they fail to provide an optimal long-term solution to this pressing issue.

In response to the challenges posed by chemical treatments, there is a growing interest in biological control strategies. Biocontrol involves the use of microorganisms to prevent or treat diseases by antagonising disease-causing agents. Unlike chemical treatments, biocontrol is environmentally friendly and does not contribute to the widespread emergence of resistant microorganisms (Pérez-Sánchez et al., 2018). One such technique that has gained traction recently is phage therapy. Phage therapy utilises the antagonistic nature of lytic bacteriophages towards bacteria to prevent or manage the proliferation of pathogenic bacteria (Nikolich and Filipov, 2020).

Bacteriophages are viruses that selectively target specific bacterial hosts, binding to them, replicating using host cellular machinery, and ultimately lysing the host cell to release their infective progeny phages. By exploiting host cellular mechanisms and inducing lysis, phages ultimately incapacitate the host bacteria (Kazi and Annapure, 2015). Phage therapy offers a promising alternative to antibiotics with several advantages. Unlike antibiotics, bacteriophages have a specific mode of action, targeting only their hosts. Their narrow host range means they do not exhibit tropism for mammalian cells and pose no threat to non-host organisms (Luong et al., 2020). Additionally, while antibiotics are associated with side effects, no adverse effects have been reported with phage therapy as phages are inherently non-toxic (Loc-Carrillo and Abedon, 2011).

Although bacterial resistance to phages has been documented, it typically arises without the development of resistance genes and is confined to the targeted bacteria. Resistance mechanisms may include host virulence attenuation through spontaneous mutation, restriction-modification systems, and adaptive immunity via the CRISPR-Cas system (Oechslin, 2018). Despite these challenges, phages possess countermeasures, such as

anti-restriction and anti-CRISPR enzymes, (Pawluk et al., 2018) and the ability to adapt to reinfect resistant bacteria (Hampton et al., 2020). The widespread presence of bacteriophages in the environment provides a significant advantage, as isolation and purification processes can be conducted rapidly under suitable conditions (Hyman, 2019). Utilising bacteriophages as a control measure has the potential to address two critical issues simultaneously: eradicating or preventing bacterial infections and mitigating antibiotics residue contamination in the food chain.

1.2 Problem Statement

Disease outbreaks in aquaculture, particularly in shrimp farming, are associated with substantial economic losses to the industry. Among the myriad challenges faced by aquaculture farmers, bacterial infections, predominantly attributed to pathogenic *Vibrio* spp., pose significant problems. In shrimp farms, infections by *Vibrio* spp. have emerged as a leading factor contributing to declining production and subsequent economic losses.

Traditionally, disease infections in aquaculture farms have been managed with antibiotics; however, this practice has inadvertently accelerated the development of AMR bacteria. The notable prevalence of AMR bacteria in Malaysia, reported as one of the highest in Southeast Asia (Suyamud et al., 2024), underscores the urgent need to explore alternative treatment modalities. Given Malaysia's diverse microenvironments and conducive climate, there exists an incredible opportunity to discover novel bacteriophages with therapeutic potential (Rajandas et al., 2021).

In Malaysia, the adaptation of phage therapy for aquaculture is still in its formative stage, with ongoing research aimed at isolating and characterising novel phages. Despite the burgeoning aquaculture industry, there is a dearth of information concerning the

identification and characterisation of bacteriophages specific to *Vibrio* spp. in Sarawak. Given the rapid expansion of shrimp production both locally and globally (Chowdhury et al., 2013; Ling, 2022), there is an urgent need to explore the potential of utilising bacteriophages that are tailored to the unique environmental conditions and microbiomes of Sarawak.

The characterisation of phage genomes presents considerable challenges, primarily due to their inherent complexity and the lack of universally conserved genomic regions, a feature typically observed in bacterial genomes. Consequently, Whole Genome Sequencing (WGS) has emerged as a crucial technique for delving into the intricacies of phage genomes, providing comprehensive insights into their molecular makeup. A thorough comprehension of a newly discovered phage genome, coupled with the establishment of phylogenetic relationship with known phages, is crucial for assessing its viability and safety in the context of phage therapy applications.

1.3 Objectives

Building upon previous research, this study hypothesised that bacteriophages possess the potential to act as natural antimicrobial agents capable of inhibiting the growth of *Vibrio* species. It has been reported that for almost every bacterial species, there exists at least one phage with the ability to specifically infect and subsequently eradicate that bacterial group (Sabouri Ghannad and Mohammadi, 2012), Given this premise, the potential of phage treatment to mitigate vibriosis in shrimp, specifically in Sarawak, warrants exploration and comprehension. Therefore, the objectives of this study are as follows:

i. To determine the prevalence of *Vibrio* spp. within a commercial shrimp farming environment.

ii. To investigate the antibiotic susceptibility profiles and the presence of virulence genes among *Vibrio* spp. strains isolated from the shrimp farm.

iii. To isolate and characterise bacteriophages exhibiting antagonistic activity against *Vibrio* species.

iv. To characterise the genomic attributes of the bacteriophages infecting *Vibrio* spp. using Whole Genome Sequencing (WGS).

1.4 Chapter Summary

In conclusion, this chapter has provided the general introduction regarding problems faced in shrimp aquaculture industry around the world, generally, and in Malaysia, particularly. This chapter also introduces the potential of using bacteriophages as therapeutic agents to mitigate diseases infections. Evidently, phage therapy stands as a promising alternative to antibiotic usage in the prevention and treatment of bacterial diseases in aquaculture products, notably shrimps. This approach aligns with the broader objective of advancing environmentally friendly technologies, which minimise adverse impacts on the environment and non-targeted organisms. Moreover, with enhanced understanding and comprehensive knowledge, the implementation of phage therapy holds the potential to mitigate losses in the shrimp aquaculture industry attributed to disease outbreaks. Furthermore, by replacing antibiotics or at least controlling their usage, phage therapy offers a strategy to curb the development of antibiotic-resistant bacterial strains.

This study holds particular significance for its potential to mitigate economic losses stemming from disease outbreaks in shrimp farming, while also addressing ecological and public health concerns arising from antibiotic overuse and subsequent antimicrobial

resistance (AMR). Moreover, it pioneers the isolation and characterisation of bacteriophages sourced from environmental and retail shrimp samples in Sarawak, Malaysia, with a targeted focus on combating *V. parahaemolyticus*. Given the prevalence of *Vibrio* spp., notably *V. parahaemolyticus,* in aquaculture environments and the specificity of bacteriophages to their bacterial hosts, the development of locally derived solutions adapted to the region's unique conditions is imperative. This research thus offers prospective insights into the potential applications of bacteriophages within Sarawak's aquaculture sector and seafood industry.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Shrimp Farming Industry in Sarawak

In line with Sarawak's goal of becoming a net food exporter by 2030, the state has set its sights on expanding its shrimp farming industry. Sarawak government is aiming to achieve a significant increase in export value of shrimp products up to RM1 billion by 2030 (Ling, 2022). This aspiration underscores the important role of shrimp farming practices in driving economic growth and enhancing food industry within Sarawak. According to the State Modernisation of Agriculture and Regional Development Minister, Datuk Seri Stephen Rundi, Sarawak is currently exporting RM400 million worth of shrimp annually, indicating a strong foundation upon which to build further growth. Additionally, this goal also highlighted the requirement of 7,000 hectares of land to meet the RM1 billion export goal with areas like Rambungan, Lawas, and Sarikei, to be explored to fulfil this target (Ling, 2022).

Sarawak's potential for shrimp aquaculture development is strongly supported by its favourable tropical climate characterised by high temperatures, heavy rainfall, and high humidity throughout the year, as well as its extensive coastline. The humid tropical climate in Sarawak, with temperatures ranging from 23 °C to 32 °C, provides an ideal environment for shrimp aquaculture (Kwang, 2001; WorldData Info, 2024). Additionally, the vast coastline of about 1,050 km in Sarawak, which is the second longest in all states in Malaysia, offers ample opportunities for aquaculture activities. Currently, most of the shrimp farms in Sarawak are located in Kuching, Samarahan, and Sarikei (Kwang, 2001; Bakewell et al., 2017). With its abundant and fairly unpolluted water resources and favourable climatic

conditions, Sarawak presents an ideal environment for the cultivation of shrimp, positioning the industry as one of the main contributors in the state's aquaculture landscape.

Furthermore, in the effort to enhance the shrimp industry in Sarawak, concerted efforts have been undertaken by the state government to fortify infrastructure support, coupled with strategic partnerships aimed at enhancing expertise and export capabilities. Notably, the state government has pledged assistance in infrastructure development through the Sarawak State Farmers' Organization (SFO) where they will provide essential resources and infrastructures vital for the advancement of the shrimp aquaculture. Concurrently, collaborations with industry stakeholders like Sea Horse Corporation Sdn. Bhd. play role in providing specialised expertise to improve shrimp production processes for export markets (Ling, 2022). Through such initiatives, Sarawak aims to augment its position within the global shrimp trade market, by utilising its abundant resources and favourable climates, as well as by implementing specialised knowledge and infrastructure support to uphold competitiveness and sustainability in the industry.

2.2 Disease Outbreaks

One of the principal challenges encountered by the shrimp aquaculture industry is the persistent threat of infectious disease outbreaks, with bacteria, particularly those belonging to the Vibrio genus, exerting a significant influence owing to their capacity to endure and thrive autonomously in aquatic environments (Kumar et al., 2021). *Vibrio* spp., including *V. parahaemolyticus, V. alginolyticus, V. cholerae, V. mimicus, V. harveyi*, and *V. vulnificus,* are recognized as severe pathogens for aquatic animals reared in aquaculture environments (Rasmussen et al., 2018; de Souza Valente and Wan, 2021). These bacteria are not only naturally abundant in marine and estuarine ecosystems but also constitute major

components of the microbiota in aquaculture facilities. Furthermore, *Vibrio* spp. often adhere to water-contact structures in hatcheries and farms, forming biofilms (Lavilla-Pitogo et al., 1990; Sampaio et al., 2022). The prevalence of these bacteria, compounded by climate changes that can foster their proliferation, poses a substantial risk to shrimp farming (González-Acosta et al., 2006; Arab et al., 2020).

The presence of bacterial pathogens, primarily from *Vibrio* species such as *V. harveyi*, *V. alginolyticus*, *V. campbellii*, and *V. parahaemolyticus*, presents a significant hurdle to shrimp health and growth, resulting in issues such as necrosis, delayed growth, anorexia, and mortality during post-larval development (Garibay-Valdez et al., 2020). Therefore, addressing these health challenges is imperative due to their detrimental effects on shrimp aquaculture.

2.2.1 Data on Past Vibriosis Outbreaks

Comprehensive data on past incidences of disease outbreaks are deemed critical within the aquaculture context as they yield valuable insights into the diseases epidemiology, encompassing its prevalence, incidence rates, and geographical distribution. This information holds particular significance given the observed rise in vibriosis cases over the last decade (Liu et al., 2018). Moreover, examining historical trends of vibriosis infections in aquaculture facilities provides perspective on their impact on both farms and seafood safety, empowering authorities to enact appropriate control and preventive strategies (Mohamad et al., 2019a). As underscored by Mohamad et al. (2019a), the scarcity of epidemiological reports on vibriosis in many tropical Asian nations impedes progress in disease control and prevention efforts. Table 2.1 below presents several documented vibriosis incidents in aquaculture facilities across Malaysia.

Table 2.1: Vibriosis incidences in aquaculture farms in Malaysia.

Month, Year	Location	Causative agents	Afflicted organisms	Overall impact	References
1990	Malaysia	Various *Vibrio* spp.	Marine fish (e.g. grouper, seabass, snapper)	Resulted in US$ 7.4 million loss in aquaculture	Amalina et al. (2019)
February 2008	Sabah, Malaysia	*Vibrio harveyi*	Asian seabass (cultured in net cages)	High mortality of young fish which greatly affected the farm production.	Ransangan and Mustafa (2009)
April and September, 2009	Sabah, Malaysia	*Vibrio harveyi*	Asian Seabass	Infection caused 100% mortality of the fish in a marine fish farm.	Mohamad et al. (2019b)
2011 - 2014	Malaysia	*Vibrio parahaemolyticus* (APHND-causing strains)	Cultured penaeid prawns and shrimps	A total loss of USD 0.49 billion in shrimp production	Kua et al. (2016); Ali et al. (2018)

Table 2.1 continued

September 2016	Selangor, Malaysia	*Vibrio harveyi* and *Vibrio alginolyticus*	Juvenile hybrid groupers	Infection caused average daily mortality of 120 fish with cumulative mortality rate of 29% within 10 days in a marine fish farm.	Mohamad et al. (2019b)

The monitoring and reporting system for aquaculture in Malaysia exhibit significant deficiencies, especially regarding the documentation of vibriosis incidence. Unlike in several Asian countries such as India, China, and Japan (de Souza Valente and Wan, 2021; Sanches-Fernandes et al., 2022), official data or reports on vibriosis in Malaysia are notably scarce, with a conspicuous lack of reports from states like Sarawak. The lack of reported incidents does not necessarily indicate the absence of vibriosis cases. Underreporting or a failure to report could mean that many cases remain unidentified.

Furthermore, there is a scarcity of documented vibriosis incidences beyond 2016, further exacerbating the knowledge gap. It has been reported that most cases of vibriosis in shrimp farms remain unknown as sampling of diseased shrimp cannot be conducted since farmers typically perform emergency harvests at the onset of diseases (Kua et al., 2016). Instead of comprehensive reports, only verbal accounts from a limited number of shrimp farms are available, highlighting a significant deficiency in systematic data collection and reporting platforms within Malaysia's aquaculture sector (Gibbons et al., 2014; Sharihan et al., 2018).

2.3 Economic Losses and Production Decline

As vibriosis infections often escalate swiftly, shrimp production may suffer declines, particularly when these infections lead to shrimp mortality. One of the most devastating diseases affecting aquaculture farms is acute hepatopancreatic necrosis disease (AHPND). As depicted in Figure 2.1 below, shrimp farms across many states in Malaysia were afflicted by AHPND from 2011 to 2014. The prevalence of AHPND cases was reported at 50%, 26%, and 73% in 2011, 2012, and 2013, respectively. These outbreaks resulted in mortality rates as high as 100% in Whiteleg shrimp (*Penaeus vannamei*) in affected ponds, occurring alarmingly rapidly (Kua et al., 2016).

Reports have also indicated that these outbreaks led to reduced shrimp production and cost the industry approximately USD 0.1 billion in 2011 alone, with a total loss of USD 0.49 billion between 2011 and 2014 (Kua et al., 2016; Loo et al., 2023). According to a 3-year study conducted by Kua et al. (2016), in Malaysia, these outbreaks caused total production reductions to 67,000 metric tonnes (MT), 55,000 MT, and 50,000 MT in 2011, 2012, and 2013, respectively, compared to 87,000 MT in 2010 (Kua et al., 2016). Presently, AHPND still persists in Malaysia, albeit at a low prevalence (Kua et al., 2016). This highlights the critical need for ongoing surveillance and systematic documentation of disease occurrences to pre-empt unwarranted disease outbreaks and mitigate economic losses within the shrimp aquaculture sector.

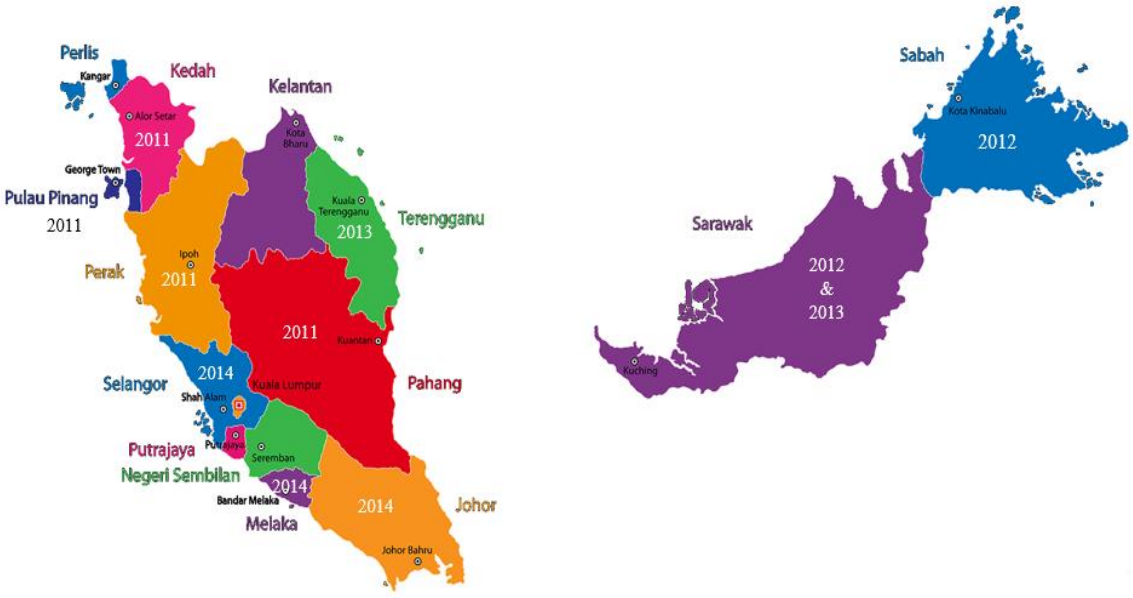


Figure 2.1: Occurrences of AHPND by year in various states in Malaysia confirmed by histopathology (Source: Kua et al., 2016).

In addition to Malaysia, AHPND outbreaks in other countries such as China, Vietnam, Thailand, the Philippines, Mexico, and South America between 2009 and 2016

resulted in a substantial reduction in global shrimp production (Kua et al., 2016; Kumar et al., 2021). This data underscores the concerns raised by diseases like AHPND, particularly when they become uncontrollable, as they can significantly damage the aquaculture industry, particularly in terms of shrimp production.

2.4 Disease and Pathogenesis of *Vibrio* species

Vibriosis encompasses a spectrum of diseases induced by pathogenic or opportunistic strains of *Vibrio* species (CDC, 2019a). Pathogenic bacteria are defined as microorganisms capable of instigating diseases in susceptible hosts, while opportunistic bacteria exploit specific conditions, particularly in immunocompromised hosts, to provoke illness (Peloux, 1985; NIH, 2007; Riccardi et al., 2019). Among the *Vibrio* spp. associated with vibriosis, notable mentions include pathogenic *V. cholerae*, as well as opportunistic species like *V. alginolyticus*, *V. vulnificus*, *V. harveyi*, and *V. parahaemolyticus* (CDC, 2019a; de Souza Valente and Wan, 2021; Haifa-Haryani et al., 2022). Table 2.2 below provides a comprehensive overview of common manifestations of vibriosis in shrimp, detailing the stages of infection and associated symptoms.

In the majority of the diseases enumerated in Table 2.2, shrimp infected by *Vibrio* spp. typically display common clinical signs, including lethargy, diminished or abnormal growth, reduced food consumption, and, in severe instances, mortality. Infections in shrimp commonly occur via multiple routes, including ingestion through feed, penetration through gills, and invasion of hepatopancreatic tissues. Once *Vibrio* spp. breach the epithelial barrier, they establish colonisation within the host's tissues, thus initiating infection in the shrimp (Chatterjee and Haldar, 2012).

Table 2.2: A list of known and reported vibriosis incidences in aquaculture farms in Malaysia.

Diseases	Causative agent(s)	Infected organism(s) and stage(s)	Clinical signs/Symptoms	Reference(s)
Zoea-2-syndrome	• *V. alginolyticus* • *V. vulnificus* • *V. mimiscus* • *V. parahaemolyticus* • *V. campbelli*	• Blue shrimp (*Penaeus stylirostris*) • Whiteleg shrimp (*Penaeus vannamei)* (Larvae stage)	• Reduction in feeding • Impairment in metamorphosis • Delayed moulting • Mortality	Sathish Kumar et al. (2017); Thangaraj et al. (2023)
Luminescent vibriosis	• *V. campbelli* • *V. harveyi* • *V. splendidus*	• Black Tiger shrimp (*Penaeus monodon*) • Whiteleg shrimp (*Penaeus vannamei*) • Indian white prawn (*Penaeus indicus)* (All stages)	• Weak and can lead to mortality • Swimming in a non-directional path • Reduction in feeding • Body and gill are dirty and dark in colour • Head and body glow in the dark • Slow growth • Infected larvae are milky in colour	Lavilla-Pitogo et al. (1990); Dash et al. (2017)

Table 2.2 continued

Loose shell syndrome	• *V. harveyi* • *V. parahaemolyticus* • *V. alginolyticus* • *V. anguillarum* • *V. metschnikovii* • *V. fluvialis*	• Black Tiger shrimp (*Penaues monodon*) • Whiteleg shrimp (*Penaues vannamei*) (Juvenile and adult stage)	• Lethargy • Shrunken hepatopancreases • Pinkish discoloration • Poor growth • Weight loss • Flaccid spongy abdomen • Gap between the muscle and shell • Mortality	Alavandi et al. (2008); AftabUddin et al. (2017)
Shell disease	• *V. alginolyticus* • *V. anguillarum*	All *Penaues* spp. (Larvae, juvenile, and adult stage)	• The affected limb of larvae or post-larvae shrimp appeared brown and ashy in colour with appearance of blisters • Problems with moulting • Affected shrimp can become cannibalistic • Mortality due to stress	Cook and Lofton (1973); Gopalan and Young (1975); Lavilla-Pitogo et al. (1990);

Table 2.2 continued

Acute Hepatopancreatic Necrosis Disease (AHPND)	*V. parahaemolyticus* (V_{APHND} strains)	• Black Tiger shrimp (*Penaeus monodon*) • Whiteleg shrimp (*Penaeus vannamei*) • Chinese white shrimp (*Penaeus chinensisa*) • Japanese Tiger shrimp (*Penaeus japonicusa*) (Post larvae stage)	• Lethargy • Anorexia and slow growth • Empty digestive tract and gut • Pale to white hepatopancreas • Soft shells • Black (melanized) spots or streaks visible within the hepatopancreas • Atrophy (shrinkage) of the hepatopancreas	Santos et al., (2020); Kumar et al. (2021);
Summer syndrome	*V. nigripulchritudo*	Western blue shrimp *(Litopenaeus stylirostris*) (Juvenile and adult stage; occurred during warm-season growout)	• Loss of appetite • Broken beard • Red beard and tail • Intestinal redness	Goarant et al. (2006)

Table 2.2 continued

White gut (feces) syndrome	• *V. harveyi* • *V. alginolyticus* • *V. anguillarum*	• Whiteleg shrimp (*Penaeus vannamei*) • Black Tiger shrimp (*Penaeus monodon*) (Juvenile and adult stage)	• Reduced feeding • Retarded growth • Distended midgut junction and midgut • White to yellow-golden contents in the midgut • Vermiform bodies in the gut or faecal strings • Empty gut and empty stomach	Sathish Kumar et al. (2022)
Bright-red syndrome	• *V. harveyi* • *V. parahaemolyticus* • *V. alginolyticus* • *V. anguillarum*	• Whiteleg shrimp (*Penaeus vannamei*) • Black tiger shrimp (*Penaeus monodon*) • Banana prawn (*Penaeus merguiensis*) (Post larvae stage; occurred mainly during rainy seasons)	• Bright red spots located bilaterally in the central region of the abdomen. • Opaque abdominal muscle • Anorexia and lethargy • Mortality	Soto-Rodriguez (2010); Cuéllar-Anjel et al., (2014)

Table 2.2 continued

Seagull syndrome	• *V. alginolyticus* • *V. harveyi* • *V. parahaemolyticus* • *V. vulnificus*	• Whiteleg shrimp *(Penaeus vannamei)* (All life stages)	• Moribund shrimp with acute bacteremia • Septic haemocytic nodules on the lymphoid organ, and heart • Loose connective tissues • Infected shrimp remain on water surface	Mohney et al. (1994); de Souza Valente and Wan (2021)

The pathogenicity of *Vibrio* spp. is associated with their ability to produce virulence factors. Virulence genes that are associated *V. parahaemolyticus* are *tlh, tdh*, and *trh* genes. Most pathogenic strains of *V. parahaemolyticus* are characterised by the presence of the *tdh* and/or *trh* genes, with the *tdh* gene found in the majority (95%) of clinical isolates, underscoring thermostable direct hemolysin (TDH) as a key virulence factor (Yáñez et al., 2015; Liu, 2003; Wang et al., 2015). Meanwhile, despite the unclear roles of the *tlh* gene on *V. parahaemolyticus* pathogenicity, its involvement in cytotoxicity and adaptation within the human gastrointestinal tract suggests that it, along with other unidentified factors, may contribute to the pathogenicity of certain *V. parahaemolyticus* strains even in the absence of the *tdh* and *trh* genes (Shinoda et al., 1991; Gotoh et al., 2010; Wang et al., 2015; Raghunanth, 2014).

Vibrio cholerae is commonly associated with several virulence genes that contribute to its pathogenicity, including those in the repeat in toxin (RTX) family, such as *rtxA* and *rtxC.* The *rtxA* gene encodes a putative cytotoxin, while *rtxC* encodes an acyltransferase, both of which are crucial for the cytotoxic effects of *V. cholerae* (Chow et al., 2001). Another key virulence gene is *ctxB*, which encodes the cholera toxin (CT), a major factor responsible for cholera syndrome and acute diarrheal diseases. (Chow et al., 2001; Takahashi et al., 2021).

Vibriosis in shrimp poses a significant concern owing to its potential to disrupt various stages of the shrimp life cycle, ranging from the egg phase to the broodstock stage (Dayang Najwa et al., 2024). Severe infections can lead to the complete decimation of shrimp populations within a short timeframe. While *Vibrio* spp. are omnipresent in the environment, their proliferation is typically constrained under normal conditions. However, environmental variables such as temperature and salinity fluctuations can foster rapid

bacterial propagation. When coupled with opportunistic factors such as compromised immunity due to environmental stressors, these conditions can precipitate outbreaks of disease in shrimp populations (Mastan and Begum, 2016; Semenza et al., 2017; Millard et al., 2021).

As previously mentioned, opportunistic *Vibrio* spp. typically pose minimal immediate threat under standard conditions. However, when there is a proliferation of infecting bacteria coupled with compromised shrimp health due to various stressors, susceptibility to vibriosis increases, potentially resulting in high mortality in severe cases of infection. Several factors contribute to stress in shrimp, including but not limited to poor water quality, overcrowding due to high stocking densities, inadequate nutrients and dissolved oxygen levels, elevated water temperatures, and low rates of water exchange (Brock and Lightner, 1990; Li et al., 2006; de Souza Valente and Wan, 2021). Consequently, it can be inferred that poor farm management practices may serve as a primary contributor to vibriosis outbreaks in shrimp farms.

2.4.1 Food Association and Vibriosis in Human

In addition to jeopardising the health of aquaculture-farmed animals, *Vibrio* spp. pose a risk to human health by contaminating aquaculture products intended for consumption. Among the most prevalent *Vibrio* spp. responsible for seafood-borne vibriosis globally are *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* (Baker-Austin et al., 2018; Bonnin-Jusserand et al., 2019). Of these, *V. parahaemolyticus* stands out as a primary causative agent of foodborne illness cases worldwide, comprising more than half (52.3%) of reported instances (New et al., 2016). This trend is also observed in Malaysia, where *V. parahaemolyticus* has been identified as a prominent cause of foodborne poisoning cases in institutional settings such as schools, universities, childcares, and nursing homes

(Letchumanan et al., 2015). Hassan et al. (2023) reported an average of 37.3 cases per year (ranging from 29 to 43 cases) of non-cholera *Vibrio* spp. isolated from patients across Malaysia between 2014 and 2020, with *V. parahaemolyticus* again emerging as the predominant pathogen, accounting for 57.8% of cases.

The detection of *Vibrio* spp. in seafood products in Malaysia has revealed high prevalences of numerous potentially pathogenic strains across various products. A study by Elhadi et al. (2004) demonstrated the presence of eight *Vibrio* spp., including *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. metschnikovii*, *V. mimicus*, *V. damsela*, and *V. fluvialis*, in shrimp, squid, crab, cockles, and mussels obtained from wet markets and supermarkets in four states in Malaysia between 1998 and 1999. Alarmingly, Kuching, Sarawak, emerged as the state with the highest prevalence of *Vibrio* spp., with 100% detection rate in shrimp samples (Elhadi et al., 2004). In a more recent investigation, Babai et al. (2020) found that the average microbial load of *V. parahaemolyticus* in red tilapias acquired from wet markets exceeded 1,100 MPN/g, with a 90% positive detection rate. While several studies have been conducted in other states such as Selangor (Elhadi et al., 2004; Lee et al., 2018; Tan et al., 2020; Venggadasamy et al., 2021), no recent studies have been found regarding the prevalence of *Vibrio* spp. in retail seafood products in Sarawak apart from these two reports. This underscores the need for further investigations to furnish comprehensive data for ongoing surveillance and management efforts.

Moreover, the elevated incidence of *Vibrio* spp. infections in Malaysia can be attributed to favourable climates and environmental conditions conducive to bacterial proliferation (Brumfield et al., 2023; Hassan et al., 2023). The natural ubiquity of *Vibrio* spp., particularly *V. parahaemolyticus*, in the marine coastal regions of Malaysia suggests that seafood serves as an almost inevitable transmission vector for these bacteria

(Letchumanan et al., 2015). Human infections with vibriosis primarily occur through the consumption of undercooked or raw seafood products contaminated with *Vibrio* spp., with less frequent transmission occurring through direct contact or exposure of open wounds to contaminated water (CDC, 2019a).

Vibriosis in humans typically presents as gastroenteritis characterised by acute dysentery, abdominal pain, watery diarrhoea, nausea, vomiting, and fever within 24 hours of consuming contaminated food (New et al., 2016; CDC, 2019a). Moreover, direct contact with contaminated water sources can result in wound and ear infections characterised by mild cellulitis and seropurulent exudate, as observed with *V. alginolyticus* infection (Chart, 2012). Common pathological features observed in infected individuals include mild erosion of the jejunum and ileum of the small intestine, gastric inflammation, and damage to internal organs (Wang et al., 2015).

While typically not severe in healthy individuals, who usually recover within approximately three days in mild infection cases, vibriosis poses a significant risk to immunocompromised individuals, where it can progress to septicaemia and severe dehydration, potentially leading to death (New et al., 2016). Additionally, particular attention must be given to *V. vulnificus* infections, as they can result in serious illness necessitating limb amputations and intensive care. Furthermore, one in five infection cases with *V. vulnificus* has been reported to result in fatality (CDC, 2019a).

One effective measure to prevent vibriosis infections in humans is through the proper handling and thorough cooking of seafood products. Consequently, due to the high prevalence of various *Vibrio* spp. in these products in Malaysia, raw consumption of seafood is not advisable. According to the acceptable standard for *V. parahaemolyticus* contamination in shellfish intended for raw consumption in Japan, the microbial load should

not exceed 100 MPN/g (Hara-Kudo and Kumagai, 2014). Similarly, the Canadian Food Inspection Agency (CFIA) has established a limit of 100 per gram for *V. parahaemolyticus* contamination in live oysters, while cooked or ready-to-eat products testing positive for *V. cholerae* are considered unsatisfactory (CFIA, 2019). Meanwhile, the Food and Drug Administration (FDA) of the United States stipulates that the safety limit for *V. parahaemolyticus* contamination in ready-to-eat fish products should not exceed 1×10^4 per gram (FDA, 2011). Unfortunately, such safety limit standards or minimum acceptable levels of *Vibrio* spp. or other bacterial contaminations in seafood are not currently established in Malaysia (Tan et al., 2020).

Given the widespread occurrence of global food poisoning incidents caused significantly by *Vibrio* spp., it is imperative for trading nations to implement effective food safety guidelines concerning the proper handling and storage of seafood to prevent cross-contamination during the exportation process (WHO, 2011; Ob et al., 2012). Furthermore, establishing an acceptable standard for *Vibrio* spp. contamination in seafood can also protect the integrity of food safety and public health. Therefore, continuous monitoring and implementation of effective surveillance strategies are crucial in preventing and controlling public health risks associated with *Vibrio* spp. contamination in food products.

2.5 Antibiotic Usage in Aquaculture

In the management and treatment of bacterial infections in aquaculture, antibiotics have traditionally been widely utilised. Since the discovery of penicillin in 1928, antibiotics have been acclaimed as a groundbreaking solution for managing infectious diseases (Katz and Baltz, 2016; Hutchings et al., 2019). Hundreds of antibiotics have been developed, effectively reducing and controlling bacterial populations, leading to a significant decrease in mortality cases in both animals and humans (Serwecińska, 2020). However, the extensive

and indiscriminate use of antibiotics has contributed to the emergence of antimicrobial-resistant (AMR) bacterial strains. Antimicrobial resistance occurs when organisms develop resistance to antibiotics, rendering them ineffective. Bacteria can acquire resistance to antibiotics through multiple and intricate mechanisms, which may evolve through mutation, or through the acquisition of mobile genetic elements (MGEs) encoding resistance factors from other bacteria via horizontal gene transfer. Consequently, antibiotic treatments become ineffective against resistant strains (Marti et al., 2014; WHO, 2023). Antimicrobial resistant bacteria pose not only a local challenge but also a significant global threat to human, animal, and environmental health (Prescott, 2014; Aslam et al., 2018). Instead of resolving bacterial infection issues, the use of antibiotics in aquaculture has precipitated a new challenge.

In Malaysia, antibiotics are commonly utilised in aquaculture as therapeutic agents, and less frequently as prophylactics and growth promoters. However, without statistics or official records detailing the type, quantity, and purpose of antibiotics used to support these claims, this depiction of antibiotic usage in aquaculture farms across Malaysia may lack accuracy (Choo, 2002). This underscores the recurring theme of the absence of reporting and documentation platforms in the Malaysian aquaculture industry. Notably, to ensure the prudent application of antibiotics in aquaculture, the Ministry of Health of Malaysia has implemented Codes of Practice under the Food Act, which serve as guidelines for controlling the presence of antibiotics in food (Choo, 2002; MOH, 2006).

2.5.1 Antimicrobial Resistance (AMR) in *Vibrio* species

Several studies have suggested that bacterial resistance did not orginally arise due to anthropogenic antibiotic use, but rather as an intrinsic defense mechanism against bioactive molecules synthesized by other antagonistic bacteria (Tamae et al., 2008; Olivares et al.,

2013; Marti et al., 2014). These defense mechanisms eventually evolved into new resistance mechanisms in environments where antibiotics are present, where they act as selectors and accelerators for the resistant bacteria (Galán, 2013). Therefore, although bacteria will inevitably develop resistance over time as it is a natural process driven by genetic changes, the emergence of AMR strains is facilitated and expedited by imprudent and uncontrolled antibiotic usage practices (WHO, 2023).

Some of the antibiotics commonly used in aquaculture farms in Malaysia include chloramphenicol, tetracycline, oxytetracycline, erythromycin, neomycin, nitrofurans, and norfloxacin (although the use of chloramphenicol, nitrofurans, and norfloxacin was later banned in Malaysia due to their adverse effects on human health) (Choo, 2002; Hassali et al., 2018; Thiang et al., 2021). The acquisition of antibiotics for farming practices typically occurs through official channels by pharmacists with appropriate credentials. However, reports indicate that farmers and traders frequently buy antibiotics from neighboring countries without appropriate labeling to meet the need to prevent significant losses in their production (Choo, 2022; Hays, 2020). Unfortunately, most Southeast Asian countries, including Malaysia, lack legislation, regulatory surveillance, and monitoring systems concerning the use of antibiotics (Thiang et al., 2021). This situation indirectly contributes to the rapid emergence of antimicrobial-resistant (AMR) bacteria due to unmitigated antibiotic use.

The prevalence of AMR bacteria in Malaysian aquaculture remains a pressing concern, particularly with shrimp emerging as a primary host susceptible to these pathogens (Haimid et al., 2022). Chitin, the predominant structural component of shrimp and other crustacean exoskeletons, has been identified as a potential intermediary surface facilitating the proliferation and dissemination of AMR through biofilm attachment, as highlighted by

Meibom et al. (2005). Furthermore, Malaysia is one of the countries in Southeast Asia with the most reports of AMR occurrence in aquaculture, along with Vietnam and Thailand (Suyamud et al., 2024). Despite generally low concentrations of antibiotics in aquatic ecosystems, studies have shown that even minimal exposure can fuel the development of antibiotic resistance in bacteria (Marti et al., 2014).

Kathleen et al. (2016) uncovered a concerning trend, revealing that over 50% of bacteria isolated from aquaculture surrounding areas and products in Sarawak exhibit high resistance to streptomycin and ampicillin. Additionally, investigations into the prevalence of *Vibrio* spp. in various tropical water samples across Peninsular Malaysia unveiled a staggering statistic: 95% of isolates demonstrated resistance to at least one antibiotic class, indicative of widespread antibiotic-resistant strains thriving in the west coast of Peninsular Malaysia. From this study, 20 distinct antibiogram patterns were also observed (You et al., 2016). Similarly, a study conducted in 2019 found a troublingly high proportion of *Vibrio* spp. (97%) isolated from groupers from nine farms in Peninsular Malaysia to be resistant to commonly used antibiotics ampicillin, penicillin G, and vancomycin. These antibiotics have been used for a long time in the industry in Malaysia, thus supporting the claim that continuous exposure to antibiotics will cause bacteria to develop resistance to them (Kathleen et al., 2016; Amalina et al., 2019).

In a recent investigation by Haifa-Hayrani et al. (2022), a striking 95% of *V. parahaemolyticus* strains isolated from diseased shrimp acquired from seven shrimp farms in Peninsular Malaysia exhibited resistance to at least three antibiotics, shedding light on the concerning nature of multidrug resistance in this context. Moreover, findings from a subsequent study in three shrimp farms in Sarawak elucidated a concerning trend, with 55% of the isolated *V. parahaemolyticus* demonstrating resistance to multiple antibiotics with 10

different antibiogram patterns observed (Elexson et al., 2023). These studies represent only a few instances of AMR in the Malaysian aquaculture landscape. It has been well-documented that occurrences of AMR among aquatic or marine organisms in Malaysia are not new.

From the standpoint of AMR dissemination, aquaculture alarmingly plays a significant role in the emergence, persistence, and transmission of resistance factors within the aquatic environment. The environment, as a whole, serves as a vast reservoir of antibiotic resistance genes (ARGs), with the aquatic environment, in particular, serving as a crucial reservoir of mobile genetic elements (MGEs) carrying these ARGs. Hence, the aquatic environment serves as a common ground for the transmission of antimicrobial resistance between bacteria. (Marti et al., 2014; Salgueiro et al., 2024). The boundaries between species in aquatic environments are narrow due to the genomic plasticity of *Vibrio* species. Horizontal gene transfer between them, even between the environmental and clinical strains, are frequent events (Marti et al., 2014; Liang et al., 2022). Notably, ARGs have been identified in shrimp farms along the Johor Strait, with the potential for transmission to other local farms situated within the same geographical area (Thiang et al., 2021). The ease with which AMR can spread from one location to another raises significant concerns, particularly when aquaculture farms are located in close proximity to each other.

2.5.2 The Presence of Residual Antibiotics in Seafood Products

In addition to fostering AMR bacterial populations, the presence of residual antibiotics in seafood products also raises concerns due to their implications on public and human health. In 2019, the United States Food and Drug Administration (USFDA) red-listed eleven shrimp exporters from Peninsular Malaysia due to the detection of antibiotics chloramphenicol and nitrofuran in one-third of their shrimp and prawn products.

Furthermore, between 2009 and 2018, 28 Malaysian exporters were red-listed after 56 shipment samples tested positive for nitrofurans (Annuar, 2020). Notably, based on Food Regulation 1985, both chloramphenicol and nitrofuran have been banned for usage in Malaysia due to their adverse implications on human health, with other countries including the United States, Canada, China, and various European countries also implementing the same bans (Bondad-Reantaso et al., 2012; Thiang et al., 2021). However, testing has revealed the presence of these residual antibiotics in seafood products, suggesting that these antibiotics are still being used illegally in some aquaculture farms in Malaysia. Nevertheless, while reports or data on antibiotic residues in meat and eggs are available (Hassali et al., 2018), none are available regarding antibiotic contamination in seafood products in Malaysia.

2.5.3 Effect of AMR Towards Human Health

Globally, 1.27 million deaths were directly attributed to AMR bacteria in 2019 alone, with an estimated total of 4.95 million deaths in the same year due to drug-resistant infections (Antimicrobial Resistance Collaborators, 2022; WHO, 2023). According to the European Centre for Disease Prevention and Control and the European Medicines Agency, an average of 25,000 deaths per year in Europe were reported due to infections caused by AMR bacteria (Marti et al., 2014). Meanwhile, in Malaysia, 3,500 deaths were reported to be caused by AMR, with an estimated total of 14,000 deaths associated with AMR-related infections in 2019 alone. Notably, the total number of AMR-related deaths in Malaysia surpasses those caused by other health problems such as diabetes, digestive and kidney diseases, chronic respiratory diseases, neurological disorders, and transport injuries (IHME, n.d.).

As a result of resistance, antibiotics become ineffective, making bacterial infections harder or impossible to treat, thereby increasing the risk of disease spread, severe illness,

disability, and even death (WHO, 2023). From an economic standpoint, the prolonged effects of AMR bacterial infections lead to more expensive treatments and longer hospital stays, affecting the productivity of individuals involved, including patients and caregivers. Furthermore, the development of new antibiotics to combat these AMR strains may take approximately 10 to 15 years, involving stringent procedures and high costs to ensure their safe use (WHO, 2022). Compounding the issue, AMR problems are not localized but can spread across boundaries between different geographical regions worldwide. This implies that AMR can be transmitted from one country to another through the trading of live animals and animal-based products (Paria et al., 2021; WHO, 2023).

In addition to the impacts of AMR on public health, residual antibiotics can also cause side effects in humans if ingested indirectly through antibiotics-contaminated food products. It has been suggested that consumption of residual antibiotics in contaminated food products can lead to various severe pathological side effects, including allergies, cancer, anaphylactic shock, kidney or renal diseases (nephropathy), bone marrow toxicity, mutagenic effects, as well as reproductive disorders. Antibiotics such as penicillin, sulfamethazine, oxytetracycline, furazolidone, chloramphenicol, nitrofurans, and gentamicin have been reported to be responsible for inducing these health issues (Bacanlı and Başaran, 2019; Arsène et al., 2022). For this reason, the use of some antibiotics, such as chloramphenicol and nitrofurans, has been banned for aquaculture in many countries around the world (Chanda et al., 2011). However, the detection of these antibiotics in food products suggests that they are still being used illegally in some countries, or the source may be from runoff from clinical facilities or wastewater treatment plants (Marti et al., 2014; Thiang et al., 2021; Bondad-Reantaso et al., 2023).

It has been predicted that the usage of antibiotics will continue to increase with the development of the aquaculture industry (Haimid et al., 2022). Estimates suggest that the total percentage of antimicrobial usage in aquaculture from 2017 to 2030 will be approximately 33%, driven by advancements in the global aquaculture industry (Haimid et al., 2022). However, this also highlights the potential for further emergence of AMR organisms. Evidently, alongside the increasing use of antibiotics, a rising trend in antibiotic resistance and treatment failures in multiple fish species has been observed (FAO, 2020). Moreover, continuous antibiotic abuse may potentially lead to the transformation of multiple drug-resistant organisms into extreme drug-resistant strains, rendering most antibiotics ineffective in controlling these organisms (Walsh, 2013; Marti et al., 2014). Additionally, the adverse effects of residual antibiotics on human and environmental health will be further exacerbated. These concerns underscore the need for alternative treatment strategies that are less invasive and overall safer. If unable to completely halt the usage of antibiotics, efforts should at least aim to minimize it.

2.6 Bacteriophages as Bio-control Agents

As antibiotics start to become ineffective in controlling and managing infectious organisms, it becomes imperative to explore alternatives to address this challenge. One such alternative that has gained traction in recent years is phage therapy. Phage therapy harnesses the natural enemy of bacteria, bacteriophages, as biological control agents. Bacteriophages, commonly referred to as phages, are viruses that infect bacteria and can replicate within them. Therefore, phages are ubiquitously present in various environments, including the human body, where bacteria reside, and aquatic environments serve as significant reservoirs for them (Batinovic et al., 2019; Łusiak-Szelachowska et al., 2020).

The first golden era of bacteriophages was overshadowed by the discovery and development of antibiotics. However, with the emergence of antibiotic-resistant bacteria, bacteriophages have once again garnered attention for their significance in the fields of biotechnology and molecular biology. Today, with advancements in science, greater knowledge and technology are available to comprehend the characteristics and mechanisms of bacteriophages (Clokie and Kropinski, 2009).

2.7 General Characteristics of Bacteriophages

The term "phages" was originally coined by Felix d'Herelle in 1917, derived from the Greek "phagein," meaning "to eat." Thus, bacteriophages, in literal terms, mean bacterial eaters. The discovery of bacterial viruses was initially made by William Twort in 1915, followed by Felix d'Herelle in 1917, who accurately described their role in causing bacterial death (Clokie and Kropinski, 2009). While all bacteriophages are viruses, not all viruses are bacteriophages. Bacteriophages exclusively replicate within bacterial cells, leaving mammalian cells unaffected, and they induce cell lysis in the process. Phages are also recognized as the most abundant organisms on Earth, with their total global abundance estimated to be on the order of 10^{31}, surpassing even the number of bacteria (Strange et al., 2021). Given their ubiquitousness in the environment, utilising their natural ability to control bacterial population is a wise strategy.

2.7.1 Structure and Classification

Bacteriophages are classified based on their phenotypic characteristics, such as their morphology, nucleic acid type, replication mode, and host organism (White and Orlova, 2019; Giri, 2021). The formal classification of viruses adheres to the International Committee on Taxonomy of Viruses (ICTV) system, which utilizes the polythetic species

concept. This concept involves defining a species based on a collection of characteristics that may or may not be present in any specific member of a group (Lefkowitz et al., 2018; Turner et al., 2023).

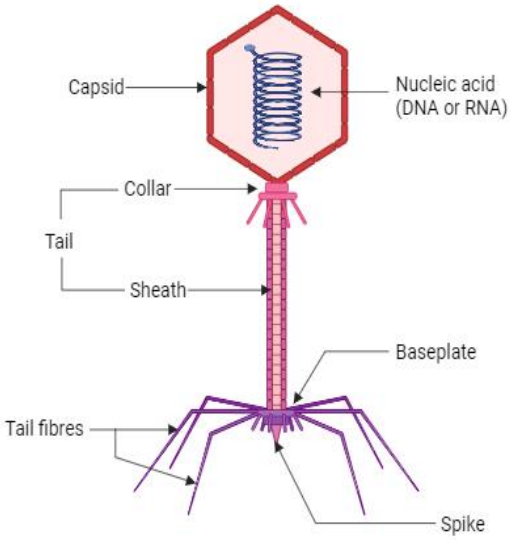


Figure 2.2: Basic structure of a tailed bacteriophage (created with https://www.biorender.com)

While bacteriophages exhibit variations in shapes and sizes, the majority of them feature a head and a tail, as depicted in Figure 2.2. The head structure, also known as the capsid, adopts a polyhedral form containing the phage's genetic material. The genome, composed of either DNA or RNA, can exist as single or double-stranded and encodes information pertaining to the proteins involved in the phage's self-replication and self-assembly processes. The size of the capsid is influenced by the size of the phage genome; hence, larger genomes correspond to larger capsids, indicative of a more complex organization (White and Orlova, 2019). Meanwhile, the tail structure typically comprises an inner hollow tube surrounded by a sheath, which may be either contractile or non-contractile.

At the end of the tail, attached to the baseplates, are the tail fibres responsible for binding the phage to its host bacteria. These tail structures play a crucial role in host recognition and attachment, as well as in injecting the phage genome into the host bacteria (Taslem Mourosi et al., 2022).

Although tailed phages belonging to the *Caudovirales* order were previously commonly classified into three families (*Myoviridae*, *Siphoviridae,* and *Podoviridae*), the International Committee on Taxonomy of Viruses (ICTV) recently abolished these groupings. These families are now simply categorised under the *Caudoviricetes* class, alongside other archaeal viruses with icosahedral capsids and double-stranded DNA as portrayed in Figure 2.3 (Turner et al., 2023). However, for the purpose of morphological distinction, these phages will be referred to as myophages, siphophages, and podophages in this chapter.

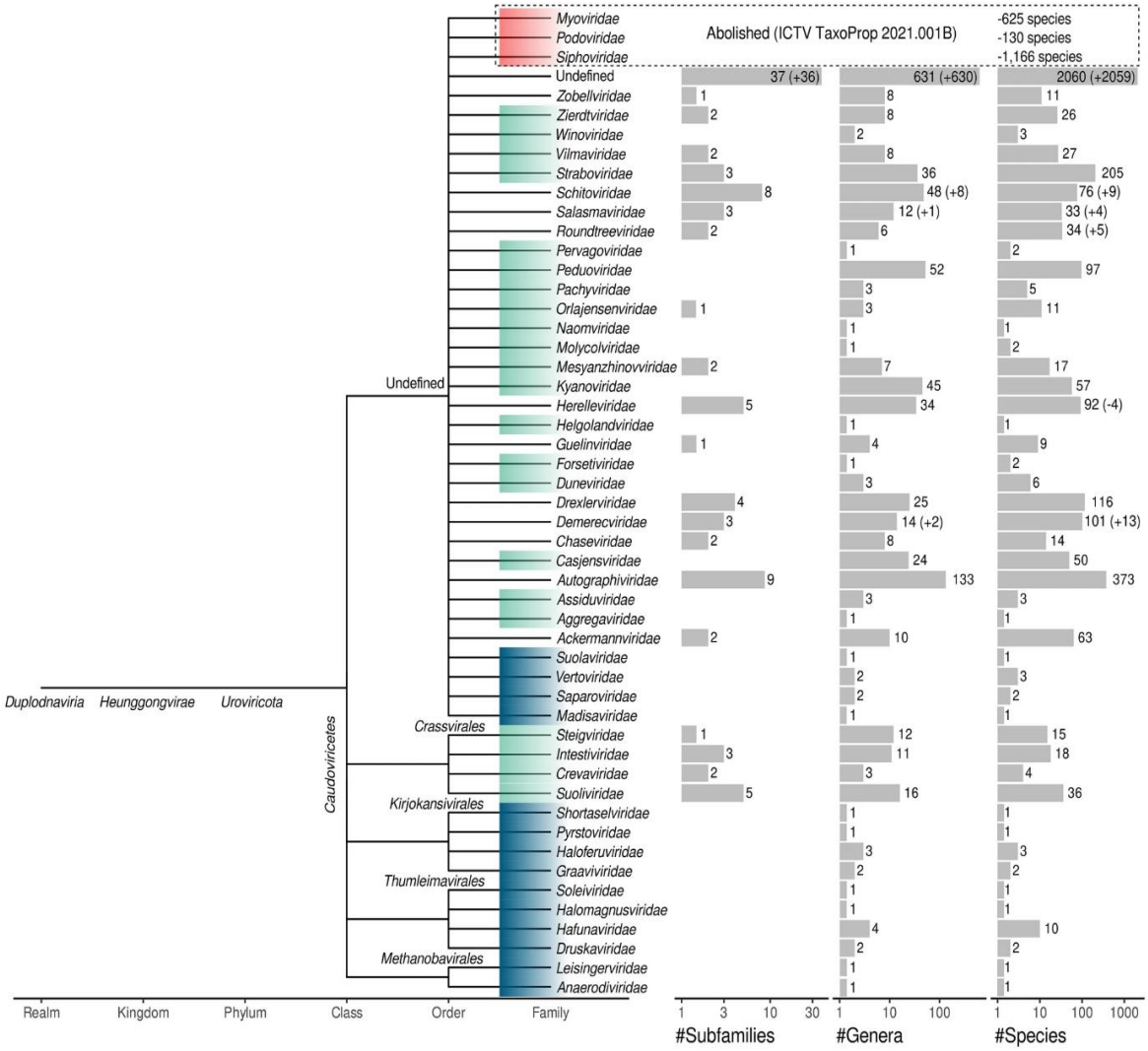


Figure 2.3: The updated taxonomic classification of the class *Caudoviricetes* as of 2021. Green and blue highlights denote the newly established bacterial and archaeal virus families, respectively, while abolished families are marked in red. Histograms adjacent to each family represent the count of subfamilies, genera, and species, with numbers in parentheses indicating the alterations documented from the ratified taxonomic proposals (Source: Turner et al., 2023).

According to Ackermann (2007), the majority (more than 96%) of bacteriophages are tailed phages. Myophages, siphophages, and podophages are tailed phages with icosahedral capsids and double-stranded DNA as their genetic material. One characteristic that sets these phages apart is their tails. Myophages are distinguished by their contractile tails, which differentiate them from other tailed phages. Siphophages, on the other hand,

possess long and flexible tails that are non-contractile. Meanwhile, unlike myophages and siphophages, podophages lack long tails, and having short, stubby ones instead (Ackermann, 2007; Nobrega et al., 2018). Figure 2.4 below illustrates the morphology of a myophage, siphophage, and podophage.

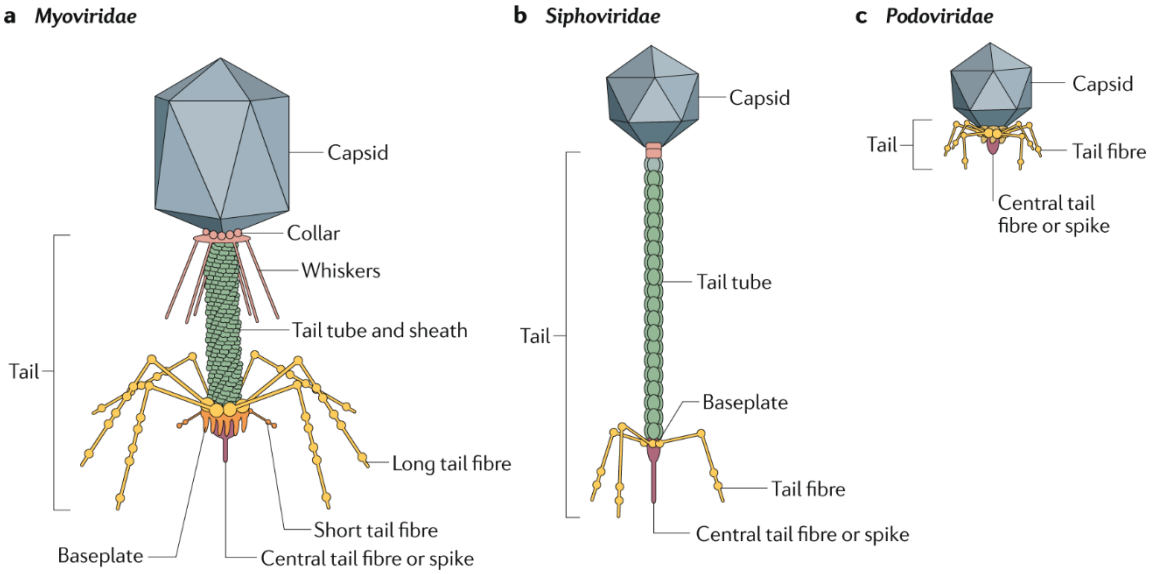


Figure 2.4: Morphology of a (A) myophage, (B) siphophage, and (C) podophage. (Source: Nobrega et al., 2018)

2.7.2 Life Cycle of Bacteriophages

As previously implied, phages are categorised as non-living entities because they are unable to reproduce without a host. Therefore, they must infect a host cell and utilise its cellular machinery and components for replicating copies of themselves (Koonin and Starokadomskyy, 2016; Naureen et al., 2020). There are two distinct life cycles of a bacteriophage: the lytic cycle and the lysogenic cycle (Figure 2.5). Each of these life cycles impacts bacterial populations and their genome composition differently.

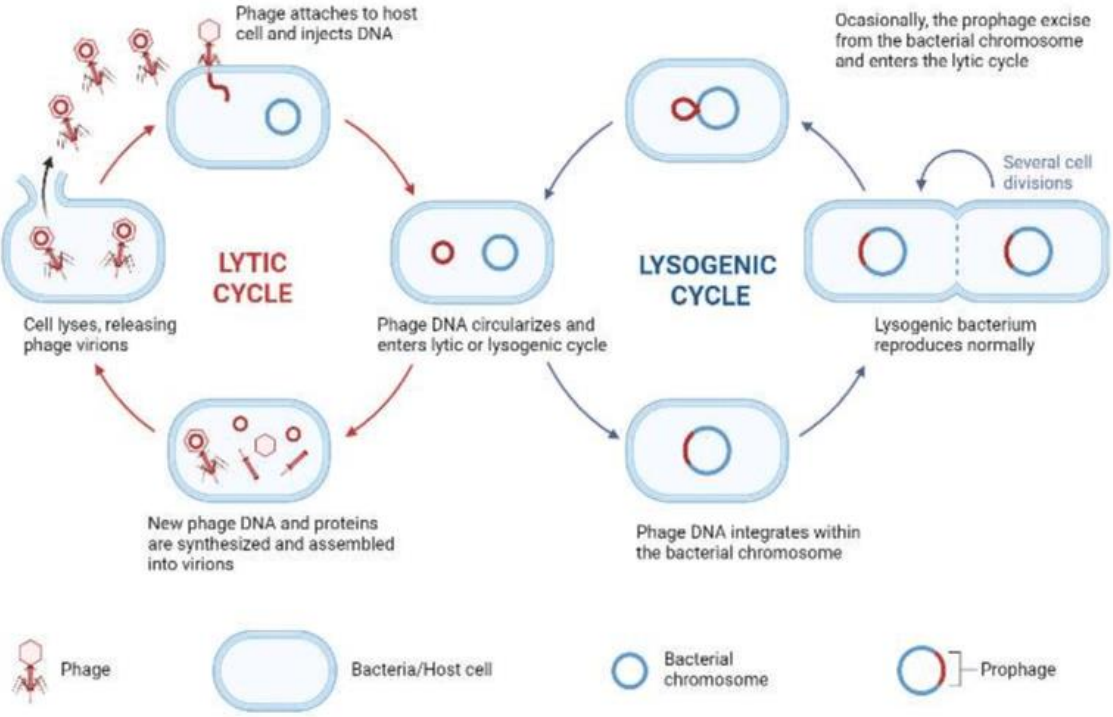


Figure 2.5: Lytic and lysogenic life cycles of a bacteriophage (Source: Alsobhi, 2021).

Lytic phages, also referred to as virulent phages, follow a lytic life cycle. In this cycle, they infect host cells, causing lysis and death of the infected cells in the process, thereby controlling bacterial population and influencing microbial diversity through phage predation (Shapiro et al., 2010). Moreover, the lytic cycle occasionally contributes to the long-term evolution of bacteria via generalised transduction. During this process, bacterial DNA may be acquired and packaged with new phage particles, transferring genetic material to other bacteria (Clokie et al., 2011; Kasman and Porter, 2022).

The lytic cycle begins with the recognition and attachment of phages to single or multiple receptors on the surface of host bacteria. Subsequently, the phage injects its genetic material (DNA or RNA) into the bacterial cell through a hole created in the cell surface via enzymatic cleavage. Replication commences as the phage shuts down bacterial DNA, RNA,

and protein synthesis, redirecting the bacterial metabolism to replicate its own genome and synthesise its own proteins and structural components instead. These components are then assembled and packaged into new phage particles called virions during the maturation stage. Enzymes produced by the phage subsequently lyse the bacterial cells by disintegrating the peptidoglycans in the cell walls, resulting in the death of the host bacteria and the release of the newly assembled bacteriophages. Depending on the specific phage, one round of infection can yield 50 to 200 virions, each virion capable of initiating new rounds of infection (Alsobhi, 2021).

Some phages, referred to as temperate or non-virulent phages, undergo a lysogenic life cycle. In contrast to the lytic cycle, the host bacteria are not killed by the phage in the lysogenic cycle. Instead, the phage integrates its genetic material into the host genome, or it exists as a plasmid within the intact host cell, following the attachment and penetration stages. This integrated DNA is replicated along with the bacterial chromosome during bacterial cell division, resulting in prophages that can be passed down to subsequent generations (Alsobhi, 2021). This life cycle can remain stable for a long period, spanning many generations, during which the host bacteria can acquire new genes, such as antibiotic-resistant or virulence genes, that can manifest as new phenotypes (Clokie et al., 2011). Through this lysogenic conversion, the genetic composition of the host bacteria is altered, indicating that lysogenic phages play a role in the evolution and genetic diversity of bacteria (Brüssow et al., 2004; Naureen et al., 2020).

While all virulent phages only undergo the lytic life cycle, temperate phages, conversely, can exhibit both life cycles depending on various factors (Alsobhi, 2021). According to Howard-Varona et al. (2017), a lysogenic phage can transition to the lytic cycle

when exposed to environmental stressors, such as ultraviolet (UV) radiation, or antibiotic treatment, leading to excision of the integrated phage DNA from the bacterial chromosome and activation of the lytic cycle. During the excision of phage DNA, some bacterial DNA may be excised concurrently, which can then be transferred to new hosts, a process known as specialized transduction (Chiang et al., 2019; Kasman and Porter, 2022). However, for the purposes of phage therapy, temperate phages may present challenges due to their potential to induce lysogenic conversion in host bacteria. To achieve the objectives of phage therapy, obligately lytic phages are the most suitable candidates, as they can effectively eliminate targeted bacteria without the risk of introducing resistance or virulence genes into the bacterial population (Loc-Carrillo and Abadon, 2011). For this reason, only lytic or virulent phages will be addressed for the remainder of this chapter. Furthermore, given the broad potential of phage applications across various fields, this chapter will focus solely on the potential applications of phages in phage therapy, aligning with the general objective of this research.

2.8 Phage Therapy versus Antibiotics

Applying bacteriophages as a treatment strategy presents several advantages compared to antibiotic treatment. The comparison between bacteriophages and antibiotics is summarized in Table 2.3 below.

Table 2.3: Comparison between bacteriophages and antibiotics as treatment strategies.

Characteristics	Bacteriophages	Antibiotics
Specificity	Only target specific bacterial strains or species, while others have a broad host range. (Diallo and Dublanchet, 2022).	Broad spectrum, affecting even the non-targeted organisms (Patangia et al., 2022).

Table 2.3 continued

Side effects	Typically, few to no adverse effects are observed as they do not impact other non-targeted organisms (Sabouri Ghannad and Mohammadi, 2012).	Disruption of the microbiota due to broad-spectrum activity, increases the risk of secondary infections and may trigger allergies and intestinal disorders in humans (Patangia et al., 2022).
Dose	Auto-"dosing," where phages can proliferate with limitations based on the host bacterial density (Loc-Carrillo and Abedon, 2011).	The dosage is contingent upon factors such as concentration, absorption, metabolism, and excretion of the antibiotics (Furfaro et al., 2018).
Resistance	Resistance can emerge due to the attenuation of host virulence. Nevertheless, this resistance can be overcome through the co-evolution of phages with their host (Borin et al., 2021).	Promote the emergence of AMR bacteria beyond the intended targets. Permanent resistance may occur in some instances (NWO, 2004).
Occurrence	Naturally occurring and ubiquitous in various environments, can be found wherever bacteria reside (Batinovic et al., 2019).	Produced naturally by bacterial soil and fungi. Manufactured in the industry through fermentation processes (Fedorenko et al., 2015).
Development process	Phages can be readily and cost-effectively produced, even in resource-limited settings. The process is typically rapid, taking only days or weeks to complete (Hitchcock et al., 2023).	New antibiotic production typically requires approximately 10 to 15 years to progress from pre-clinical to clinical trials. The process is also expensive (WHO, 2022).

Table 2.3 continued

Mode of action	Bactericidal	Bacteriostatic or bactericidal
Anti-Biofilm	Has anti-biofilm properties (Chang et al., 2022)	No anti-biofilm properties (Sharma et al., 2019)

In conclusion, the specificity, evolutionary adaptation, general safety, natural occurrence, low cost and ease of production, ability to strictly induce bacterial death, and anti-biofilm properties render phages a promising alternative to antibiotics in the treatment of bacterial infections. However, unlike antibiotics, the application of bacteriophages is still underdeveloped, with only a few trial studies conducted (Romero-Calle et al., 2019). Further research is needed to fully grasp the potential of phages in controlling bacterial infections and to devise effective strategies for their utilisation.

2.8.1 Specificity to Bacterial Hosts

One characteristic that makes phages suitable as bacterial control agents is their specificity toward their host bacteria. Unlike antibiotics, which affect a broad spectrum of organisms, including the non-targeted ones, bacteriophages target only their specific host bacterium or bacteria, leaving the rest of the microbiome unaffected (Kasman and Porter, 2022). Most bacteriophages have a narrow host range, with their specificity extending only to a single bacterial species or even to a specific strain of the species. According to Bertozzi Silva et al. (2016), the viral specificity of the phage ensures that a specific phage can only attach to certain strains or species of bacteria. This specificity of bacteriophages is determined by the adhesion structures found on the cell wall of the bacteria, or even on their pili or the flagella in some cases.

The receptor-binding proteins (RBPs) of the phage recognize and bind to the receptors of the bacteria in a three-step process. In the first step, phages randomly encounter their hosts through collisions due to diffusion in the medium as well as Brownian motion. In the second step, a reversible interaction occurs between the phage RBP and the bacteria's primary receptor to keep the phage and bacteria close before an irreversible binding occurs with a secondary and more specific receptor in the third step. The third step is usually mediated by enzymatic cleavage, which triggers changes in the confirmation of the phage molecules, allowing the injection of the phage's genetic material into the host (Bertozzi Silva et al., 2016; Leprince and Mahillon, 2023).

While most phages are reported to be species- or strain-specific, there are also those with a broad host range, indicating their ability to infect different species or even genera of bacteria. For instance, bacteriophage KVP40, isolated in Japan, demonstrates the ability to infect eight *Vibrio* spp., including *V. cholerae*, *V. parahaemolyticus*, *V. natriegens*, and *Photobacterium leiognathid* (Miller et al., 2003). Additionally, Vibriophage vB_ValM_PVA8, isolated from sewage water in China, can infect both *V. alginolyticus* and *V. parahaemolyticus* (Fu et al., 2023). From the perspective of phage therapy, broad host range bacteriophages may offer greater advantages than narrow host range phages due to their capacity to infect multiple host bacterial species simultaneously (Ross et al., 2016).

However, the challenge posed by the narrow host range in most phages can be overcome by employing phage cocktails (Loc-Carrillo and Abedon, 2011). In multiple-species cocktails, various phages with different host targets are combined to provide a broad spectrum of activity, targeting more than one species (Abedon et al., 2021). Such cocktails are commonly employed for the treatment of generalized diseases caused by different species or genera of bacteria. For instance, Pyophage-type cocktails, capable of targeting

multiple species across various bacterial genera, including *Enterococcus*, *Escherichia*, *Proteus*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*, have been utilised in the treatment of skin and soft tissue infections (McCallin et al., 2018; Leitner, 2021; Abedon et al., 2021).

Meanwhile, in single-species cocktails and single-strain cocktails, multiple phages targeting the same species but different strains, and the same strain, respectively, are combined to enhance their effectiveness (Abedon et al., 2021). An example of a single-species cocktail is demonstrated in the study of Gu et al. (2012), where a cocktail containing three phages (GH-K1, GH-K2, and GH-K3), each with different but overlapping host strains of *Klebsiella pneumoniae*, exhibited better success in reducing the bacterial load in infected mice than when a monophage treatment was used. Additionally, in a study by Mateus et al. (2014), a combination of two phages with the ability to infect *V. parahaemolyticus* resulted in a higher reduction in the bacterial load compared to when each phage was used separately.

Hence, phages utilised for therapy are frequently combined in cocktails to augment their efficacy. Nonetheless, there are currently no official or validated guidelines for formulating mixed phage preparations. To create cocktails with optimal therapeutic potential, numerous factors pertaining to the candidate phages, including their morphology, characteristics, life cycle, and infection profiles, must be taken into account (Molina et al., 2022). Consequently, cocktails should not be composed of random phages, as their collective activities may potentially diminish rather than enhance efficacy.

2.8.2 Bacteriophage Resistance

One of the challenges encountered when utilising bacteriophages is the potential development of bacterial resistance towards them. However, this resistance primarily arises

from host attenuation rather than the acquisition of resistance genes. Genome sequencing and variant analysis have revealed that most bacterial resistance to phages stems from spontaneous mutations in genes responsible for producing phage receptors (Labrie et al., 2010; Yen et al., 2017). These mutations can alter bacterial receptors, trigger the production of extracellular polysaccharides, or synthesise analogs (competitive inhibitors) for receptor-binding proteins, thus obstructing phage attachment to host bacteria (Kim et al., 2020; Hasan and Ahn, 2022). For instance, *Escherichia coli* K-12 developed resistance to T-even-like bacteriophages through point mutations, rearrangements, and insertions in their OmpA outer membrane protein, which serves as the phage receptor (Morona et al., 1984; Koebnik et al., 1991).

Nevertheless, pathogenic bacteria acquiring resistance to phages due to receptor modifications may experience attenuation in their virulence or even lose their pathogenic properties (León and Bastías, 2015). This is because phage adsorption receptors, integral to bacterial cell surface components, often act as virulence factors for the bacteria (Mateus et al., 2014). Moreover, structural modifications may lead to reduced fitness in resistant bacteria compared to their non-resistant counterparts (León and Bastías, 2015). It has been reported that phage-resistant bacterial strains are more susceptible towards antibiotics treatment since the same mutations that disrupt phage binding also disrupt the receptor protein's activity as a component of the bacterial drug efflux pump (Hyman, 2019).

However, unlike antibiotic resistance, phages can counter bacterial resistance through co-evolution and adaptive strategies (León and Bastías, 2015). Phages and their host bacteria have engaged in a continual evolutionary arms race over many years, each developing defence and counterstrategies, respectively (Koderi Valappil et al., 2021). This ongoing evolutionary dynamic contributes to phages adaptation in nature and their ability to

overcome bacterial resistance (Klumpp et al., 2012). Phages can adapt to changes in their bacterial hosts by recognising new or modified receptors, prompting modifications to the receptor-binding sites in their tail fibres to counteract these changes. For instance, *E. coli*'s λ phages adapt by evolving the associated gene, allowing them to bind to an alternative receptor (OmpF) when the structure of the original outer membrane protein (LamB) is altered (Meyer et al., 2012). Similarly, phage ϕHP3 has been observed to modify its putative tail spike protein to overcome resistance in *E. coli* (Salazar et al., 2021). Consequently, when bacteria develop resistance to phages, the phages employ counterstrategies to overcome the resistance.

Alternatively, the development of bacterial resistance to phages can be prevented or minimised through the application of phage cocktails, particularly those comprising phages targeting different types of bacterial receptors, as opposed to monophage treatment. In addition to enhancing their infective capacity against bacteria, phage cocktails can impede the emergence of monophage-resistant strains (Mateus et al., 2014; Kim et al., 2020). By employing a cocktail of phages with diverse receptor specificities, it becomes significantly more challenging for bacteria to mutate and develop cross-resistance simultaneously against all of them, and the bacteria may remain susceptible to the remaining phages even if they are resistant to one (Abedon et al., 2021). A study conducted by Kim et al. (2020) demonstrated that their phage cocktail, comprising different types of *V. coralliilyticus* phages (phage pVco-5, pVco-7, and pVco-14), effectively controlled the pVco-7-resistant variants and prevented their infection in Pacific oyster larvae. This indicates that despite the previous resistance of the bacterial strain to one of the phages, the combined action of the three phages successfully overcame the phage resistance. Furthermore, the infective activity of the phage

cocktail was also superior to that observed when employing pVco-5 and pVco-14 as monophage treatments (Kim et al., 2020).

2.9 Current State of Research on Bacteriophages in Malaysia

While study of bacteriophages has been done for a long time in other countries such as the United States, China, and other European countries, this field is fairly new in Malaysia. In recent years, published papers from Malaysia have discussed the studies of phages in clinical, livestock, aquaculture, and agriculture sectors (Adamu Ahmad et al., 2016; Guang-Han et al., 2016; Thung et al., 2020; Sasireigga et al., 2021), but only a limited number of them were located in Sarawak. Furthermore, no published paper on Vibriophages from Sarawak was found.

Overall, while the study of bacteriophages may have been relatively new in Malaysia compared to some other countries, it's likely that with increasing global awareness and recognition of the potential of bacteriophages, as well as because of its status as one of the countries with the highest AMR occurrence in Southeast Asia, Malaysia is also beginning to invest more resources and attention into this field of research (Carascal et al., 2022; Suyamud et al., 2024). Given the abundance of resources in Sarawak, it is important to explore the phage populations here that can potentially be utilised and tailored for local applications.

2.9.1 Published Studies and Findings on Vibriophages in Malaysia

As part of a burgeoning research field in Southeast Asia, particularly in Malaysia, a three-phase strategy was proposed by Carascal et al. (2020) to initiate a phage revolution in this region. The first phase entails intensifying efforts to discover and characterise new phages. The second phase suggests the creation and implementation of laboratory protocols and clinical guidelines for evaluating phage activity. Meanwhile, in the third phase,

regulatory standards for therapeutic phage applications in various fields, such as agriculture, aquaculture, the food industry, and the medical field, should be adapted (Carascal et al., 2020).

From available publications, it is evident that Malaysia is currently in the first phase, with recent years producing more findings regarding newly isolated phages. According to the ASEAN Microbial Database (AMIBASE), Malaysia contributed to the second highest record of phages at 187 records in Southeast Asia, with the majority of the records are from biomedical-related phage research (Carascal et al., 2022). Table 2.4 below provides a summary of the findings relating to Vibriophages isolated in Malaysia. As more studies are conducted on these locally sourced phages, the complexities of phage-host dynamics that are unique to this region can be better understood, thus opening new avenues for promising biotechnological applications and therapeutic development.

Table 2.4: Vibriophages isolated from various sources and locations in Malaysia.

Bacteriophage(s)	Host	Source (location)	Description	Reference
VPUSM 4, VPUSM 7, and VPUSM 8	*V. cholerae* O1 El Tor Inaba	Environmental water samples (Kelantan)	Myophages isolated from different water sources characterised by indistinguishable features, including narrow host range, similar morphologies, and identical genomic characteristics, suggesting that they may either represent a single phage or several very closely related phages.	Al-Fendi et al. (2014)
VpKK5	*V. parahaemolyticus*	Coastal sand sediment (Sabah)	A siphophage with a narrow host range and is capable of completely lysing the fish-pathogenic strain of *V. parahaemolyticus*.	Lal et al. (2016a)
ValKK3	*V. alginolyticus*	Marine sediment (Sabah)	A myophage that is specific to *V. alginolyticus* ATCC® 17749™, which is pathogenic to both fish and humans.	Lal et al. (2016b)
Vp33, Vp22, Vp21, and Vp02	*V. parahaemolyticus*	Seafood samples (Selangor)	Podophages with a narrow host range but exhibited the ability to lyse multiple strains (23/126) of *V. parahaemolyticus*, including four clinical strains.	Tan et al. (2021)
Vp08 and Vp11	*V. parahaemolyticus*	Seafood samples (Selangor)	Siphophages capable of lysing multiple environmental strains (44/126) of *V. parahaemolyticus*.	Tan et al. (2021)

Table 2.4 continued

VA-WOI	*V. alginolyticus*	Wild oysters from markets (Kelantan)	A siphophage specifically lysing strains of *V. alginolyticus*, originally isolated from farmed Asian seabass, indicating a narrow host range.	Zin et al. (2024)

2.10 Methodology for Bacteriophage Isolation and Characterisation

Bacteriophages to be isolated are characterised to assess their suitability for phage therapy. The protocol for bacteriophage isolation is typically rapid and straightforward, adhering to established procedures introduced by d'Herelle, which have been employed by numerous phage researchers since their inception (Ross et al., 2016). These procedures can be tailored to meet specific research goals and requirements. The general steps involved in phage isolation and characterisation are detailed below.

2.10.1 Identification of Bacterial Hosts and Samples

Phage isolation is typically performed using a single bacterial strain as the parental host, which is then tested against other strains to determine its host range. Although this method often results in the isolation of phages with narrow host ranges, the isolation of broad host range phages has also been reported (Ross et al., 2016). A broad host range can be facilitated if other bacteria share similar types of receptors as the isolation host, although exceptions exist, such as phages equipped with two sets of tail fibres (Hyman, 2019). When the objective is to isolate phages with a broad host range, employing a culture containing a mixture of multiple bacterial hosts may increase the likelihood of isolating phages capable of infecting multiple strains or species compared to using a single host (Jensen et al., 1998; Ross et al., 2016). Additionally, bacterial strains that are easily cultured in the laboratory and are not overly pathogenic should be considered for practical reasons. Furthermore, to isolate phages for therapeutic applications, bacterial hosts should be screened for lysogeny to avoid the isolation of temperate phages, which are unsuitable for this purpose (Hyman, 2019). Ultimately, phage isolation procedures can be customised based on the specific objectives of the research.

Another aspect to be considered in phage isolation is the selection of appropriate sources. Phages are ubiquitous in environments where their bacterial hosts reside. Consequently, samples may be collected from environmental sources such as water, soil, and sediment (Al-Fendi et al., 2014; Lal et al., 2016a; Samir et al., 2022), clinical samples including blood, ascitic fluid, urine, cerebrospinal fluid, and serum (Brown-Jaque et al., 2016), or from sewage and raw faecal matter (Wirjon et al., 2016; Dakheel et al., 2019). A fundamental principle in phage hunting is that phages are often located where their hosts are found (Strathdee et al., 2023).

Depending on whether the samples are solid or liquid, debris and contaminants must be physically removed. Typically, centrifugation and filtration through low-protein-binding membrane filters (made from materials such as polytetrafluoroethylene, polypropylene, polycarbonate, polysulfone, or cellulose acetate) with pore sizes of 0.2 µm or 0.45 µm are employed to remove unwanted contaminants and particles, while minimally impacting phage yield (Van Twest and Kropinski, 2009; Göller et al., 2020; Van Charante et al., 2021).

2.10.2 Phage Enrichment

The subsequent phase in the phage isolation protocol involves enriching phages by using their host bacteria. Throughout this stage, enrichment biases can be employed to select phages that align with the primary objective of the isolation. For instance, phages intended for therapeutic purposes should be strictly lytic, possess exceptional capacity to infect the targeted bacteria, and exhibit favourable infection profiles and stability (Loc-Carillo and Abedon., 2011; Weber-Dąbrowska et al., 2016). Therefore, various factors including bacterial host, media type (nutrient composition, pH, salinity, osmolarity, etc.), incubation

conditions and parameters, and numerous other variables must be taken into account (Van Charante et al., 2021).

During the enrichment procedure, host bacteria are cultured overnight in suitable liquid media. Commonly utilised media for phage enrichment include Tryptic Soy Broth (TSB) and Luria-Bertani Broth (LB) (Van Twest and Kropinski, 2009). Typically, the strength of the media is increased to enhance the likelihood of successful phage isolation and to broaden the variety available for selection (Glonti and Pirnay, 2022). For instance, Lal et al. (2016a) employed double-strength (2x) TSB to enrich their phages. Utilising concentrated medium for enrichment also yields phages with shorter latency periods if they are already present in high titres, a desirable characteristic from a phage therapy perspective (Khan Mirzaei and Nilsson, 2015).

Alternatively, Ghugare et al. (2017) proposed another method for successfully isolating phages when their concentrations in samples are very low. In this method, instead of mixing the phage samples with the host bacteria in a standard enrichment culture, the bacterial cells are immobilised and concentrated on a 0.45 μm pore filter, and the phage samples are immediately passed through the filter. Subsequently, the filter is incubated in a culture medium, and it was observed that the recovery of phages (if they are present in the samples) is higher than when using the standard enrichment method (Ghugare et al., 2017). Hence, the selection of the enrichment methods depends on the concentration of the phages in a sample.

Furthermore, supplementing salt, such as magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$), in the media during the enrichment step is also essential. This is because cations (Mg^{2+} or Ca^{2+}) facilitate the attachment of the phages to their host cells and increase phage propagation to a certain limit (Yazdi et al., 2020; Mhone et al., 2022). According to

Yazdi et al. (2020), phages grown with their host bacteria in a media containing $MgCl_2$ or $CaCl_2$ showed a significant increase in their adsorption rate by approximately 20% compared to the control without cations. The presence of cations in the media stabilises the interaction between phages and their hosts, leading to higher yields during the isolation process due to the enhanced infectivity of the phages (Yazdi et al., 2020).

2.10.3 Isolation, Confirmation, and Purification of Phages

After the enrichment of phages is performed (typically overnight), the samples are ready for phage isolation. The phage-bacteria mixture is centrifuged to eliminate the bacteria cells or other larger particles, resulting in a phage lysate containing only the enriched phages. Sometimes, in addition to centrifugation, chloroform is added to lyse the remaining bacteria cells in the filtrate to maximise the release of phages (Hyman, 2019). However, this step is often unnecessary, especially when targeting rare lipid-containing phages or filamentous phages, as it can cause damage to the phage particles (Hyman, 2019; Van Charante et al., 2021). Therefore, this step is optional depending on the type of phages to be isolated.

Prior to phage isolation and purification, the presence of phages needs to be confirmed first. Table 2.5 below summarises all the commonly used methods to detect phage presence. The spot test method is the most commonly utilised for the initial screening of lytic phages, as it offers a straightforward approach (Stenholm et al., 2008). Positive results manifest as confluent spots, indicative of bacterial lysis caused by multiple phages. This method, characterised by its simplicity and expediency, facilitates rapid confirmation and economises on plate usage by allowing multiple lysates to be spotted on a single lawn (Van Charante et al., 2021).

Table 2.5: Bacteriophage detection methods (Hyman, 2019).

Methods	Description	Advantages	Disadvantages
Spot testing (Stenholm et al., 2008)	A lawn of host bacterium is prepared on an agar plate, and 5-20 µL of phage lysate is spotted on the lawn. The plate is then incubated under appropriate conditions. The presence of a lysis zone indicates the presence of phages.	Simple and fast. It allows testing of multiple phage lysates on the same bacterial plate, thus saving on the number of media used.	Can only be used for bacteria that can grow to confluence on agar media. False positives are a potential result if other bactericidal components are present.
Plaque assay/ Double-layer agar assay (Kropinski et al., 2009)	Phage lysates are increasingly diluted and then mixed with host bacteria before being plated on an agar surface using soft agar overlay. The presence of plaques after incubation indicates the presence of phages.	Can visualize productive phage growth and differentiate between lytic and temperate phages, as the presence of clear plaques indicates lytic phages, and turbid plaques indicates temperate phages.	Only effective for host bacteria capable of confluence growth on agar media. There is potential for false negatives since some phages are unable to form plaques due to limited diffusion in agar or low productivity.

Table 2.5 continued

Culture lysis (McLaughlin, 2007)	Phage lysate is incubated with host bacteria in a broth medium. The presence of phages is indicated by the reduction or loss of culture turbidity due to cell lysis. Metabolic dyes are sometimes employed to assess the metabolic activities of the host bacteria.	Excellent for bacteria that cannot grow to confluence on agar media and allows for spectrophotometric measurement of turbidity in single tubes or multiple well plates.	Prone to false-positives due to non-productive lysis and the presence of other bactericidal components in the culture. Additionally, cell debris may interfere and inactivate free phages. Hosts that develop phage resistance may cause false negatives since the turbidity of the culture remains unchanged.
Routine test dilution (RTD) (Redmond and Ward, 1966)	Phage lysates are diluted to achieve just less than confluent lysis on agar media.	Effective for phages that form small indistinct plaques or phages that cannot form distinct plaques on agar media.	Can produce false-positive results if the phage lysates are not diluted enough.

Spot testing and plaque assay are commonly employed in tandem by many phage researchers, with spot testing utilised as the initial assessment followed by plaque assay for more precise analysis (Hyman, 2019). Subsequently, plaque assay becomes essential to confirm the presence of phages, considering the potential for false positives. Such occurrences may arise due to the presence of other bactericidal compounds, such as secondary metabolites within the sample (Van Charante et al., 2021). Moreover, plaque assay facilitates the isolation of well-defined plaques for further purification step. In this purification phase, individual and well-separated plaques are picked using sterile pipette tips or loops and dispensed in buffers. To obtain a phage lysate containing a singular phage type, the plaque undergoes a minimum of three rounds of purification, a procedure considered adequate for achieving pure phage isolates (Hyman, 2019; Van Charante et al., 2021).

2.10.4 Phage Characterisation

After several rounds of plaque purification, it is anticipated that pure phages have been obtained. The subsequent step involves studying and determining the characteristics or properties of the phages. This phase is crucial for assessing whether the phage is suitable and aligned with the intended purpose of isolation. Commonly screened properties when isolating new phages include high lysis activity or any measure of phage virulence activity against the targeted bacteria, absence of lysogenic potential, lack of antimicrobial resistance and virulence genes, host range, and stability in storage as well as in various growth conditions (Fernández et al., 2019; Hyman, 2019; Duyvejonck et al., 2021). The methods typically employed (although contingent on the study's objectives) to evaluate the properties of isolated phages are outlined in Table 2.6 below.

Table 2.6: Methods commonly employed for characterising phages.

Method	Objective	Description of method
Phage morphology analysis	To determine the morphology of the phage, which will help classify it under the appropriate group.	Examination of phage morphology is commonly conducted using a transmission electron microscope (TEM). The sample is prepared by depositing a drop of phage lysate onto a TEM grid and allowing it to fix before staining with a negative stain. Subsequently, the grid is viewed using TEM to observe the morphology of the phage (Ackermann, 2009a).
Host range specificity test	To ascertain the infection profile of the phage, assessing whether they exhibit a narrow or broad host range.	Phages undergo testing against multiple strains or species of bacteria distinct from their parental hosts, employing either well-established laboratory strains or environmentally and clinically isolated strains. The phages are then assessed for their lytic activity against the bacteria using either the spot testing or plaque assay method. (Kutter, 2009; Hyman, 2019).
One-step growth	To determine the growth rate and replication kinetics of the phage.	In a one-step growth experiment, a bacterial culture is initially infected with the phage at the optimal multiplicity of infection (MOI). Samples are taken at various time intervals throughout the infection cycle, and the number of viable phages in the culture is assessed using a plaque assay or other appropriate method. Plotting the phage concentration over time allows for the determination of the burst size and the latent period of the phage. (Abedon and Yin, 2009; Hyman and Abedon, 2009).

Table 2.6 continued

Adsorption curve	To determine the kinetics of phage attachment to host cells and the efficacy of phage-host interactions.	The determination of a phage's adsorption curve involves measuring the rate at which phage particles attach to their host bacterial cells over time. In this method, a known concentration of phages is combined with a bacterial culture. At various intervals, samples are collected, and the unadsorbed phages are separated by centrifugation or filtration. The remaining phages, bound to the bacterial cells, are quantified using a suitable technique such as plaque assay. Plotting the percentage of unadsorbed phages against time allows for the construction of the adsorption curve, typically depicting an initial rapid adsorption phase followed by a slower phase as host receptors become saturated (Hyman and Abedon, 2009).
Phage stability assay	To assess the viability of phages under varying conditions.	Phage stability tests are conducted to assess the persistence and viability of bacteriophages under different environmental conditions. The procedure typically involves subjecting phage samples to various parameters or stressors (temperature, pH, salinity, etc.) and then evaluating their infectivity or survival over time. These tests provide crucial insights into the resilience of phages under different environmental conditions, aiding in the optimization of storage protocols and phage application procedures (Fortier and Moineau, 2009; Duyvejonck et al., 2021.

Table 2.6 continued

Phage genome sequencing	To understand the genetic composition and diversity of phages.	Phage genome sequencing is performed using Whole Genome Sequencing (WGS). From this method, the gene functions of the phage can be determined by comparing them with other available phages. It can also be determined whether the isolated phage is novel or not. Furthermore, the presence or absence of antimicrobial-resistance and virulence genes, as well as lysogens can be determined (Klumpp et al., 2012; Turner et al., 2021).

Before the characterisation process, the phage titre is determined using the plaque assay, although extremely precise measurement is not imperative (Hyman and Abedon, 2009). This step quantifies the concentration of viable phages in a sample or stock, typically expressed as plaque-forming units per millilitre (PFU/mL). Ensuring an adequate phage concentration is crucial to effectively infecting host bacteria, as low titres result in diminished lysis activity (low productivity) (Hyman, 2019).

Additionally, the multiplicity of infection (MOI) of phages is often determined before other characterisation methods, although it is optional in some cases. MOI refers to the ratio of the number of phage particles (PFU) to the number of host bacterial cells (CFU) during an infection experiment (Hyman, 2019). Optimal MOI values are utilised to study individual infection events, such as phage-host interactions (adsorption curve) and phage growth and replication profiles (one-step growth). However, in phage therapy, reliance on MOI is deemed impractical due to fluctuations in bacterial densities between bacterial challenge and phage application (Abedon, 2016). Thus, MOI determination is typically reserved for laboratory-based phage characterisation.

It is essential to recognise that certain characteristics such as phage titre, MOI, one-step growth, and adsorption curve only represent phage properties when conducted in controlled environments. They may not accurately reflect their attributes in *in vivo* applications, such as in patients or animals, as well as in biofilms and other phage applications (Hyman, 2019).

2.11 The Importance of Whole Genome Sequencing (WGS) of Bacteriophages

The sizes of phage genomes vary greatly, ranging from approximately 3,000 nucleotides for single-stranded RNA viruses of *E. coli* (Friedman et al., 2009) to as large as

500 kbp for a *Bacillus megaterium* phage (Hatfull and Hendrix, 2011). The diversity in phage genomes is evident in the unusual events of finding extensive nucleotide sequence similarities in current databases, unless the phage has known closely related phages or prophages infecting the same hosts (Hatfull and Hendrix, 2011). Even phages infecting the same host can exhibit significant genome diversity. Indeed, metagenomic DNA data reveals that viruses represent the largest reservoir of unexplored genetic diversity on the planet, even larger than the rest of chromosomes (Pope et al., 2011; Klumpp et al., 2012; Dávila-Ramos et al., 2019).

Consequently, the molecular characterisation of phages presents unique challenges due to the complex nature and mosaicism of their genomes. Evidently, there has only been a small progress in the number of complete phage genomes in available databases, such as the National Centre for Biotechnological Information (NCBI), in recent years (Klumpp, 2012). Technological advancements, fortunately, have led to the emergence of various sequencing methods that made phage genome sequencing more accessible, and thus offering deeper insights into the genomes. Among these, Next Generation Sequencing (NGS) has become the preferred technology for comprehensive analysis of phage genome properties in a process known as Whole Genome Sequencing (WGS). This multifaceted approach offers valuable insights into various functional aspects of the genome, including the presence of virulence genes, integration or excision genes, and other factors associated with the phage's lysogenic life cycle and transduction potential. Furthermore, it enables the identification of phylogenetic relationships with other phages in existing databases, all at a relatively rapid pace and reasonable costs (Hatfull and Hendrix, 2011; Hyman, 2019).

Ultimately, WGS is acknowledged as a pivotal method for phage characterisation, especially for phages designated for therapeutic purposes, since meticulous genome characterisation is imperative for the regulatory approval of phage applications in healthcare and the food industry (Klumpp et al., 2012).

CHAPTER 3

PREVALENCE OF *Vibrio parahaemolyticus, Vibrio cholera,* AND *Vibrio alginolyticus* IN WHITE-LEG SHRIMP (*Penaeus vannamei*) FARM IN SARAWAK

3.1 Overview

The shrimp industry stands as a significant contributor within Southeast Asia's aquaculture sector, with Malaysia alone boasting an estimated total area of marine shrimp farms spanning between 5,100 and 5,200 hectares as of 2012, and a market value totalling RM1.13 billion in 2020 (DOF, 2012; Gilbert, 2023). Despite its economic potential, the industry grapples with persistent challenges, chief among them being disease outbreaks, such as vibriosis, caused by *Vibrio* species. These heterotrophic bacteria are pervasive in both freshwater and marine environments, thriving as free-living cells or symbionts across a diverse range of habitats. To date, approximately 110 species within the *Vibrio* genus have been identified, many of which exhibit pathogenic traits towards both animals and humans (Farmer and Janda, 2015). *Vibrio* spp. infections in humans typically arise from exposure to contaminated water or the consumption of undercooked seafood carrying the bacteria (Dutta et al., 2021). Of notable concern, *V. parahaemolyticus* stands out as a prevalent culprit behind food poisoning cases in Malaysia (Abdullah Sani et al., 2013). Such infections pose severe health risks, especially to vulnerable demographics like the elderly and children, often leading to complications such as septicaemia and even fatalities (Song et al., 2020).

Some *Vibrio* species, like *V. cholerae*, are notorious for their pathogenic and infectious nature, while others, such as *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus*, are considered opportunistic pathogens (de Souza Valente and Wan, 2021; Haifa-Haryani et al., 2022). Shrimp afflicted with *Vibrio* spp. often display clinical symptoms such

as lethargy, reduced moulting, abnormal swimming patterns, decreased food consumption, and potential anorexia, evidenced by empty guts and an off-white body coloration (de Souza Valente and Wan, 2021). Notably, *V. parahaemolyticus*, known to induce acute hepatopancreatic necrosis disease (AHPND), leaves affected shrimp with empty stomachs and midguts, alongside a hepatopancreas displaying a pallid to white appearance (Hong To et al., 2020). Conversely, *V. alginolyticus* instigates "flaming bacterial disease," causing infected shrimp to emit a luminescent glow (Alifia et al., 2021). Additionally, select strains of *V. cholerae* have been linked to *Vibrio*-induced bacteraemia, manifesting in diminished haemocyte count and lethargy among afflicted crustaceans (de Souza Valente and Wan, 2021). Severe vibriosis outbreaks in shrimp can even lead to mortality.

While *Vibrio* spp. are ubiquitous in the environment, their proliferation remains manageable at low levels. However, shifts in environmental factors like temperature and salinity can precipitate rapid bacterial growth, instigating vibriosis outbreaks capable of decimating entire shrimp populations in affected ponds, and resulting in substantial production losses (Mastan and Begum, 2016; Semenza et al., 2017). Moreover, contamination of seafood products with elevated *Vibrio* spp. concentrations poses a significant threat to consumer health. Hence, early detection and quantification of *Vibrio* spp. are imperative for monitoring aquaculture outbreak dynamics, assessing potential public health hazards, and mitigating economic repercussions.

Due to the pervasive threat posed by *Vibrio* spp. infections, early detection and vigilant monitoring of these bacteria are imperative to curb disease transmission. Among the methods commonly employed for swift bacterial detection in environmental settings is the Most Probable Number (MPN) method. This statistical approach offers an estimation of

microbial loads within a sample (Rubini, 2023). Utilising the principle of statistical probability, the MPN method estimates the likelihood that a specified number of bacteria will yield particular outcomes when subjected to testing across a series of tubes or wells containing growth media (de Man, 1983). The standard procedure for MPN assessment involves the inoculation of a succession of tubes or wells with diluted portions of the bacterial suspension from the original sample. Following an incubation period, typically overnight, the presence or absence of bacterial growth, indicated by media turbidity, is assessed. The obtained results are then cross-referenced with MPN statistical tables, providing an estimated range of bacterial loads with the highest probability of yielding positive outcomes. Widely applied in various fields, including the evaluation of bacterial quantities in water, milk, seafood, as well as environmental and clinical samples (Moussa et al., 2020; Aryal, 2023; Rubini et al., 2023), the MPN method is recognized as a dependable approach for approximating bacterial concentrations, although it may not yield an exact number of the total bacterial population (FDA, 2000).

Compared to other enumeration methods, such as viable plate counts, MPN-mPCR allows for the rapid processing of many samples at the same time, which helps to reduce the processing time (Russo et al., 2014). This technique also helps to reduce the amount of Petri dishes and costly selective media used for the enumeration process Moreover, plating on thiocitrate bile salt (TCBS) agar will not accurately differentiate *V. cholerae* from *V. alginolyticus,* and *V. parahaemolytocus* from other non-sucrose fermenting *Vibrio* species. Therefore, presumptive isolates will need to be tested using biochemical tests which can be energy- and time-consuming (Noorlis, 2011). The MPN-mPCR technique also provides a more sensitive and rapid detection and enumeration than other plating methods. Without the

need to isolate pure isolates, the possibility of getting false negatives through the MPN-mPCR method is lower than the MPN-plate method (Miwa et al., 2013).

Currently, there is a scarcity of data on the prevalence of *Vibrio* spp. in aquaculture farms in Sarawak, particularly within shrimp farms. Similar to other states in Malaysia, shrimp farmers in Sarawak confront significant challenges, with disease outbreaks posing a major threat. However, the absence of official data presents a hurdle, as most incidents are communicated verbally and not formally reported (Baker-Austin et al., 2020). With Sarawak aiming to elevate shrimp production to RM1 billion in export value by 2030, these disease challenges could significantly impede progress (Ling, 2020). Furthermore, the lack of official data on disease incidents and *Vibrio* spp. prevalence in Sarawak, attributed to the absence of surveillance systems, under-reporting, or incidents going unreported, obstructs a comprehensive understanding of the extent of these challenges and hampers the formulation of effective strategies for disease management and prevention in Sarawak's shrimp farming industry (Baker-Austin et al., 2018).

In Malaysia, including Sarawak, White-leg shrimp (*Penaeus vannamei*) dominates the shrimp farming industry, accounting for 72% of production (Chowdhury et al., 2013). Due to its prominence, this study focuses on a farm specializing in the cultivation of this species. The objective of this study is to identify and isolate *Vibrio* spp. from various sources including water, sediment, shrimp, and effluent samples gathered from a White-leg shrimp farm located in Kuching, Sarawak. Specifically, the research aims to determine the apparent prevalence of three *Vibrio* species: *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*, within samples extracted from two ponds situated in the coastal shrimp farm.

3.2 Materials and Methods

3.2.1 Sample Collection

Samples were collected from Persatuan Nelayan Kawasan (PNK) Satang Biru, Lembaga Kemajuan Ikan Malaysia (LKIM), Telaga Air, Sarawak (0"N 110°11'51., 1°40'59, 1). Four sample types (water, sediment, shrimp, and effluent) were collected from two shrimp ponds (P1 and P2) every two weeks, commencing from the stocking period until the harvesting period, spanning approximately three months. Both earthen shrimp ponds encompassed a surface area of one hectare with a depth of one meter. Sampling was conducted between August and October 2020 for P1 and October to December 2020 for P2, with each sampling event occurring between nine to ten o'clock in the morning. The entire sampling period encompassed the warm season transitioning into the onset of the rainy season.

The sampling method was derived from the methodology outlined by Kaysner et al. (1990) with adaptations. Forty-eight (n=48) samples, consisting of twelve each of water (n=12), sediment (n=12), shrimp (n=12), and effluent (n=12), were acquired throughout the sampling duration. During each sampling event, each sample type was collected from three distinct locations (sampling points) to constitute triplicates. Surface water and effluent samples, each amounting to at least 1 L, were collected in sterile polypropylene bottles at each sampling point. Shrimp were captured using a net and transferred to sterile plastic bags. The shrimp were captured starting from the post-larvae stage, progressing through the juvenile and sub-adult stages until reaching the harvestable adult stage by the conclusion of the sampling period. Meanwhile, sediment was gathered using an ethanol-sterilized polyvinyl chloride (PVC) pipe and stored in sterile 50 mL microcentrifuge tubes. All

samples were promptly transported to the laboratory in an ice box and processed within 24 hours of collection (Kaysner et al., 1990).

In addition to sample collection, the physical and chemical parameters (temperature, salinity, and pH) of the pond water were measured and recorded. These parameters were selected due to their common association with factors influencing the distribution of *Vibrio* species (de Souza Valente and Wan, 2021). Water temperature and pH were measured on-site during each sampling event using the Fisherbrand™ accumet™ AP125 Portable pH/Ion/mV/Temperature Meter Kit (Thermo Fischer Scientific, U.S.). Water salinity was assessed using a salinity refractometer (Hisamatsu, Japan). Measurements were conducted in triplicate at three distinct locations within the ponds and expressed as mean values.

3.2.2 Enrichment and MPN Method

Before enrichment, the triplicate water and effluent samples were combined in equal volumes in sterile microcentrifuge tubes. Similarly, the triplicate sediment and shrimp samples were homogenized using a sterilized conventional blender. Subsequently, one gram (for solid samples) or 1 mL (for liquid samples) of each sample was thoroughly mixed with 9 mL of alkaline peptone water (APW) (Merck, Darmstadt, Germany) and pre-enriched through overnight incubation at 37 °C. This step is necessary to increase the sensitivity of the MPN method. This ensures that even low levels of microorganisms can be detected, which is crucial for accurate enumeration, especially in samples where the microbial load is expected to be low (Jacobson et al., 2017).

The resulting suspension was then serially diluted 10-fold from 10^{-1} to 10^{-5} in APW. Utilising the three-tube most probable number (MPN) method outlined in the FDA's Bacteriological Analytical Manual (BAM), 1 mL of each dilution was transferred into

triplicate MPN tubes containing 10 mL of APW broth. Subsequently, all tubes were incubated aerobically at 37 °C for 24 hours. Prior to genomic DNA extraction from the MPN tubes following incubation, the turbidity of the tubes was observed (FDA, 2000). Sterile APW broth served as the blank control throughout the experiment.

3.2.3 Bacterial DNA Extraction

Bacterial DNA extraction from the turbid MPN tubes was conducted using the boiled-cell method with slight adjustments (Queipo-Ortuño, 2008). One milliliter of each culture was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. Subsequently, the supernatant was carefully removed, and the resulting pellet was resuspended in 100 µL of sterile distilled water. The suspension was then subjected to boiling for 10 minutes followed by rapid cooling at -21 °C for 5 minutes. The DNA extract was subjected to centrifugation again at 10,000 rpm for 10 minutes and subsequently stored at -21 °C for further analysis.

3.2.4 Multiplex Polymerase Chain Reaction (mPCR)

Multiplex PCR was conducted using three sets of primers that specifically targeted *V. parahaemolyticus, V. cholerae*, and *V. alginolyticus*, respectively, as designed by Kim et al. (2015). The primers used were as shown in Table 3.1 below. Each 15 µL of the PCR mixture consisted of the following components: 7.5 µL exTEN 2X PCR Master Mix (1st BASE), 0.6 µL of each primer with a concentration of 10 µM, 2.0 µL DNA template, and 1.9 µL sterile distilled water.

Table 3.1: Sequences, sources, and expected amplicon sizes of primer pairs used to target each *Vibrio* spp. in this study (Kim et al., 2015).

Target species	Target gene[a]	Primer name	Primer sequence (5'→ 3')	Amplicon size (bp)
Vibrio parahaemolyticus	NC_004605.1 (c1155272-1154856)	VP 1155272 F	AGCTT ATTGG CGGTT TCTGT CGG	297
		VP 1155272 R	CKCAA GACCA AGAAA AGCCG TC	
Vibrio cholerae	NC_002506.1 (c634002-633547)	VC C634002 F	CAAGC TCCGC ATGTC CAGAA GC	154
		VC C634002 R	GGGGC GTGAC GCGAA TGATT	
Vibrio alginolyticus	NZ_CH902589.1 (1198230–1198616)	VA 1198239 F	ACGGC ATTGG AAATT GCGAC TG	199
		VA 1198239 R	TACCC GTCTC ACGAG CCCAA G	

[a] Reference sequence number and gene position in Genbank (NCBI) database.

PCR amplification was performed using the T100™ Thermal Cycler (Bio-Rad, USA) following the specified conditions: initial denaturation at 94 °C for 5 minutes; 30 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds; and a final extension step at 72 °C for 10 minutes (Kim et al., 2015). Subsequently, the PCR products were subjected to electrophoresis on a 1.5% (w/v) agarose gel at 80 V for one hour, with 1X Tris-Borate-EDTA (TBE) as the buffer.

The GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific, USA) was used as the molecular weight marker. A mixture of *V. parahaemolyticus* ATCC 27969, *V. cholerae* KCDC 13589, and *V. alginolyticus* ATCC 17749 served as the positive control, while sterile APW processed concurrently with the samples was utilised as the negative control to substitute the DNA template.

Following multiplex PCR analysis, MPN values were determined based on the MPN table for three tubes and expressed as MPN/mL for water and effluent samples and MPN/g for sediment and shrimp samples (de Man, 1983).

3.2.5 Statistical Analysis

Data analysis was conducted with GraphPad Prism (version 9.5.1, Dotmatics, San Diego, CA). The prevalence of *Vibrio* spp. was calculated along with a 95% confidence interval (CI). Fisher's exact test was employed to compare the overall prevalence of the three *Vibrio* spp., with statistical significance set at $P < 0.05$. To investigate the association between environmental and chemical parameters (i.e., temperature, pH, and salinity) and the load of each *Vibrio* spp., Spearman's rank correlation coefficient (rs) was computed using PAST 4.13 software (University of Oslo, Norway).

3.3 Results

3.3.1 Prevalence Percentages of *Vibrio* species

The anticipated sizes for the PCR products of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* are 297 bp, 154 bp, and 199 bp, respectively. Agarose gel electrophoresis (AGE) of the PCR products revealed bands consistent with the expected sizes (Figure 3.1). Through optimization steps, an annealing temperature of 60 °C proved to be optimal for specific amplifications using the three primer sets. Conversely, PCR products generated at

annealing temperatures below 58 °C failed to produce any observable bands during AGE. From this result, it was found that annealing temperature of 60°C is ideal for these three primers sets because it balances their binding specificity and efficiency to the complementary DNA sequences, leading to successful amplification. Temperatures below 58°C are insufficient to achieve effective primer binding, resulting in no observable PCR products.

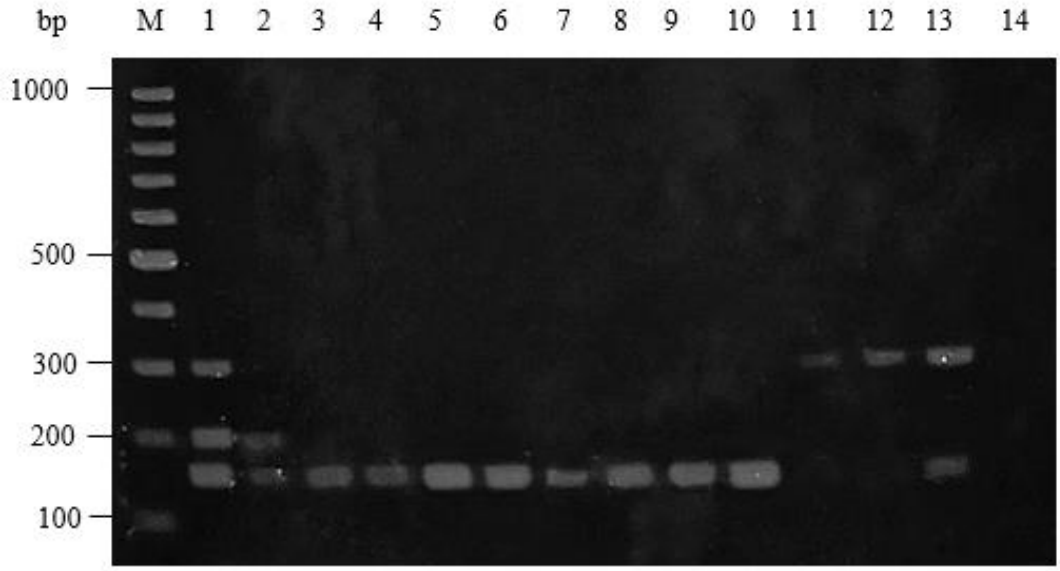


Figure 3.1: Gel electrophoresis of multiplex PCR of *Vibrio* spp. through amplification of random sequences for identification of *V. parahaemolyticus* (297 bp), *V. alginolyticus* (199 bp), and *V. cholerae* (154 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (cocktail of *V. parahaemolyticus* ATCC 27969, *Vibrio cholera* KCDC 13589, and *V. alginolyticus* ATCC 17749); Lane 2 – 13: representative positive samples; Lane 14: negative control.

Based on the presence of positive PCR bands, the prevalence of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* in four types of samples collected from the shrimp farm, spanning from the stocking period to the harvesting period, was summarised in Table 3.2. Throughout the sampling period in the shrimp farm, a total of 48 samples were collected from two ponds across six samplings. Upon examination of all samples, the overall

prevalence of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* was determined to be 97.92% (n=47, 95% CI: 89.10 to 99.89%), 47.92% (n=23, 95% CI: 34.47 to 61.67%), and 25.0% (n=12, 95% CI: 14.92 to 38.78%), respectively. Notably, among the three *Vibrio* spp., *V. parahaemolyticus* exhibited the highest prevalence, whereas *V. alginolyticus* demonstrated the lowest prevalence ($P<0.05$).

Table 3.2: Prevalence of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* in water, sediment, shrimp, and effluent samples taken from stocking to harvesting period based on MPN-mPCR method.

Sample	**Prevalence (%)**			
	n[a]	***V. parahaemolyticus***	***V. cholerae***	***V. alginolyticus***
Water	12	100.0[b] (75.75 – 100)[c]	58.33 (31.95 – 80.67)	0.0 (0 – 24.25)
Sediment	12	100.0 (75.75 - 100)	33.33 (13.81 – 60.94)	0.0 (0 – 24.25)
Shrimp	12	91.67 (64.61 – 99.57)	16.67 (2.96 – 44.80)	66.67 (39.06 – 86.19)
Effluent	12	100.0 (75.75 - 100)	83.33 (55.20 – 97.04)	33.33 (13.81 – 60.94)
TOTAL	48	97.92 (89.10 – 99.89)	47.92 (34.47 – 61.67)	25.0 (14.92 – 38.78)

[a] Number of samples.
[b] Percentage of positive samples.
[c] 95% confidence interval (%)

In comparing samples, *V. parahaemolyticus* emerged as the most prevalent in all four sample types. Within water samples, the prevalence of *V. parahaemolyticus* and *V. cholerae* stood at 100% (n=12, 95% CI: 75.75 to 100%) and 58.33% (n=7, 95% CI: 31.95 to 80.67%),

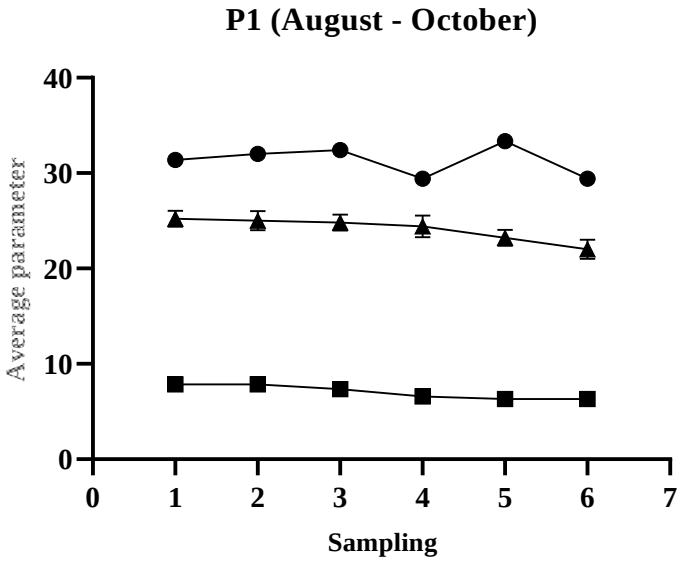
respectively, while *V. alginolyticus* was undetected. Among sediment samples, *V. parahaemolyticus* again dominated, with 100% (n=12) positive detection, followed by *V. cholerae* at 33.33% (n=4, 95% CI: 13.81 to 60.94%). Conversely, *V. alginolyticus* remained undetected in sediment samples. In shrimp samples, *V. alginolyticus* exhibited the second highest detection rate after *V. parahaemolyticus*, with a prevalence of 66.67% (n=8, 95% CI: 39.06 to 86.19%). In effluent samples, *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* were all positively detected, with prevalences of 100% (n=12, 95% CI: 75.75 to 100%), 83.33% (n=10, 95% CI: 55.2 to 97.04%), and 33.33% (n=4, 95% CI: 6.6 to 60.0%), respectively.

Statistical analysis revealed significant differences in the overall prevalence among the three *Vibrio* spp. ($P < 0.05$). However, the prevalence of *V. parahaemolyticus* between sample types was not significantly different ($P > 0.05$). The prevalence of *V. cholerae* significantly differs between sediment and effluent ($P < 0.05$), as well as between shrimp and effluent samples ($P < 0.05$). Meanwhile, the prevalence of *V. alginolyticus* significantly differed between shrimp and water samples ($P < 0.05$) and between sediment and shrimp samples ($P < 0.05$).

3.3.2 Physical and Chemical Parameters

Three physical and chemical parameters of the water samples were measured and recorded throughout the sampling period. Based on the graphs below (Figure 3.2), the water temperatures for both P1 and P2 remained in the range of 29 °C - 34 °C from stocking to harvesting. The salinity in P1 was in the range of 22 – 26 ppt, while the salinity in P2 was in a slightly lower range of 20 – 25 ppt. Meanwhile, the pH levels for water in both ponds ranged in the acidic to neutral pH range (pH 5.9 – pH 7.9).

A



B

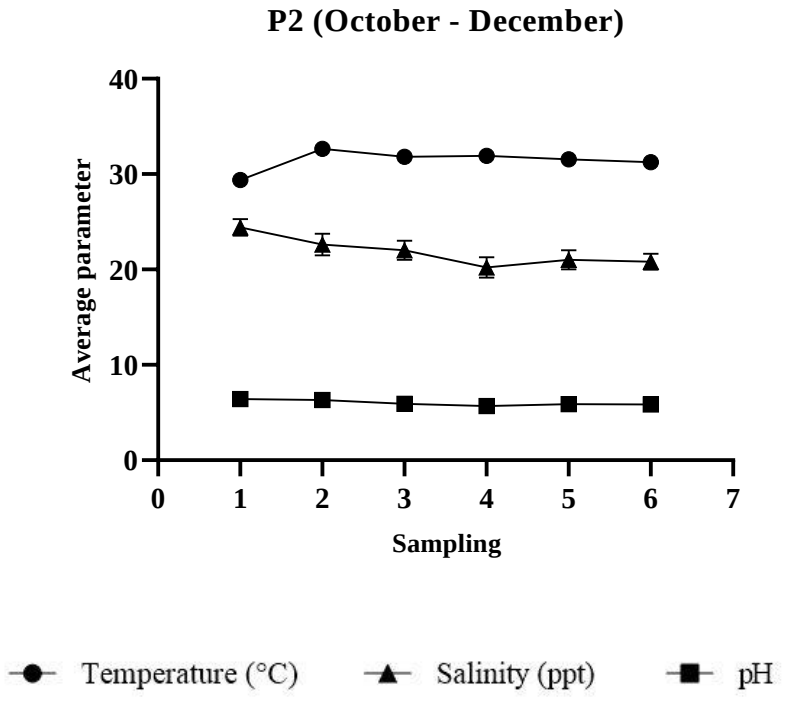


Figure 3.2: Average temperature, salinity, and pH of pond water in (A) P1 and (B) P2 throughout one shrimp production cycle.

3.3.3 Most Probable Number (MPN) of *Vibrio* spp. in P1

Based on the MPN-mPCR method, the highest concentration of *V. parahaemolyticus* in water, sediment, and shrimp samples at P1 from the stocking (Sampling 1) to the harvesting (Sampling 6) period exceeded 1,100 MPN/mL (Table 3.3). Although a slight decrease in the detection of *V. parahaemolyticus* was observed in these samples after the initial sampling, the concentration eventually rose to more than 1,100 MPN/mL by the final sampling. In effluent samples, the highest concentration of *V. parahaemolyticus* was detected during Sampling 5, reaching a maximum concentration of 110 MPN/mL. Comparatively, the concentration of *V. parahaemolyticus* in effluent samples at P1 was the lowest among the four sample types.

Meanwhile, the highest and lowest concentrations of *V. cholerae* in water samples were >1,100 MPN/mL and <3 MPN/mL, respectively. The concentration of *V. cholerae* increased to >1,100 MPN/mL in the second sampling but then declined, remaining low at <3 MPN/mL until harvesting. In sediment and shrimp samples, the highest detection was observed in Sampling 2 at maximum concentration of 38 MPN/g and 180 MPN/mL, respectively. However, the concentrations of *V. cholerae* in both samples decreased in subsequent weeks, remaining at <3 MPN/g until harvesting. Unlike *V. parahaemolyticus*, the concentration of *V. cholerae* in effluent samples was among the highest among the four sample types, reaching >1,100 MPN/mL. Although the concentration decreased in Sampling 3 and 4, it increased again until harvesting, reaching a maximum value of 1,000 MPN/mL.

In both water and sediment samples, the concentration of *V. alginolyticus* detected was <3 MPN/mL and <3 MPN/g, respectively, and remained low throughout the sampling period. In shrimp samples, *V. alginolyticus* was detected in Sampling 1 with the highest maximum concentration of 4,100 MPN/g. However, the concentration decreased to <3

MPN/g in Sampling 5 and 6. Meanwhile, in effluent samples, the concentration of *V. alginolyticus* increased in Sampling 5 until it reached a maximum concentration of 94 MPN/mL during harvesting.

From Figure 3.3, it was noted that the concentration of *V. parahaemolyticus* in water, sediment, and shrimp samples increased to its highest level towards harvesting, surpassing that of the other two *Vibrio* spp. Only in effluent samples did the concentration of *V. parahaemolyticus* become the lowest among *V. cholerae* and *V. alginolyticus* by the end of the sampling period. The concentration of *V. cholerae* in water, sediment, and shrimp samples steadily decreased and remained low towards the end of the sampling period, unlike *V. parahaemolyticus*. However, in effluent samples, the concentration of *V. cholerae* increased until it surpassed *V. parahaemolyticus* and *V. alginolyticus*. Concurrently, the concentration of *V. alginolyticus* in water and sediment samples remained the lowest among the three *Vibrio* spp. from stocking to harvesting. In shrimp samples, *V. alginolyticus* initially had the highest detected concentration but steadily decreased towards harvesting to become one of the lowest *Vibrio* spp. detected. Conversely, the concentration of *V. alginolyticus* in effluent samples increased towards harvesting, becoming the second highest *Vibrio* spp. detected in the samples.

Table 3.3: Microbial load of *Vibrio* spp. (MPN/g or MPN/mL) in samples collected from P1 throughout one production cycle.

Sampling	Sample	*V. parahaemolyticus*			*V. cholerae*			*V. alginolyticus*		
		Min[a]	Med[b]	Max[c]	Min	Med	Max	Min	Med	Max
1	**Water**	420	>1,100	-	45	160	420	-	<3	9.5
	Sediment	210	1,100	4,100	3.6	11	38	-	<3	9.5
	Shrimp	-	<3	9.5	-	<3	9.5	180	1,100	4,100
	Effluent	8.7	36	9.4	4.5	16	42	-	<3	9.5
2	**Water**	18	93	420	420	>1,100	-	-	<3	9.5
	Sediment	40	210	430	3.6	11	38	-	<3	9.5
	Shrimp	1.3	7.4	20	9	43	180	3.7	15	42
	Effluent	1.3	7.2	42	420	>1,100	-	-	<3	9.5
3	**Water**	180	1,100	4,100	1.3	7.2	18	-	<3	9.5
	Sediment	4.5	16	42	-	<3	9.5	-	<3	9.5
	Shrimp	8.7	36	94	3.6	14	42	1.3	7.2	18
	Effluent	4.5	15	42	1.7	3.6	18	-	<3	9.5

Table 3.3 continued

4	**Water**	180	1,100	4,100	-	<3	9.5	-	<3	9.5
	Sediment	4.5	15	42	-	<3	9.5	-	<3	9.5
	Shrimp	3.6	11	38	-	<3	9.5	0.17	3.6	18
	Effluent	8.7	35	94	-	<3	9.5	-	<3	9.5
5	**Water**	420	>1,100	-	0.17	3.6	18	-	<3	9.5
	Sediment	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Effluent	8.7	38	110	40	210	430	0.15	3	11
6	**Water**	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Sediment	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Effluent	4.5	15	42	90	290	1,000	8.7	35	94

[a] Minimum MPN/g or MPN/mL value; [b] Median MPN/g or MPN/mL value; [c] Maximum MPN/g or MPN/mL value

(- = No minimum or maximum value)

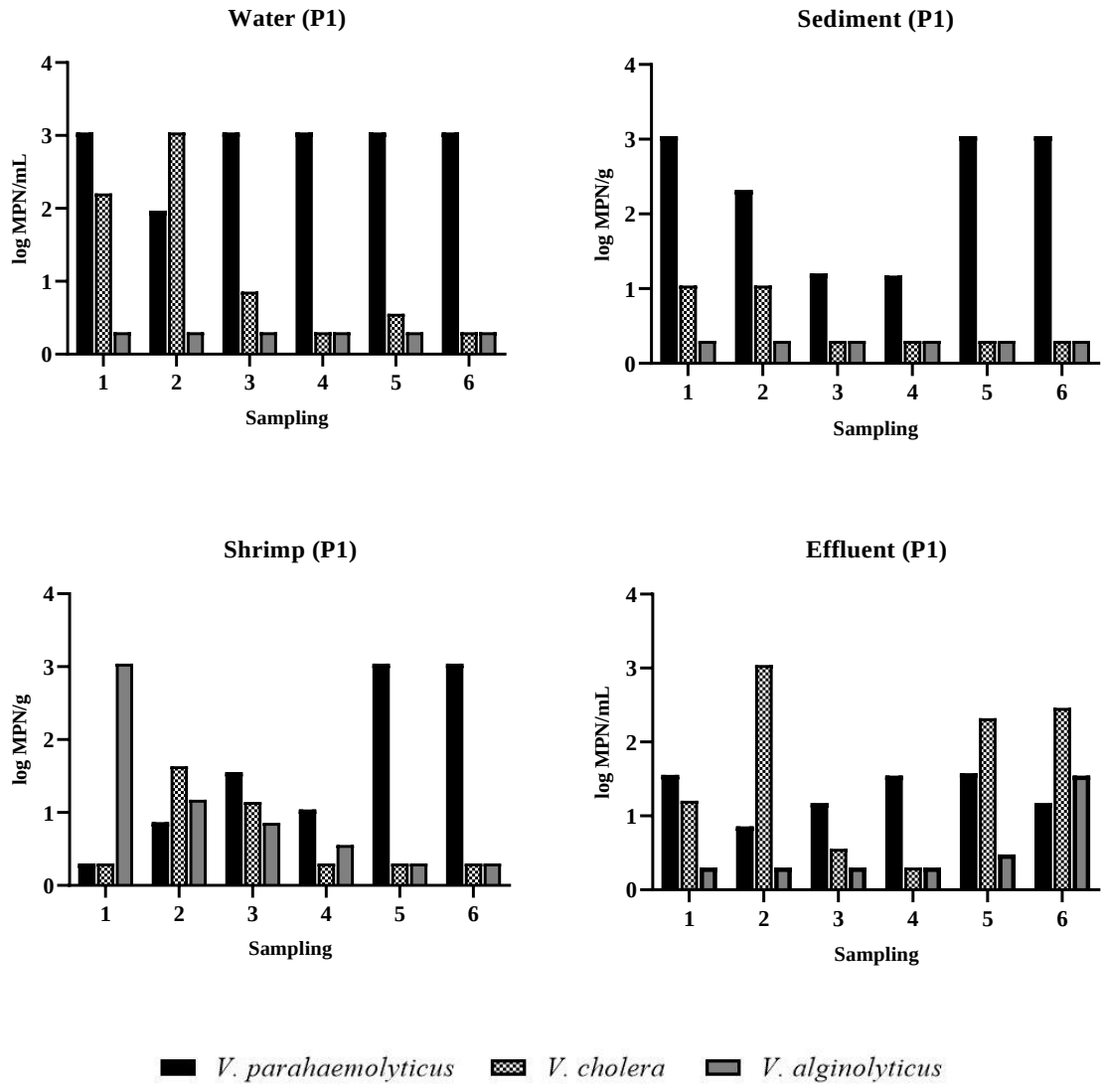


Figure 3.3: Concentration of *Vibrio* spp. in water, sediment, shrimp, and effluent samples collected from P1. The concentrations are expressed in log median of MPN value. For values with no minimum or maximum limit, the log median is derived from the lowest or highest estimated number as shown in Table 3.3.

3.3.4 Most Probable Number (MPN) of *Vibrio* spp. in P2

Similar to P1, the highest concentration of *V. parahaemolyticus* in water samples collected from P2 was >1,100 MPN/mL (Table 3.4). The concentration remained relatively constant throughout the sampling period, from stocking to harvesting. In sediment samples, the highest concentration of *V. parahaemolyticus* detected was as high as >1,100 MPN/g. However, a decrease was observed in Sampling 3, with a maximum value of 94 MPN/g of *V. parahaemolyticus* detected. The concentration increased again in the following weeks and remained constant at >1,100 MPN/g until harvesting. Similar to shrimp samples from P1, the concentration of *V. parahaemolyticus* detected in shrimp samples from P2 started low at maximum value of 42 MPN/g but eventually increased to >1,100 MPN/g towards the harvesting. Meanwhile, the maximum concentration of *V. parahaemolyticus* in the effluent samples increased from 420 MPN/mL in Sampling 1 to >1,100 MPN/mL towards Sampling 6.

The highest concentration of *V. cholerae* in water samples was >1,100 MPN/mL, which is on Sampling 2 as seen in Table 3.4. However, the concentration decreased until <3 MPN/mL towards harvesting. Meanwhile, the highest maximum concentration of *V. cholerae* in sediment and shrimp samples were 18 MPN/mL and 9.5 MPN/mL, respectively. Similar to water samples, the concentration of *V. cholerae* in both samples then decreased to <3 MPN/mL or MPN/g towards harvesting. On the other hand, the concentration of *V. cholerae* in the effluent samples increased to >1,100 MPN/mL in Sampling 3 and decreased to a maximum concentration of 18 MPN/mL during harvesting.

The concentrations of *V. alginolyticus* in both water and sediment samples remained constant at <3 MPN/mL or MPN/g from stocking to harvesting (Table 3.4). Meanwhile, in shrimp samples, *V. alginolyticus* was detected at a maximum concentration of 42 MPN/g in

Sampling 1 but subsequently decreased and remained low at <3 MPN/g until harvesting. Similarly, the concentration of *V. alginolyticus* in effluent samples in Sampling 1 was relatively high at a maximum concentration of 1,000 MPN/mL, even higher than both *V. parahaemolyticus* and *V. cholerae*. However, the concentration decreased and remained low at <3 MPN/mL until the last sampling.

From the graphs (Figure 3.4), it was observed that the concentration of *V. parahaemolyticus* in all four sample types increased to MPN value of >1,100 MPN/mL or >1,100 MPN/g towards harvesting. In addition, *V. parahaemolyticus* had the highest detection compared to the other two *Vibrio* spp. being studied. Meanwhile, the concentration of *V. cholerae* in water, sediment, and shrimp samples remained relatively low compared to *V. parahaemolyticus*. Although increments were observed in the middle, the concentration decreased towards the end of sampling. In effluent samples, the concentration of *V. cholerae* started high, even higher than the concentrations of *V. parahaemolyticus* and *V. alginolyticus*. However, the concentration decreased towards harvesting. Additionally, *V. alginolyticus* has the lowest overall concentration in all four samples towards harvesting. Although the concentrations started high in shrimp and effluent samples, unlike *V. parahaemolyticus, V. alginolyticus* loads decreased towards the end of the production cycle.

Table 3.4: Microbial load of *Vibrio* spp. (MPN/g or MPN/mL) in samples collected from P2 throughout one production cycle.

Sampling	Sample	*V. parahaemolyticus*			*V. cholerae*			*V. alginolyticus*		
		Min[a]	Med[b]	Max[c]	Min	Med	Max	Min	Med	Max
1	**Water**	420	>1 100	-	0.15	3	9.6	-	<3	9.5
	Sediment	8.7	29	94	-	<3	9.5	-	<3	9.5
	Shrimp	3.7	15	42	-	<3	9.5	3.6	14	42
	Effluent	37	150	420	180	1 100	4 100	90	290	1 000
2	**Water**	420	>1 100	-	-	<3	9.5	-	<3	9.5
	Sediment	420	>1 100	-	-	<3	9.5	-	<3	9.5
	Shrimp	8.7	35	94	-	<3	9.5	-	<3	9.5
	Effluent	420	>1 100	-	1.3	7.4	20	3.7	15	42
3	**Water**	180	1 100	4 100	0.15	<3	9.6	-	<3	9.5
	Sediment	8.7	35	94	1.2	6.1	18	-	<3	9.5
	Shrimp	4.5	15	42	-	<3	9.5	-	<3	9.5
	Effluent	180	1 100	4 100	420	>1 100	-	-	<3	9.5

Table 3.4 continued

4	**Water**	180	1 100	4 100	0.15	<3	11	-	<3	9.5
	Sediment	420	>1 100	-	1.3	7.2	18	-	<3	9.5
	Shrimp	37	150	420	-	<3	9.5	-	<3	9.5
	Effluent	420	>1 100	-	8.7	36	94	-	<3	9.5
5	**Water**	180	1 100	4 100	-	<3	9.5	-	<3	9.5
	Sediment	420	>1 100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1 100	-	-	<3	9.5	-	<3	9.5
	Effluent	180	1 100	4 100	3.6	14	42	-	<3	9.5
6	**Water**	180	1 100	4 100	-	<3	9.5	-	<3	9.5
	Sediment	420	>1 100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1 100	-	-	<3	9.5	-	<3	9.5
	Effluent	420	>1 100	-	1.3	7.2	18	-	<3	9.5

[a] Minimum MPN/g or MPN/mL value; [b] Median MPN/g or MPN/mL value; [c] Maximum MPN/g or MPN/mL value

(- = No minimum or maximum value)

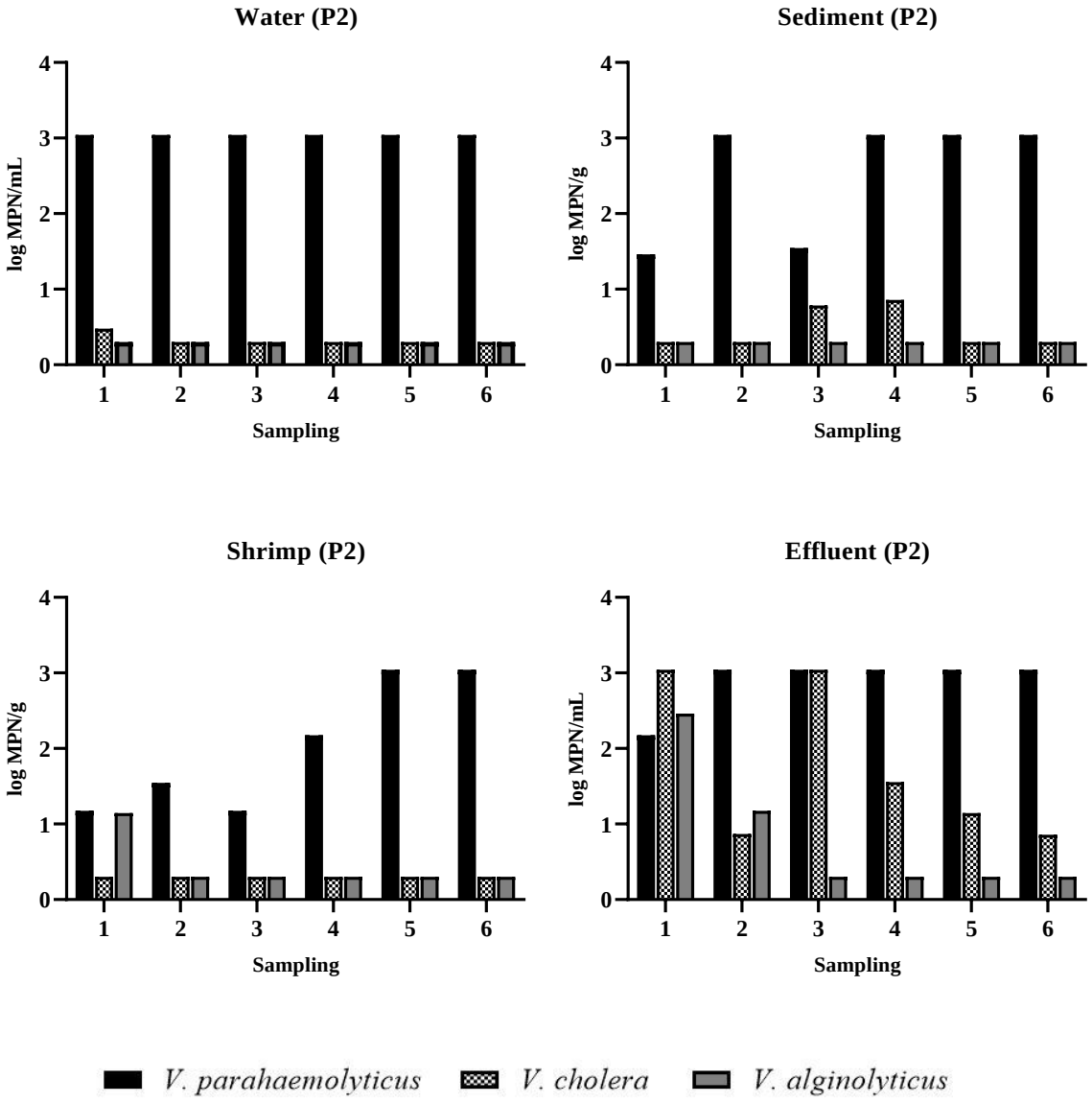


Figure 3.4: Concentration of *Vibrio* spp. in water, sediment, shrimp, and effluent samples collected from P2. The concentrations are expressed in log median of MPN value. For values with no minimum or maximum limit, the log median is derived from the lowest or highest estimated number as shown in Table 3.4.

3.3.5 Spearman's Correlation Analysis

Spearman's statistical analysis was employed to assess the correlation between parameters and each *Vibrio* species. In P1, the temperature exhibited a weak negative correlation with *V. parahaemolyticus* population, a positive correlation with *V. cholerae* population, and no correlation with *V. alginolyticus* population. The pH of pond water in P1 was slightly negatively correlated with *V. parahaemolyticus* population, positively correlated with *V. cholerae*, and not correlated with *V. alginolyticus* population. Additionally, water salinity displayed a weak negative correlation with *V. parahaemolyticus* population, a significant positive correlation with *V. cholerae* population, and no correlation with *V. alginolyticus* population in P1 (Figure 3.5A).

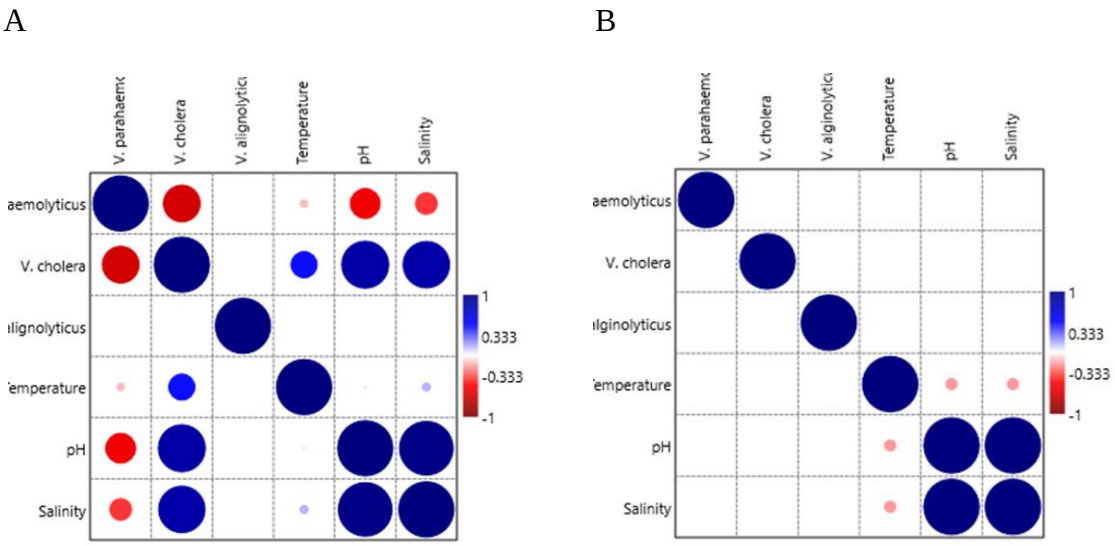


Figure 3.5: Spearman's rs analysis matrix showing the correlation between the three environmental and chemical parameters with each *Vibrio* spp. in (A) Pond 1 and (B) Pond 2. A rs of +1 (blue) represents a perfect association of ranks, a rs of zero (blank) shows no association between ranks and a rs of -1 (red) indicates a perfect negative association of ranks. The closer rs is to zero, the weaker the association between the ranks.

In contrast, temperature did not exhibit any significant correlation with the concentrations of the three *Vibrio* spp. studied. Furthermore, there was no apparent relationship between pH values and the presence of all three *Vibrio* spp. in P2. Similarly, no discernible correlation emerged between water salinity and the occurrence of these *Vibrio* spp. in P2. (Figure 3.6B).

3.4 Discussion

Based on the finding of this study, *V. parahaemolyticus* is the most prevalent among the three *Vibrio* spp., with the highest load in all four samples exceeding 10^3 MPN/g or MPN/mL. Moreover, while *V. cholerae* and *V. alginolyticus* concentration decreased in most of the samples towards the harvesting period, *V. parahaemolyticus*, on the other hand, exhibited increments in all samples towards harvesting. The dominance of *V. parahaemolyticus* over *V. cholerae* and *V. alginolyticus* in all four samples may indicate that *V. parahaemolyticus* is illustrating the "pathogen advantage theory". According to this theory, one pathogen species may have a competitive advantage over other species in their natural ecosystem, commonly brought by temperature rise. This situation will eventually lead to the overabundance of that domineering species (Colwell, 2004; Thornstenson and Ullrich, 2021). In this case, *V. parahaemolyticus* was able to exploit available resources better than *V. cholerae* and *V. alginolyticus*, resulting in the positive growth of *V. parahaemolyticus* and reduced growth of *V. cholerae* and *V. alginolyticus* (McLaren and Callahan, 2020).

In shrimp samples, the overall load of the three *Vibrio* spp. was low during the stocking period compared to the other samples. However, as the production cycle progressed, exposure to environmental factors and contamination with *Vibrio* spp. that are

already present in nature caused the counts to increase towards harvesting (Kim and Lee, 2017). *V. cholerae*, while not considered a serious pathogen to shrimps, with only a few studies reporting their ability to cause vibriosis in shrimps, can still cause infection in humans through consumption of contaminated products (Gopal et al., 2005; Haldar et al., 2007; Cao et al., 2015). It has been reported that about 42% of *V. cholerae* infections in humans are caused by consuming seafood products (Chen et al., 2022). Meanwhile, *V. alginolyticus* is an opportunistic pathogen, indicating that once contamination in the host crosses a certain threshold, vibriosis can occur, and mortality is a possible outcome (Brown et al., 2012). The minimum bacterial load of *Vibrio* spp. that can cause vibriosis varies depending on several factors, including the specific *Vibrio* spp., the health and immune status of the host, and the route of exposure. Additionally, individual susceptibility to vibriosis can differ. In our study, the incidence of *V. alginolyticus* contamination in all samples, particularly in shrimp samples when harvested, is still low, making it less likely to cause vibriosis.

Despite the low counts of both *V. cholerae* and *V. alginolyticus*, the concentration of *V. parahaemolyticus* in shrimp samples during the harvesting is concerningly high. While it is impossible to get shrimps that are entirely free of *Vibrio* spp. contamination due to their ubiquitousness in the marine environment, there are standard acceptable contamination levels set by every country for export purposes. For example, Crustacean seafood products for export to the United States must have less than 10^4 MPN/g of *V. parahaemolyticus* contamination. In Australia and New Zealand, the contamination level of *V. parahaemolyticus* in their imported crustacean products must be less than 10^3 MPN/g (Lokkhumlue and Prakitcaiwattana, 2014). In raw or improperly cooked seafood products,

pathogenic bacteria such as *Vibrio* spp. can become highly accumulated, rendering the seafood unsafe for consumption (Harrison et al., 2022).

One of the factors that may contribute to the increase in the microbial load of *Vibrio* spp. in the ponds is the high temperature, which is typically prevalent between August and December. Malaysia has a tropical climate with a fairly slight variation in the average monthly temperature. With a mean annual temperature of 26.4 °C, Malaysia experiences high temperatures all year round (World Bank Climate Change Knowledge Portal, n.d.). The relatively high temperature in the shrimp farm can explain the high occurrence of *Vibrio* spp. in both P1 and P2, with average temperatures in the range of 29 °C to 34 °C throughout the sampling period. Our results show that temperatures in this range correlate with the presence of culturable *Vibrio* spp. at high concentration, particularly *V. parahaemolyticus*.

However, according to Spearman's statistical analysis, temperature exhibits varying correlations with different *Vibrio* spp. populations. The temperature in P1 has a weak negative correlation with *V. parahaemolyticus* population, a positive correlation with *V. cholerae* population, and no correlation with *V. alginolyticus* population. Meanwhile, the temperature in P2 does not correlate with all three *Vibrio* spp. These statistical results are in contrast with the findings in our study, as well as in many reported studies. This observation may be due to the only slight changes in the temperature throughout the sampling period. Environmental conditions, such as temperature, can vary spatially and temporally within a sampling site. Therefore, even if correlations exist between environmental parameters and microbial populations, these correlations may not be consistently observed across all samples or sampling events (Carr et al., 2019). Furthermore, one factor rarely act in isolation,

implying that other factors, including but not limited to pH and salinity, also affect the proliferation of *Vibrio* species in the ponds (de Souza Valente and Wan, 2021).

Many studies reported the correlation between temperature and growth patterns of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* in coastal environments (Vezzulli et al., 2016; Thornstenson and Ullrich, 2021; Williams et al., 2022). It has been shown that temperatures of 20 °C and above are optimum for the growth of *Vibrio* spp. in marine water. Furthermore, many studies have shown that *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* have optimum growth at 37 °C and can still grow at a wide temperature range between 20 °C - 42 °C (Ravel et al., 1995; Gu et al., 2016; Sheikh et al., 2022). In the case of *V. parahaemolyticus*, it has been observed that the increase in temperature leads directly to an increase in growth (Kim et al., 2012a). This observation supports our finding, where the concentration of *V. parahaemolyticus* in all samples, especially in water, increased and remained high at $>10^3$ MPN/mL when the temperature in P1 and P2 remained in the high range of >30 °C. Meanwhile, temperatures less than 15 °C have been reported to induce a viable but non-culturable (VBNC) state in *Vibrio* spp. (Baker-Austin et al., 2018). Although this condition is not a concern for coastal farming since the temperature in Malaysia remains relatively high throughout the year, it may raise concern during post-harvesting and importing, when the seafood needs to be kept on ice or frozen to prevent spoilage. Although bacteria in the VBNC state become dormant and are not detectable using conventional culture methods, they remain viable. In addition, some functions such as metabolic activity, antibiotic resistance, specific gene expression, virulence, and pathogenicity are sustained (Fernández-Delgado et al., 2015).

In addition to temperature, salinity is another factor that can affect the growth patterns of *Vibrio* spp. in marine ecosystems (Sampaio et al., 2022). The salinity of water samples taken from both P1 and P2 fell in the range of 20 – 25 ppt throughout the sampling period. This range of salinity is favourable for the growth of shrimps in the coastal shrimp farm (Chu and Brown, 2021). Unfortunately, this range is also optimum for the growth of *V. parahaemolyticus. V. parahaemolyticus* is reported to have rapid growth in coastal water with salinity in the range of 15 to 25 ppt (Givens et al., 2014). The results also indicate that as the pond water salinity in both P1 and P2 decreased slightly towards harvesting, the overall load of the three *Vibrio* spp., notably *V. parahaemolyticus,* increased. It has been reported that a decrease in salinity, especially in brackish water, encourages the growth and spread of *Vibrio* spp. (Baker-Austin et al., 2018). However, statistical analysis showed a weak negative correlation between water salinity with *V. parahaemolyticus* population, a significant positive correlation with *V. cholerae* population, and no correlation with *V. alginolyticus* population in P1. In P2, no correlation was observed between water salinity with all three *Vibrio* spp. The result of this statistical analysis may be due again to the only slight changes in the salinity, as well as the combined effects of other environmental and chemical factors towards the microbial population (de SouzaValente and Wan, 2021). The consistency in water salinity in both ponds can also be attributed to the farm's adherence to recommended physio-chemical standards that create an environment conducive to the healthy growth of the shrimp.

Another factor that can significantly affect the growth and survival of *Vibrio* spp. is the pH of water. Most *Vibrio* spp. prefer slightly acidic to neutral pH levels (pH 6.5-7.5) (Ross and Schreiber, 1998). This information supported our observation, where the overall growth of the three *Vibrio* spp., especially *V. parahaemolyticus,* is rapid from stocking to

harvesting, while the pH of water in both ponds remained in the range of pH 5.9 – 7.9. While this pH range is considered optimum for their growth, some *Vibrio* spp. such as *V. cholerae*, was reported to be able to tolerate a wide range of pH, including alkaline pH as high as pH 9.0 (Nhu et al., 2021). However, according to statistical analysis, the pond water pH is slightly negatively correlated to *V. parahaemolyticus* population, positively correlated to *V. cholerae*, and no correlation with *V. alginolyticus* population in P1. No correlation was observed again between pH values with all three *Vibrio* spp. in P2.

In general, pH levels below pH 6.5 might inhibit the growth of *Vibrio* spp. since acidic conditions can denature proteins and disrupt cellular processes (Kayser and Kayser, 2007). On the other hand, pH levels above pH 7.5 were reported to affect the growth of *Vibrio* spp. by interfering with the bacterial enzymes and other proteins required for their cellular processes, thus preventing their proper functioning (Kieliszek and Błażejowski, 2005). For *V. parahaemolyticus* studies, they can grow at a wide range of pH values, with optimal growth occurring at a pH of 7.0 – 8.0. However, *V. parahaemolyticus* can also survive and grow at lower pH values, as low as pH 4.0 (Suzuki et al., 2014).

In addition to affecting the growth and survival of *Vibrio* spp., the pH of water can also influence the expression of certain virulence factors, such as toxins and enzymes, which can contribute to the pathogenicity of these bacteria (Kim et al., 2008). For example, *V. cholerae* has been shown to produce higher levels of *cholera* toxin (CTX) and toxin-coregulated pilus (TCP), two of the main virulence factors, at low pH levels (Faruque and Nair, 2010; Weber and Klose, 2011). In a study conducted by Whitaker et al. (2010), it was observed that acidic conditions induced the expression of lysine decarboxylase, an enzyme responsible for regulating the bacterial acid stress response system in *V. parahaemolyticus*

O3:K6. This mechanism enables the bacteria to survive in acidic conditions, thereby enhancing their virulence potential (Whitaker et al., 2010). Overall, the pH of water can significantly impact the presence and activity of *Vibrio* spp. in aquatic environments, with optimal pH levels being critical for their growth and survival.

The overall growth of *V. cholerae* and *V. alginolyticus* decreased towards the end of sampling, while *V. parahaemolyticus* showed increment or constant high concentration throughout the sampling. Both *V. cholerae* and *V. alginolyticus* might have been outcompeted by the rapidly growing *V. parahaemolyticus* that could utilise the resources better (Thornstenson and Ullrich, 2021). This statement was in agreement with Caburlotto et al. (2010), who stated that another reason for the different growth patterns is the difference in the sensitivity of the different *Vibrio* spp. towards environmental conditions, including temperature, salinity, and pH. In this case, *V. parahaemolyticus* may be more sensitive to environmental factors than *V. cholerae* and *V. alginolyticus*.

In addition to being sensitive to environmental and chemical parameters, overfeeding has been identified as a potential factor contributing to the abundance of *Vibrio* spp. in shrimp ponds. Overfeeding leads to the formation of nutrient-rich sludge, creating conditions favorable for the proliferation of harmful microorganisms, including *Vibrio* species (Horowitz and Horowitz, 2020). However, in the Telaga Air shrimp farm, no sludge formation was observed throughout the sampling period. Additionally, information from the manager revealed the implementation of a well-timed feeding schedule using automatic timers to prevent overfeeding of the shrimp. Further studies are necessary to thoroughly investigate the feeding conditions and their relationship with *Vibrio* spp. in the farm. In environmental samples, *V. parahaemolyticus* is often reported to be the dominant species

isolated, especially in brackish waters. Such is the case that the number of other *Vibrio* spp. isolated in a sample is used to evaluate the presence and risk of *V. parahaemolyticus*. A study by Ruiz-Cayuso et al. (2021) found that for each *Vibrio* spp. detected, the odds of isolating *V. parahaemolyticus* increased by 2.84-fold. This study proved and supported the findings by Blackwell and Oliver (2008), who also reported the correlation between *V. parahaemolyticus* with the total *Vibrio* spp. and other coliforms such as *Escherichia coli* isolated from an environmental sample (Ruiz-Cayuso et al., 2021). The dominance of *V. parahaemolyticus* against other *Vibrio* spp. is due to their ability to withstand a wide range of temperatures and salinities, as well as to adapt and thrive in various environments (Janda and Abbott, 2010). This characteristic allows *V. parahaemolyticus* to outcompete other *Vibrio* spp. in marine and freshwater environments.

According to the project manager of PNK Satang Biru shrimp farm, a complete harvest of adult *P. vannamei* from each pond can yield approximately seven to eight metric tons of shrimp in one production cycle. During the last sampling (the 6th sampling) conducted at the time of harvesting adult shrimps, the average size of the harvested shrimp was approximately 130 mm long. Overall, it took approximately 90 to 100 days for the post-larvae shrimp to grow to a harvestable size. While the direct effect of the high presence of the *Vibrio* spp. was not observed on the overall growth and production of the shrimp in the shrimp farm, the risk for infection is still present. Without stressors, *Vibrio* spp., as an opportunistic pathogen, can reach significant concentration without triggering the onset of vibriosis (Newman, 2022). However, the ability of *Vibrio* spp. to cause disease should not be overlooked, considering the relationship between the shrimp and the stressors (de Souza Valente and Wan, 2021). In addition, accumulation of *Vibrio* spp. in shrimp products can

still pose health risks to consumers when not processed or cooked appropriately (Kaysner et al., 2004).

The findings in our study raise concern since the microbial load in the harvested shrimp product exceeds the standards acceptable in most importing countries. However, early detection of *Vibrio* spp. in shrimp farms, especially during stocking, is crucial because it helps to prevent the spread of the bacteria to other shrimps and can lead to a more effective treatment plan. If *Vibrio* spp. is not detected until it has spread throughout the farm, it might lead to significant loss of shrimps and finances for the farm. Additionally, *Vibrio* spp. can cause serious health problems for humans who consume contaminated shrimp, so early detection and treatment can protect public health. Early detection also allows for more targeted and potentially less disruptive treatment methods rather than implementing broad and potentially harmful measures if the infection has spread widely.

Shrimp farmers should pay attention to their management practices to ensure the quality of their products and to prevent the spread of diseases. Good practices include controlling water quality, preventing overcrowding, and maintaining good hygiene. Continuous real-time monitoring of the critical control points (HACCP) in shrimp aquaculture is also essential to ensure food safety and prevent disease outbreaks. Overall, good farm management is the key to preventing vibriosis and protecting the health and well-being of both the shrimps and the seafood consumers.

3.5 Chapter Summary

V. parahaemolyticus, *V. cholerae*, and *V. alginolyticus* were successfully detected and enumerated in all four sample types in both ponds, starting from stocking until the harvesting period. This study has shown that *V. parahaemolyticus* is the most dominant

species compared to *V. cholerae* and *V. alginolyticus* in the Telaga Air shrimp farm, suggesting that the ability of *V. parahaemolyticus* to exploit available resources more effectively than *V. cholerae* and *V. alginolyticus* contributes to the elevated *V. parahaemolyticus* load in the shrimp farm. This becomes a concern because *V. parahaemolyticus* is a well-recognized human pathogen associated with seafood-related infections, and its prevalence in shrimp farms can have implications for food safety. In the future, prevalence studies can be extended to encompass more shrimp farms across Sarawak, providing a more comprehensive dataset on the prevalence and distribution of *Vibrio* species.

The study's outcomes, serving as a valuable model, highlight the need for ongoing monitoring of *Vibrio* spp. prevalence in shrimp farms across Sarawak and addressing the current lack of comprehensive data on the subject in the region. This study also underscores the importance of employing the MPN-multiplex PCR technique for the surveillance of *Vibrio* spp. in shrimp farms. The method offers several advantages, including enhanced sensitivity and rapid simultaneous processing of multiple samples, which reduces processing time. The efficiency of this technique positions it as a promising tool for ongoing monitoring efforts, providing critical data for the prevention and control of vibriosis outbreaks in shrimp farms, thus maintaining the sustainability of the shrimp industry.

CHAPTER 4

ANTIBIOTIC RESISTANCE AND VIRULENCE GENE PROFILES OF *Vibrio parahaemolyticus, Vibrio cholera,* AND *Vibrio alginolyticus* ISOLATED FROM WHITE-LEG SHRIMP FARM

4.1 Overview

Controlling the population of *Vibrio* spp. presents significant challenges attributable to their pathogenicity and antibiotic resistance. These bacteria are renowned for their capacity to induce diseases not only in marine organisms but also in humans (Sampaio et al., 2022). Furthermore, the emergence of antibiotic-resistant strains of *Vibrio* spp. is increasingly worrisome, given the diminished efficacy of conventional treatments employing broad-spectrum antibiotics. Moreover, *Vibrio* spp. is acknowledged for possessing the highest growth rate among bacteria, and their adeptness at thriving in various environmental conditions, as previously discussed in Chapter 3, contributes to their resilience and persistence (Baker-Austin et al., 2020; Abioye et al., 2021).

In the aquaculture sector, a diverse array of antibiotics has traditionally been employed by farmers to address bacterial infections, particularly vibriosis, attributable to *Vibrio* species. Especially within shrimp cultivation, antibiotics such as oxytetracycline, tetracycline, quinolones, sulfonamides, enrofloxacin, norfloxacin, gentamicin, and trimethoprim are permitted and routinely utilised to mitigate the incidence of morbidity and mortality among shrimp populations (Haifa-Haryani et al., 2022). In Malaysia, antibiotics are administered either via inclusion in feed additives or through immersion baths, serving as both prophylactic measures and therapeutic interventions for infected organisms (Ibrahim et al., 2010; Amalina et al., 2019). Nevertheless, due to the indiscriminate or excessive application of antibiotics, their efficacy has begun to wane with the emergence of an

escalating number of antimicrobial-resistant (AMR) bacterial strains. Furthermore, the inadvertent dissemination of AMR bacteria may be facilitated across different geographic regions through the import and export of live aquatic animals and animal-derived products (Rodgers et al., 2011; Paria et al., 2021).

In addition to the antibiotic resistance profile, the identification and monitoring of virulence genes in *Vibrio* spp. can also contribute to understanding the bacteria's pathogenicity and aiding in future disease management. As noted in the preceding chapter (Chapter 3), *Vibrio* spp. infections have been documented as major contributors to instances of foodborne illness in Malaysia. *Vibrio parahaemolyticus*, in particular, is reported to be the cause of the highest seafood-associated gastroenteritis in various Asian countries (Elmahdi et al., 2016). Consumption of *Vibrio*-contaminated food products can result in gastroenteritis and may even lead to septicaemia and mortality in immunocompromised individuals (Abdullah Sani et al., 2013; Song et al., 2020). Additionally, the global occurrence of *V. parahaemolyticus* prompts efforts to understand their pathogenicity and their interactions with humans and aquatic animals (Wang et al., 2015). Although the majority of environmental isolates of *Vibrio* spp. are non-pathogenic, their genomes are highly dynamic, meaning that non-pathogenic strains can easily acquire virulence genes from pathogenic strains through horizontal gene transfer (Xu et al., 2017). Consequently, *Vibrio* spp. maintain an extensive reservoir of virulence genes, even in non-pathogenic strains (Zoqratt et al., 2018; Abdelaziz Gobarah et al., 2022).

Antibiotic resistance poses a substantial challenge to public health and economic stability. These issues underscore the urgent need for effective strategies to combat antibiotic resistance, as it is a growing threat with far-reaching implications. Efforts to mitigate these

challenges require a multifaceted approach, including an improved understanding of *Vibrio* spp. biology, enhanced surveillance and monitoring, and the development of alternative treatment modalities to combat infections caused by these bacteria. The emergence of resistant strains not only compromises the efficacy of antibiotic treatment but also raises concerns about the potential for increased morbidity, mortality, and healthcare risks and costs (WHO, 2023). Moreover, antibiotic resistance transcends national borders, necessitating collaborative efforts across countries and sectors to address this pressing issue (Muteeb et al., 2023). As recognized by the WHO, antibiotic resistance poses a significant threat to global health security, emphasizing the critical importance of proactive measures to mitigate its impact (WHO, 2023).

Therefore, it is imperative to ascertain and oversee the dissemination of AMR strains within a farm to curtail their propagation and aid in disease management. Consequently, this chapter will delve into the profiles of antibiotic resistance and virulence genes of three *Vibrio* spp. isolated from the Telaga Air shrimp farm.

4.2 Materials and Methods

4.2.1 Isolation and Preparation of Bacteria

Bacterial cultures that were enriched in the previous chapter (Chapter 3) were spread and streaked on differential and selective media, Thiosulfate–citrate–bile salts–sucrose (TCBS) agar and CHROMAgar™ *Vibrio*, to acquire single colonies. On TCBS agar, *Vibrio parahaemolyticus* would appear as green colonies, while *V. cholerae* and *V. alginolyticus* would appear as yellow colonies. The differentiation of *Vibrio* spp. on TCBS agar is primarily based on their ability to ferment sucrose, which is one of the key components of the medium. *Vibrio parahaemolyticus* cannot ferment sucrose. As a result, it does not

produce acid to lower the pH, so the colour of the medium does not change to yellow. Instead, the colonies of *V. parahaemolyticus* retain the green colour of the TCBS agar, resulting from the alkaline pH maintained by the medium. *Vibrio cholerae* and *V. alginolyticus* can ferment sucrose, which leads to the production of acid. The acid lowers the pH of the medium, causing the pH indicator (bromothymol blue and thymol blue) in the agar to turn yellow (Nigro and Steward, 2015).

Meanwhile, on CHROMAgar™ *Vibrio*, *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* appear as mauve, blue, and milky white colonies, respectively. The appearance of different *Vibrio* spp. on CHROMAgar™ *Vibrio* is based on the incorporation of chromogenic substrates into the medium. These substrates are specific to enzymatic activities characteristic of certain *Vibrio* spp., leading to the development of distinct colony colours.

Based on these morphologies, 10 isolates (n=10) of each *Vibrio* spp. were selected and cultured in Alkaline Peptone Water (APW). The presumptive *V. parahaemolyticus* isolates were assigned codes TA01 – TA10, *V. cholerae* isolates as TA11 – TA20, and *V. alginolyticus* isolates as TA21 – TA30. Multiple rounds of culturing and streaking on selective media were conducted to ensure the isolation of pure cultures. In addition to morphological characterisation, PCR was performed using species-specific primer sets mentioned in Chapter 3 (Table 3.1) to confirm the species of each isolate.

4.2.2 Antibiotic Susceptibility Test (AST)

Once the identity of each isolate was confirmed, the susceptibility of each isolate to different antibiotics was assessed using the disc diffusion assay, as outlined by the Clinical and Laboratory Standards Institute (Hudzicki, 2009; Clinical and Laboratory Standard

Institute, 2015). The bacterial isolates were cultured in 10 mL Mueller-Hinton Broth (MHB) supplemented with 2% NaCl at 37 °C for 24 hours.

The following day, the overnight cultures were adjusted to a 0.5 McFarland standard. Using sterile cotton swabs, the cultures were spread onto Mueller-Hinton agar (MHA) plates supplemented with 2% NaCl and allowed to dry for five minutes. After the plates dried, antibiotic discs were placed on the agar surface in triplicate using sterile forceps. The discs were positioned at approximately equal distances from each other to ensure accuracy. The antibiotics utilised in this susceptibility test included Ampicillin (AMP, 10 µg), Chloramphenicol (C, 30 µg), Penicillin G (P, 10 IU), Amoxicillin-clavulanic acid (AMC, 30 µg), Amikacin (AK, 30 µg), Erythromycin (E, 15 µg), Tetracycline (TE, 30 µg), Ceftazidime (CAZ, 30 µg), Ciprofloxacin (CIP, 5 µg), Cephalotin (KF, 30 µg), Norfloxacin (NOR, 10 µg), Gentamicin (CN, 10 µg), Rifampicin (RD, 5 µg), Imipenem (IPM, 10 µg), Kanamycin (K, 30 µg), Nalidixic acid (NA, 30 µg), Bacitracin (B, 10 IU), and Meropenem (MEM, 10 µg) (Oxoid, Hampshire, United Kingdom). These antibiotics are commonly employed to treat *Vibrio* spp. infections in aquaculture and clinical settings (Elmahdi et al., 2016). All plates were then incubated at 37 °C for 24 hours.

Following incubation, the diameter of the antibiotic inhibition zone was measured, and the average was calculated based on triplicate measurements. The level of susceptibility was determined according to the guidelines in the Clinical and Laboratory Standards Institute's Document M-45 for *Vibrio* species (Clinical and Laboratory Standard Institute, 2015). From the susceptibility results, the Multiple Antibiotic Resistant (MAR) indices of the isolates were determined using the following formula:

$$\text{MAR index} = \frac{\text{Number of antibiotics the isolate was resistant to}}{\text{Total number of antibiotics tested}} \qquad \text{Equation 4.1}$$

4.2.3 Polymerase Chain Reactions (PCR) for Virulence Genes Detection

The DNA of each bacterial isolate was extracted using the boiled-cell method as previously described in Chapter 3 (Queipo-Ortuno, 2008). Multiplex and singleplex PCRs were conducted using nine primer sets targeting different virulence genes of *Vibrio* spp., as outlined in Table 4.1.

Table 4.1: List of primers targeting different virulence genes used in this study.

Virulence gene	Primer name	Primer sequence (5' → 3')	Product size (bp)	Annealing temperature (°C)	Reference
tdh	F-*tdh*	GTA AAG GTC TCT GAC TTT TGG AC	269	58	Bej et al. (1999)
	R-*tdh*	TGG AAT AGA ACC TTC ATC TTC ACC			
trh	F-*trh*	TTG GCT TCG ATA TTT TCA GTA TCT	500	58	Bej et al. (1999)
	R-*trh*	CAT AAC AAA CAT ATG CCC ATT TCC G			
tlh	tl-F	AAA GCG GAT TAT GCA GAA GCA CTG	450	58	Bej et al. (1999)
	tl-R	GCT ACT TTC TAG CAT TTT CTC TGC			

Table 4.1 continued

pirA	AP3-F	ATG AGT AAC AAT ATA AAA CAT GAA AC	333	53	Sirikharin et al. (2015)
	AP3-R	GTG GTA ATA GAT TGT ACA GAA			
V. cholerae-associated *toxS*	F-*toxS*	CCA CTG GCG GAC AAA ATA ACC	640	52	Sechi et al. (2000)
	R-*toxS*	AAC AGT ACC GTA GAA CCG TGA			
V. cholerae-associated *toxR*	F-*toxR*	TTT GTT TGG CGT GAG CAA GGT TTT	595	52	Sechi et al. (2000)
	R-*toxR*	GGT TAT TTT GTC CGC CAG TGG			
rtxA	*rtxA*-F	CTG AAT ATG AGT GGG TGA CTT ACG	417	55	Chow et al. (2001)
	rtxA-R	GTG TAT TGT TCG ATA TCC GCT ACG			
rtxC	*rtxC*-F	CGA CGA AGA TCA TTG ACG AC	263	55	Chow et al. (2001)
	rtxC-R	CAT CGT CGT TAT GTG GTT GC			
ctxB	ctx B_2	GAT ACA CAT AAT AGA ATT AAG GAT G	460	55	Chow et al. (2001)
	ctx B_3	GGT TGC TTC TCA TCA TGG AAC CAC			

The detection of *tdh*, *trh*, and *tlh* genes was carried out using a multiplex PCR method adapted from Bej et al. (1999), with minor adjustments. Each PCR reaction mixture (25 µL) comprised 12.5 µL of exTEN 2X PCR Master Mix (1st BASE), 1.25 µL of each 10 µM primer, 2.0 µL of DNA extract, and 3.0 µL of sterile distilled water. PCR conditions included initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min (Bej et al., 1999). A clinical isolate of *V. parahaemolyticus* (from laboratory stock), previously confirmed to possess the *tlh*, *tdh*, and *trh* genes, was used as the positive control.

The detection of the *pirA* gene was performed using a singleplex PCR under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 30 sec, extension at 72 °C for 40 sec, and a final extension at 72 °C for 7 min. The PCR mixture consisted of 7.5 µL of exTEN 2X PCR Master Mix (1st BASE), 1.0 µL of each forward and reverse AP3 primer (10 µM concentration), 2.0 µL of DNA extract, and 5.5 µL of distilled water (Sirikharin et al., 2015). An environmental isolate of *V. parahaemolyticus* (from laboratory stock), previously confirmed to possess the *pirA* genes, was used as the positive control.

Meanwhile for the detection of *V. cholerae*-associated *toxR* and *toxS* genes, a multiplex PCR was performed following the method described by Sechi et al. (2004). The PCR mixture included 12.5 µL of exTEN 2X PCR Master Mix (1st BASE), 1.0 µL of each 10 µM primer, 2.0 µL of DNA template, and 4.5 µL of sterile distilled water. PCR conditions were similar to those used for detecting *tdh*, *trh*, and *tlh* genes, with the annealing temperature adjusted to 52 °C and the final extension extended to 20 min (Sechi et al., 2004).

A clinical isolate of *V. cholerae* that has been confirmed for the presence of *toxR* and *toxS* genes was used as the positive control.

Additionally, detection of *rtxA*, *rtxC*, and *ctxB* genes was conducted using a multiplex PCR mixture containing 12.5 µL of exTEN 2X PCR Master Mix (1st BASE), 0.6 µl of each primer (10 µM concentration), 2.0 µL of DNA extract, and 6.9 µL of distilled water. The PCR protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min (Chow et al., 2001). A clinical isolate of *V. cholerae* that was confirmed for the presence of *rtxA*, *rtxC,* and *ctxB* genes was used as the positive control.

In all PCRs mentioned above, sterile distilled water (dH_2O) was used as the negative control. The reactions were conducted using the T100^{TM} Thermal Cycler (Bio-Rad, USA). PCR products were visualized through agarose gel electrophoresis (AGE) with a 1.5% gel concentration. Electrophoresis was carried out at 80 V for 2 hours, and the gels were then stained with Ethidium bromide (EtBr) for one hour before visualization using a safeVIEW-MINI2 Blue Light Transilluminator (Cleaver Scientific, Warwickshire, United Kingdom). The presence of bands was observed and recorded.

4.3 Results

4.3.1 Isolation of *Vibrio* species

When plated on selective and differential media, such as TCBS and CHROMAgar^{TM} *Vibrio*, different *Vibrio* spp. bacterial colonies appear with different coloured morphologies (Figure 4.1). *Vibrio parahaemolyticus* appeared as green colonies on TCBS agar, while *V. cholerae* and *V. alginolyticus* both appeared as yellow colonies (Figure 4.1A). Meanwhile,

V. parahaemolyticus, *V. cholerae*, and *V. alginolyticus* appeared as mauve, blue, and milky white colonies, respectively, on CHROMAgar™ *Vibrio* (Figure 4.1B). Based on PCR, it had been confirmed that each of the selected isolates were indeed their respective *Vibrio* species (data not shown).

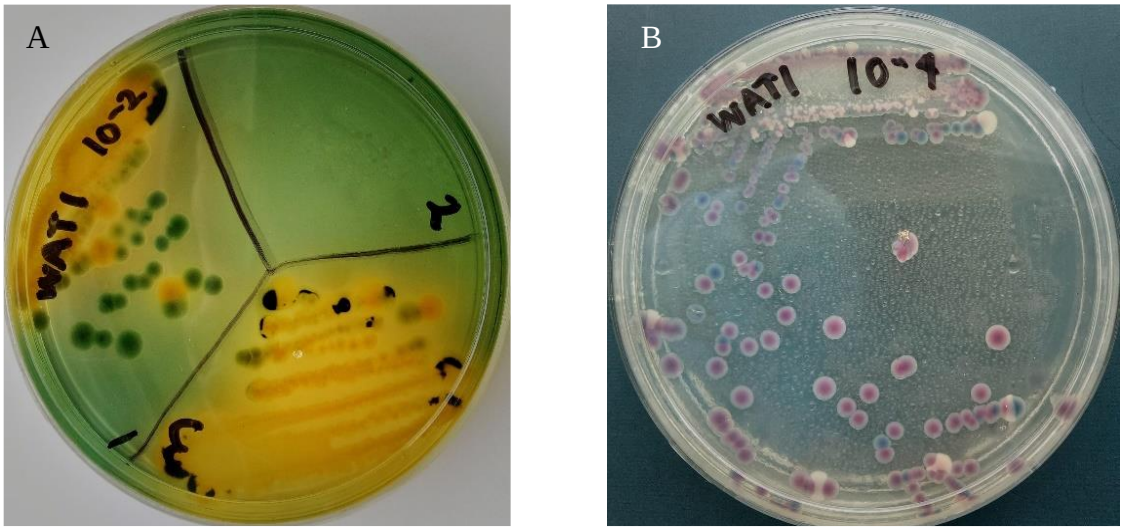


Figure 4.1: *Vibrio* spp. appearances on TCBS and CHROMAgar™ *Vibrio*. (A) *Vibrio parahaemolyticus* appears as green colonies, while V. cholerea and *V. alginolyticus* appear as yellow colonies on TCBS agar. (B) *Vibrio parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* appear as mauve (purple), blue, and white colonies, respectively on CHROMAgar™ *Vibrio*

4.3.2 Antibiotic Susceptibility Test (AST) of *Vibrio* species

The antibiotic susceptibility test was performed on 10 isolates of each of the three *Vibrio* spp., totalling 30 isolates (n = 30). The responses of the isolates were categorised as susceptible (can be treated with the antibiotic), intermediate (may be treated with adjusted dosage), and resistant (cannot be treated with the antibiotic) based on guidelines from the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standard Institute,

2015; CDC, 2019b). The test revealed varying responses of each isolate to the 18 tested antibiotics.

Analysis of Table 4.2 below indicates that Ceftazidime, Meropenem, Gentamicin, Tetracycline, Nalidixic acid, Norfloxacin, Ciprofloxacin, and Chloramphenicol were 100% effective against all isolates of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*. Amoxicillin-clavulanate was effective against 100% of *V. parahaemolyticus* and *V. cholerae* isolates, while Erythromycin was 100% effective against all isolates of *V. parahaemolyticus* and *V. alginolyticus*. Conversely, based on the AST test results, it was observed that 100% of *V. parahaemolyticus* and *V. alginolyticus* isolates were completely resistant to Penicillin G and Bacitracin, whereas 100% of *V. cholerae* isolates exhibited resistance to Penicillin G.

Based on observations made during the measurement of the zone of inhibition, it was noted that a few isolates exhibited colony growth within the existing zone of inhibition, as depicted in Figure 4.2 below. Following several repeated tests, consistent results were obtained. Therefore, these isolates were classified as resistant to the antibiotics, irrespective of the diameter of the inhibition zone. This growth within the zone of inhibition indicated the presence of resistant mutants (Hudzicki, 2009).

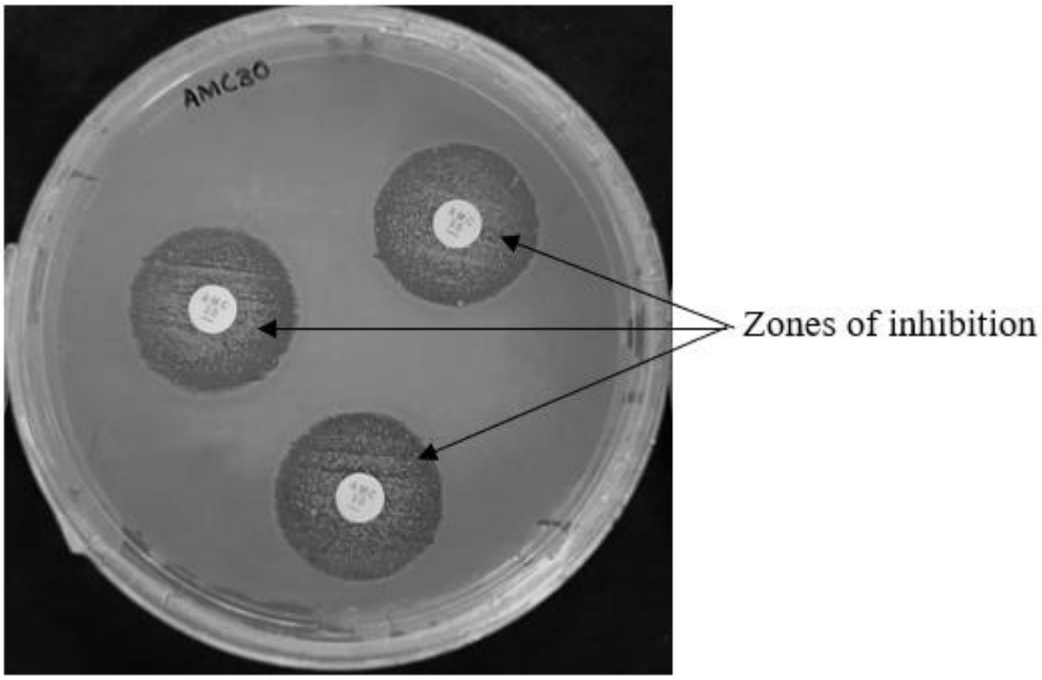


Figure 4.2: Colonies growth in zone of inhibition may indicate that the bacterium is resistant towards the antibiotic

Table 4.2: Antibiotic resistance profile of *Vibrio* spp. isolated from Telaga Air shrimp farm against 18 antibiotics.

Antimicrobial Class	**Antibiotic**	**Abbreviation**	**Number (%) of response of isolates to antibiotic**								
			***Vibrio parahaemolyticus* (n=10)**			***Vibrio cholera* (n=10)**			***Vibrio alginolyticus* (n=10)**		
			S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
PenicillinS and β-lactam/ β-lactamase inhibitor combinations	Ampicillin	AMP10	2 (20)	5 (50)	3 (30)	6 (60)	2 (20)	2 (20)	6 (60)	1 (10)	3 (30)
	Amoxicillin-clavulanate	AMC30	10 (100)	-	-	10 (100)	-	-	9 (90)	-	1 (10)
	Penicillin G	P10	-	-	10 (100)	-	-	10 (100)	-	-	10 (100)
Cephalosporins/Cephems	Ceftazidime	CAZ30	9 (90)	1 (10)	-	10 (100)	-	-	10 (100)	-	-
	Cephalothin	KF30	-	3 (30)	7 (70)	2 (20)	1 (10)	7 (70)	4 (40)	-	6 (60)
Carbapenems	Imipenem	IPM10	8 (80)	-	2 (20)	6 (60)	-	4 (40)	2 (20)	5 (50)	3 (30)
	Meropenem	MEM10	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-

Table 4.2 continued

Aminoglycosides	Amikacin	AK30	3 (30)	3 (30)	4 (40)	9 (90)	1 (10)	-	10 (100)	-	-
	Gentamicin	CN10	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
	Kanamycin	K30	3 (30)	6 (60)	1 (10)	7 (70)	3 (30)	-	8 (80)	2 (20)	-
Tetracyclines	Tetracycline	TE30	9 (90)	1 (10)	-	10 (100)	-	-	10 (100)	-	-
Quinolones	Nalidixic acid	NA30	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
	Norfloxacin	NOR10	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
Fluoroquinolones	Ciprofloxacin	CIP5	5 (50)	5 (50)	-	10 (100)	-	-	10 (100)	-	-
Phenicols	Chloramphenicol	C30	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
Macrolides	Erythromycin	E15	4 (40)	6 (60)	-	2 (20)	7 (70)	1 (10)	2 (20)	8 (80)	-

Table 4.2 continued

Ansamycins	Rifampicin	RD5	2 (20)	5 (50)	3 (30)	-	1 (10)	9 (90)	2 (20)	2 (20)	6 (60)
Polypeptides	Bacitracin	B10	-	-	10 (100)	2 (20)	-	8 (80)	-	-	10 (100)

4.3.3 Multiple Antibiotic Resistance (MAR) Profiles

The antibiotic resistance profiles and MAR (Multiple Antibiotic Resistance) indices of *V. parahaemolyticus* isolates from the shrimp farm are detailed in Table 4.3 below. Eight distinctive antibiotic resistance patterns were observed among the *V. parahaemolyticus* isolates, with 2 isolates (*V. parahaemolyticus* TA01 and TA10) exhibiting the same resistance pattern of P-B, and another 2 isolates (*V. parahaemolyticus* TA02 and TA03) displaying an antibiotic resistance pattern of P-AK-KF-B. The remaining isolates exhibited varying patterns. Isolate TA01 and TA10 were respectively isolated from water and effluent samples, while isolate TA02 and TA03 were both isolated from water samples. The MAR indices of the *V. parahaemolyticus* isolates ranged from 0.11 to 0.39. Pattern P-B demonstrated the lowest MAR index (0.11), whereas pattern AMP-P-AK-KF-RD-IPM-B, belonging to isolate TA07 previously isolated from a shrimp sample, exhibited the highest MAR index (0.39).

Table 4.3: The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) indices of *V. parahaemolyticus* isolates.

Isolate	Source	Antibiotic resistance profiles*	MAR index
TA01	Water	PB	0.11
TA02	Water	PAKKFB	0.22
TA03	Water	PAKKFB	0.22
TA04	Sediment	AMPPKFRDB	0.28
TA05	Sediment	PKFRDB	0.22
TA06	Sediment	AMPPAKKFKB	0.33
TA07	Shrimp	AMPPAKKFRDIPMB	0.39
TA08	Shrimp	PAKB	0.17

Table 4.3 continued

TA09	Effluent	PKFIPMB	0.22
TA10	Effluent	PB	0.11

*Antibiotics: Ampicillin (AMP); Penicillin G (P); Amoxicillin-clavulanate (AMC); Amikacin (AK); Erythromycin (E); Cephalothin (KF); Rifampicin (RD); Imipenem (IPM); Kanamycin (K); Bacitracin (B)

In Table 4.4 below, the antibiotic resistance profiles and MAR (Multiple Antibiotic Resistance) indices of ten *V. cholerae* isolates from the shrimp farm are presented. Seven distinctive antibiotic resistance patterns were observed, with three isolates (*V. cholerae* TA11, TA12 and TA19) exhibiting the same resistance pattern of P-KF-RD-IPM-B, while another 2 isolates (*V. cholerae* TA16 and TA18) displayed a resistance pattern of P-RD-B. The remaining isolates showed varying patterns from each other. The MAR indices of the *V. cholerae* isolates ranged from 0.11 to 0.33. Similar to the *V. parahaemolyticus* isolates, pattern P-B exhibited the lowest MAR index (0.11), while pattern AMP-P-E-KF-RD-B demonstrated the highest MAR index (0.33).

Table 4.4: The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) indices of *V. cholerae* isolates.

Isolate	Source	Antibiotic resistance profiles*	MAR index
TA11	Water	PKFRDIPMB	0.28
TA12	Water	PKFRDIPMB	0.28
TA13	Water	AMPPEKFRDB	0.33
TA14	Sediment	AMPPKFRDB	0.28
TA15	Sediment	PKFRDIPM	0.22
TA16	Sediment	PRDB	0.17
TA17	Shrimp	PKFRD	0.17

Table 4.4 continued

TA18	Shrimp	PRDB	0.17
TA19	Effluent	PKFRDIPMB	0.28
TA20	Effluent	PB	0.11

*Antibiotics: Ampicillin (AMP); Penicillin G (P); Amoxicillin-clavulanate (AMC); Amikacin (AK); Erythromycin (E); Cephalothin (KF); Rifampicin (RD); Imipenem (IPM); Kanamycin (K); Bacitracin (B)

Meanwhile, for *V. alginolyticus* isolates, eight different antibiotic resistance patterns were observed (Table 4.5). Among them, three isolates, namely TA24 and TA25 isolated from shrimps, and TA29 from effluent, exhibited the same patterns of P-RD-B. The MAR indices of the ten *V. alginolyticus* isolates ranged from 0.17 to 0.33. Isolate TA22, with the antibiotic resistance pattern of AMP-P-AMC-KF-IPM-B, exhibited the highest MAR index (0.33). Conversely, antibiotic resistance patterns P-RD-B, P-KF-B, and AMP-P-B displayed the lowest MAR index (0.17).

Table 4.5: The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) indices of *V. alginolyticus* isolates.

Isolate	**Source**	**Antibiotic resistance profiles***	**MAR index**
TA21	Shrimp	AMPPKFRDB	0.28
TA22	Shrimp	AMPPAMCKFIPMB	0.33
TA23	Shrimp	PKFIPMB	0.22
TA24	Shrimp	PRDB	0.17
TA25	Shrimp	PRDB	0.17
TA26	Effluent	PKFRDB	0.22
TA27	Effluent	PKFB	0.17
TA28	Effluent	PKFRDIPMB	0.28

Table 4.5 continued

TA29	Effluent	PRDB	0.17
TA30	Effluent	AMPPB	0.17

*Antibiotics: Ampicillin (AMP); Penicillin G (P); Amoxicillin-clavulanate (AMC); Amikacin (AK); Erythromycin (E); Cephalothin (KF); Rifampicin (RD); Imipenem (IPM); Kanamycin (K); Bacitracin (B)

4.3.4 Detection of Virulence Genes in *Vibrio* species

Virulence genes detection was performed on all 30 *Vibrio* spp. isolates by using primer sets that target the specific virulence gene sequences. Table 4.6 below summarised the results of the PCR where presence of specific bands was observed and recorded.

Table 4.6: Detection of virulence genes in *Vibrio* spp. isolates using PCR.

***Vibrio* spp.**	**Isolate**	**Virulence gene**								
		tdh	***trh***	***tlh***	***toxS***	***toxR***	***toxA***	***rtxA***	***rtxC***	***ctxB***
Vibrio parahaemolyticus	TA01	-	-	+	+	+	-	-	-	-
	TA02	-	-	+	+	+	-	-	-	-
	TA03	-	-	+	+	+	-	-	-	-
	TA04	-	-	+	+	+	-	-	-	-
	TA05	-	-	+	+	+	-	-	-	-
	TA06	-	-	+	+	+	-	-	-	-
	TA07	-	-	+	+	+	-	-	-	-
	TA08	-	-	+	+	+	-	-	-	-
	TA09	-	-	+	+	+	-	-	-	-
	TA10	-	-	+	+	+	-	-	-	-

Table 4.6 continued

Vibrio cholera	TA11	-	-	-	+	+	-	+	+	-
	TA12	-	-	-	+	+	-	+	+	-
	TA13	-	-	-	+	+	-	+	+	-
	TA14	-	-	-	+	+	-	+	+	-
	TA15	-	-	-	+	+	-	+	+	-
	TA16	-	-	-	+	+	-	+	+	-
	TA17	-	-	-	+	+	-	+	+	-
	TA18	-	-	-	+	+	-	+	+	-
	TA19	-	-	-	+	+	-	+	+	-
	TA20	-	-	-	+	+	-	+	+	-
Vibrio alginolyticus	TA21	-	-	-	-	-	-	-	-	-
	TA22	-	-	-	-	-	-	-	-	-
	TA23	-	-	-	-	-	-	-	-	-
	TA24	-	-	-	-	-	-	-	-	-
	TA25	-	-	-	-	-	-	-	-	-
	TA26	-	-	-	-	-	-	-	-	-
	TA27	-	-	-	-	-	-	-	-	-
	TA28	-	-	-	-	-	-	-	-	-
	TA29	-	-	-	-	-	-	-	-	-
	TA30	-	-	-	-	-	-	-	-	-

(+): positive detection; (-): no detection

PCR was conducted on ten *V. parahaemolyticus* isolates to detect all of the virulence genes listed in Table 4.1. Based on the PCR results, the presence of *tlh*, *toxS*, and *toxR* genes was observed in all ten *V. parahaemolyticus* isolates (Table 4.6). In Figure 4.3 below, a band with a size of 450 bp was detected in all *V. parahaemolyticus* isolates, consistent with the expected size of the *tlh* gene. However, no bands corresponding to the expected sizes of *trh* and *tdh* genes were identified. In Figure 4.4, two bands were observed in each isolate, with sizes of 640 bp and 596 bp, matching the anticipated sizes of *toxS* and *toxR*, respectively. Meanwhile, the detection of the *pirA* gene, which contributes to some strains of *V. parahaemolyticus* the ability to cause Acute Hepatopancreatic Necrosis Disease (AHPND), yielded negative results for all *V. parahaemolyticus* isolates (Figure 4.5).

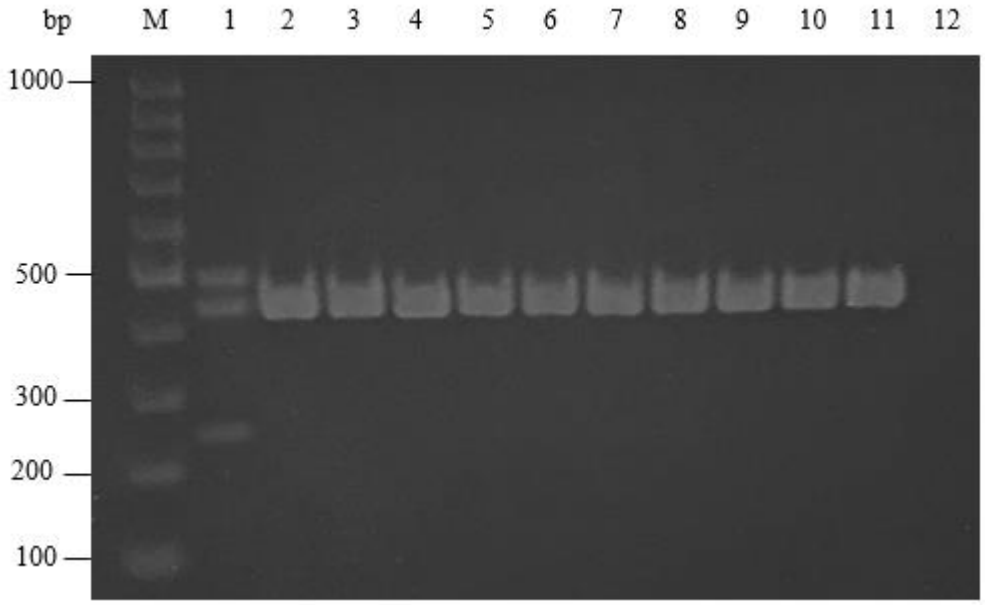


Figure 4.3: PCR result for the detection of *tdh* (269 bp), *trh* (500 bp), and *tlh* (450 bp) virulence genes in *V. parahaemolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. parahaemolyticus* TA01 – TA10; lane 12: negative control (sterile dH_2O).

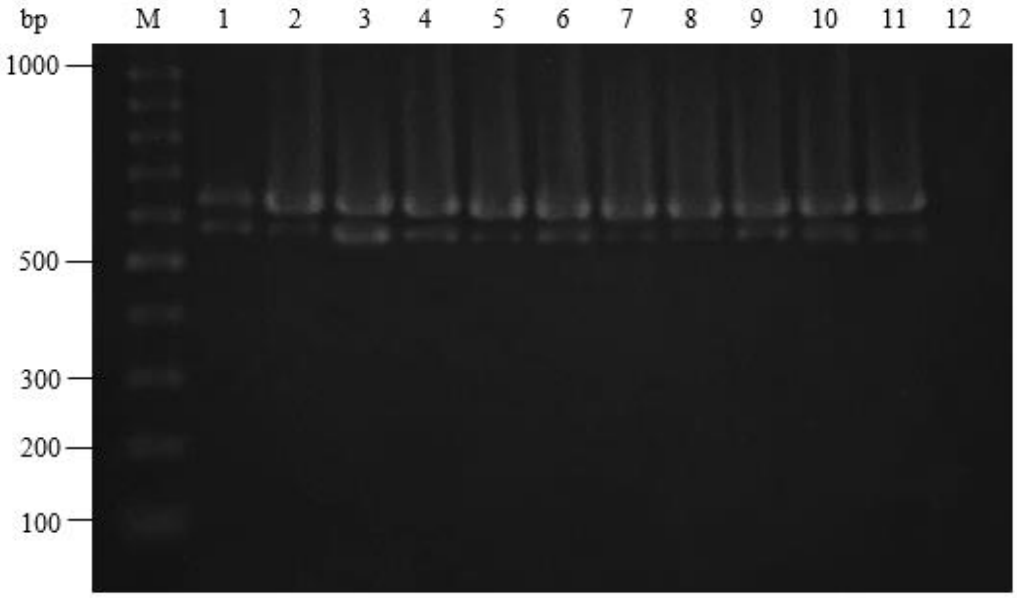


Figure 4.4: PCR result for the detection of *toxR* (596 bp) and *toxS* (640 bp) virulence genes in *V. parahaemolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. parahaemolyticus* TA01 – TA10; lane 12: negative control (sterile dH_2O).



Figure 4.5: PCR result for the detection of *pirA* (333 bp) virulence genes in *V. parahaemolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. parahaemolyticus* TA01 – TA10; lane 12: negative control (sterile dH_2O).

Meanwhile, in the *V. cholerae* isolates, four virulence genes were detected positively in all ten isolates (Table 4.6). As depicted in Figure 4.6 below, two bands with sizes of 640 bp and 595 bp, corresponding to the expected sizes of the *toxS* and *toxR* genes, respectively, were observed for each isolate. Moreover, in Figure 4.7, two positive bands corresponding to the *rtxA* (417 bp) and *rtxC* (263 bp) genes were observed for each *V. cholerae* isolate, while *ctxB* (460 bp) was not detected in any of the isolates.

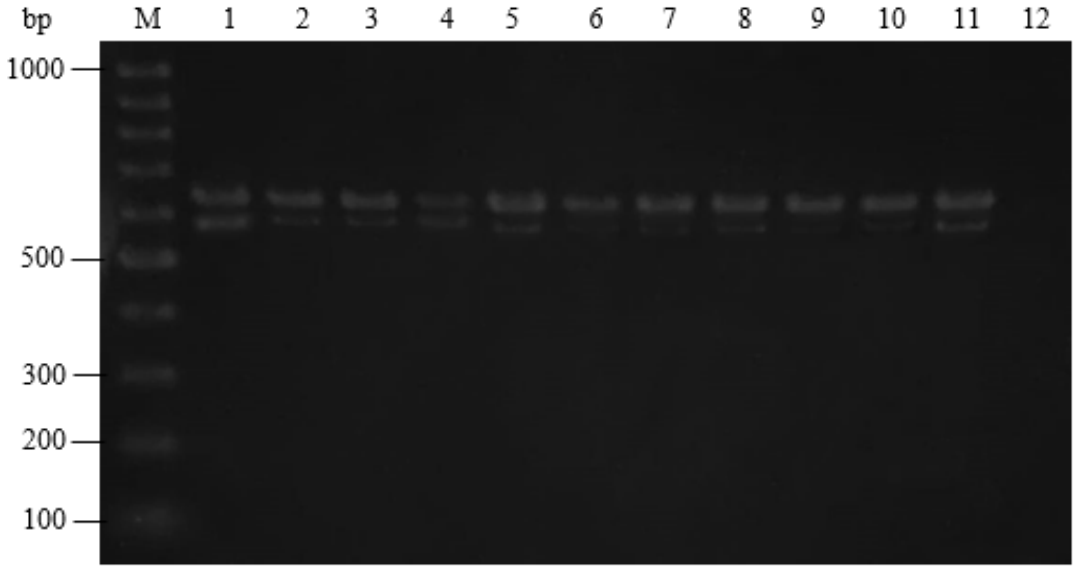


Figure 4.6: PCR result for the detection of *toxR* (595 bp) and *toxS* (640 bp) virulence genes in *V. cholerae* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. cholerae* TA11 – TA20; lane 12: negative control (sterile dH_2O).

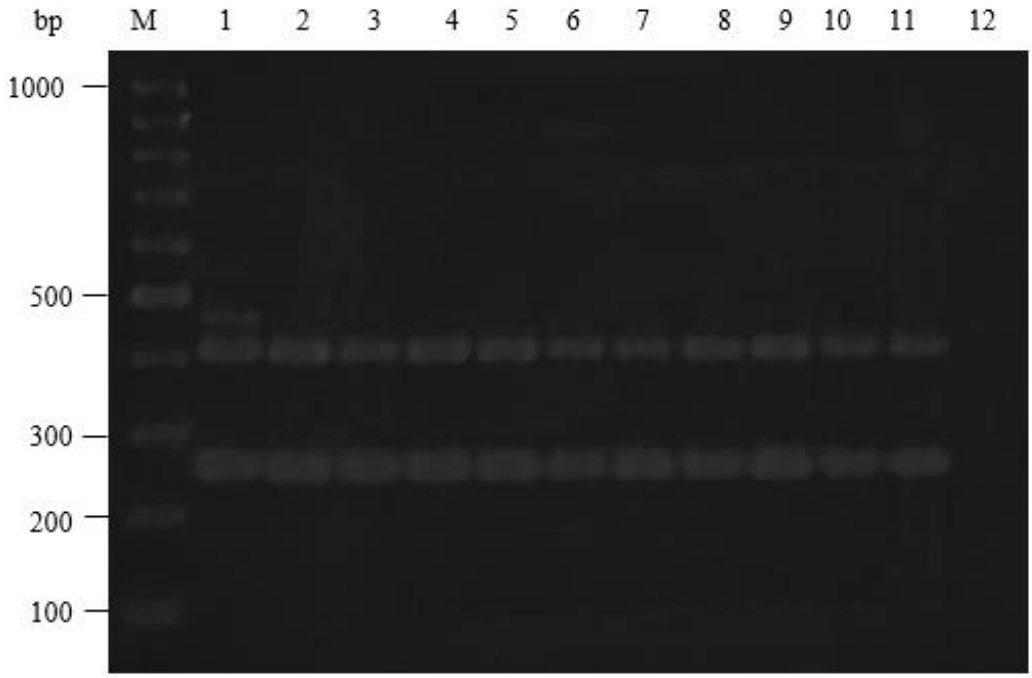


Figure 4.7: PCR result for the detection of *rtxA* (417 bp), *rtxC* (263 bp) and *ctxB* (460 bp) virulence genes in *V. cholerae* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. cholerae* TA11 – TA20; lane 12: negative control (sterile dH_2O).

No positive detection was observed for the nine targeted virulence genes in the *V. alginolyticus* isolates (Table 4.6). As depicted in Figure 4.8, no bands were detected for all ten isolates in the detection of the *toxR* and *toxS* genes (similar results were observed with the other virulence genes). This indicates that none of the isolated *V. alginolyticus* harbour the targeted virulence genes.

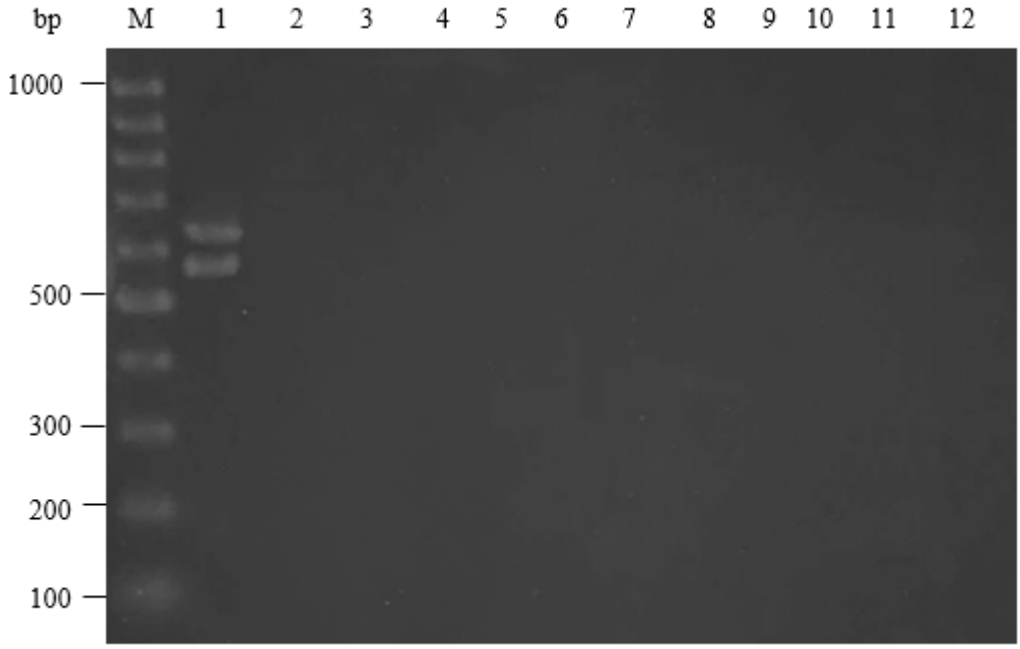


Figure 4.8: PCR result for the detection of *toxR* (595 bp) and *toxS* (640 bp) virulence genes in *V. alginolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. alginolyticus* TA21 – TA30; lane 12: negative control (sterile dH_2O).

4.4 Discussion

Bacteria often develop resistance to antimicrobials due to excessive usage. In this study, resistance to at least two antibiotics was observed in all 30 *Vibrio* spp. isolates. The occurrence of antimicrobial resistance (AMR) is frequently documented in regions with extensive antibiotic use, such as outbreak areas (Kumar et al., 2009; Amalina et al., 2019). Specifically for *Vibrio* spp., their resistance to a variety of antibiotics commonly used in aquaculture has been steadily increasing over the years, leading to diminished effectiveness of antibiotic treatments (Letchumanan et al., 2015).

The antibiotic susceptibility test revealed that all *Vibrio* spp. isolates exhibited multiple resistance to at least two antibiotics. These findings parallel those of an antibiotic resistance study conducted in cultured shrimp farms in the Philippines, where *Vibrio* spp.

isolated from ponds employing antibiotics displayed resistance to multiple antibiotics (Tendencia and De la Peña, 2001; Elmahdi et al., 2016). The MAR indices of the *Vibrio* spp. isolated from Telaga Air shrimp farm ranged from 0.11 to 0.39, with the majority (60%) of the total isolates having MAR indices exceeding 0.2. Despite the farm manager's assertion that antibiotic treatment was not administered in the shrimp farm, the presence of isolates with MAR indices exceeding 0.2 suggests they may have originated from a contaminated source with extensive antibiotic use. This suggests the existence of residual antibiotics originating from wastewater or nearby agricultural and aquaculture activities (Hanna et al., 2023). Additionally, certain antibiotics demonstrate stability and can persist in the environment long after their use has ceased, leading to contamination by the accumulated residual antibiotics (Larsson, 2014; Lundborg and Tamhankar, 2017).

Numerous studies have highlighted the high resistance of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* to ampicillin (Oh et al., 2011; Elmahdi et al., 2016; Abioye et al., 2023). Al-Othrubi et al. (2014) reported that over 50% of *V. parahaemolyticus* isolated from cockles and shrimp samples from various Malaysian markets displayed high resistance to ampicillin. Similarly, Amalina et al. (2019) found that the majority of *Vibrio* spp. isolated from cultured groupers in farms across Peninsular Malaysia exhibited high resistance to ampicillin. However, in this investigation, only 30% of *V. parahaemolyticus* and *V. alginolyticus* isolates, and 20% of *V. cholerae* isolates, exhibited resistance to ampicillin, with the remaining isolates displaying susceptibility or intermediate resistance to the antibiotic. Notably, *V. parahaemolyticus* exhibited a higher proportion of isolates with intermediate resistance (50%) compared to the other two *Vibrio* spp., indicating that the dosage of the ampicillin antibiotics needs to be adjusted to treat infection caused by some of

the *V. parahaemolyticus* strains (CDC, 2019b). Overall, only a small percentage of isolates from the Telaga Air shrimp farm displayed resistance to ampicillin.

In this investigation, antibiotics belonging to specific classes remained highly effective at controlling all *Vibrio* spp. isolates, including phenicols (Chloramphenicol), tetracyclines (Tetracycline), cephalosporins (Ceftazidime), fluoroquinolones (Ciprofloxacin), quinolones (Norfloxacin and Nalidixic acid), aminoglycosides (Gentamicin), and carbapenems (Meropenem). These antibiotics operate through diverse mechanisms to combat bacterial infections. Phenicol, tetracycline, and aminoglycoside antibiotics hinder protein synthesis, with phenicols targeting the 50s ribosomal subunit, and tetracyclines and aminoglycosides affecting the 30s ribosomal subunit (Kapoor et al., 2017). Notably, tetracycline is often recommended for treating severe *Vibrio* spp. infections due to its capacity to impede the synthesis of pathogenic extracellular enzymes, particularly proteases and lipases (Elmahdi et al., 2016). Meanwhile, cephalosporins and carbapenems inhibit cell wall synthesis or function, thereby disrupting bacterial growth and multiplication (Kapoor et al., 2017). Additionally, fluoroquinolones and quinolones hinder DNA replication and synthesis, and impede cell division by interfering with the function of DNA gyrase and topoisomerases (Hooper and Jacoby, 2016; Correia et al., 2017).

In contrast, both penicillin G and bacitracin exhibited complete ineffectiveness against the *Vibrio* spp. isolates. Bacteria often undergo natural genetic changes over time, leading to their development of antimicrobial resistance. However, the rapid emergence and dissemination of these multidrug-resistant (MDR) strains are exacerbated by human activities, particularly through the excessive use and misuse of antimicrobials in humans, animals, and plants (WHO, 2023). The spread of resistance genes among bacteria in aquatic environments can occur swiftly through horizontal gene transfer. Several studies have

documented the discovery of resistance genes in clinical isolates of *Vibrio* spp. that were previously identified in environmental isolates, suggesting genetic exchange between clinical and environmental strains (Elmahdi et al., 2016).

The isolates underwent screening to detect the presence of virulence genes alongside assessing their susceptibility to antibiotics. The detection of the *tlh* gene in all *V. parahaemolyticus* isolates, contrasted with its absence in the *V. cholerae* and *V. alginolyticus* isolates, suggests high conservation of this gene in *V. parahaemolyticus*, often utilised as a signature molecular marker for the species (Gutierrez West et al., 2013). Nevertheless, several studies have indicated the presence of the *tlh* gene in other *Vibrio* spp. apart from *V. parahaemolyticus.* As reported by Wang et al. (2007), the *tlh* gene is widespread in various *Vibrio* spp., including *V. alginolyticus, V. harveyi*, *V. vulnificus*, and *V. anguillarum* (Yáñez et al., 2015). Consequently, the *tlh* gene may not be as exclusive to *V. parahaemolyticus* as previously assumed, potentially diminishing its precision as a species-specific marker.

Most pathogenic strains of *V. parahaemolyticus* possess *tdh* and/or *trh* genes in addition to the *tlh* genes (Yáñez et al., 2015). In clinical isolates, *tdh* gene was detected in majority (95%) of the isolates, making TDH as the one of the main virulence factors produced by *V. parahaemolyticus* (Liu, 2003; Wang et al., 2015). The absence of *tdh* and *trh* genes that encode thermostable direct hemolysin (TDH) and its homolog, the TDH-related hemolysin (TRH) respectively in all isolates may suggest that the isolates were non-pathogenic or have low pathogenicity in human, as the two genes were commonly used as the indicator for *V. parahaemolyticus* pathogenicity (Bej et al., 1999; Gutierrez West et al., 2013). These results align with numerous studies indicating that the majority of environmental strains of *V. parahaemolyticus* are non-pathogenic, with the *tdh* and/or *trh* genes being detected only in low frequency in these strains (Carburlotto et al., 2009).

However, the emergence of pathogenic *Vibrio* spp. continues to increase every year resulting in increment in infection cases (Elmahdi et al., 2016). There are also evidences that suggested *V. parahaemolyticus* acquired *tdh* genes through horizontal gene transfer (Okuda et al., 2001).

While the precise roles of the *tlh* gene in *V. parahaemolyticus* pathogenicity remain unclear, it encodes for phospholipase A2 and thermolabile hemolysin, both of which have been reported to induce lysis of human erythrocytes (Shinoda et al., 1991; Wang et al., 2015). Furthermore, its expression has been demonstrated to be upregulated in environments resembling those of the human intestines, suggesting its involvement in the adaptation, survival, and virulence of *V. parahaemolyticus* within the host gastrointestinal tract (Gotoh et al., 2010; Yáñez et al., 2015). This notion is further supported by findings indicating that HeLa, Chang liver, and RAW264.7 cells treated with purified TLH protein exhibit severe cytotoxicity, which is dose and time-dependent, thereby suggesting that TLH shares similar biological functions with the TDH and TRH toxins (Wang et al., 2012; Wang et al., 2015). Therefore, despite the unclear roles of TLH proteins in *V. parahaemolyticus* pathogenicity, it is premature to rule out these *V. parahaemolyticus* isolates as non-pathogenic simply because the *tdh* and *trh* genes were not detected. Moreover, some studies report that 10% of clinical isolates of *V. parahaemolyticus* lack the *tdh* and *trh* genes but still exhibit high cytotoxicity to gastrointestinal cells, suggesting that there are other virulence factors that contributed to their pathogenicity (Raghunanth, 2014).

One of the diseases causing significant losses in shrimp farms is Acute Hepatopancreatic Necrosis Disease (AHPND). This disease is induced by specific strains of *V. parahaemolyticus* (VP_{AHPND}) carrying the plasmid (pVA1) containing *pirA* and *pirB* genes, encoding binary toxins PirA and PirB (Sirikharin et al., 2015; Dhar et al., 2019;

Hossain et al., 2020). The presence of both toxin subunits is necessary for the manifestation of AHPND pathologies, and these toxins induce hepatopancreatic necrosis in infected shrimp, leading to rapid progression and 100% mortality in severely infected ponds (Sirikharin et al., 2015; Hossain et al., 2020). For *pirA* gene detection, the AP3 detection method has been widely used in previous studies and is recommended for confirming AHPND-positive strains (Han et al., 2015; Hossain et al., 2020). Compared to other VP_{AHPND} detection methods (AP1 and AP2), the primer sets employed in the AP3 method have shown the highest sensitivity and specificity (Sirikharin et al., 2015; Hossain et al., 2020). In this study, none of the *Vibrio* spp. isolates, particularly the *V. parahaemolyticus* isolates, exhibited the presence of the *pirA* gene, indicating that the isolates are not AHPND-causing strains. However, continuous monitoring is essential as there have been reported cases where the acquisition of *pirA* and *pirB* gene through horizontal gene transfer has transformed non-VP_{AHPND} strains into VP_{AHPND} strains (Dhar et al., 2019). Through the acquisition plasmid containing these genes through transformation, transduction, or conjugation, *V. parahaemolyticus* may acquire new traits, which is the ability to cause AHPND.

In all *V. cholerae* isolates, two out of three virulence genes typically associated with pathogenicity were detected: the *rtxA* and *rtxC* genes belonging to the repeat in toxin (RTX) family. This family encompasses gene clusters including *rtxA*, encoding a putative cytotoxin, *rtxC*, encoding an acyltransferase, and *rtxB* and *rtxD*, involved in producing proteins responsible for toxin transportation (Chow et al., 2001). Chow et al. (2001) demonstrated a concurrent relationship between positive detection of *rtxA* and *rtxC* genes and positive results in HEp-2 cytotoxicity assays in clinical and environmental *V. cholerae* isolates, and vice versa, demonstrating the integrity between the genotypic and phenotypic expressions of these genes across strains. Supporting this association, strains with deleted RTX gene

clusters showed negative results in PCR detection and cytotoxicity assays (Chow et al., 2001). These findings suggest that the presence of both *rtxA* and *rtxC* in *V. cholerae* isolates from the Telaga Air shrimp farm indicates their pathogenic potential.

Additionally, the *ctxB* gene is a fundamental component that encodes the cholera toxin (CT), responsible for cholera syndrome characterised by acute diarrheal diseases (Chow et al., 2001; Takahashi et al., 2021). Cholera toxin is regarded as the primary virulence factor in *V. cholerae* and is frequently associated with strains of significant pandemic potential (Chow et al., 2001). All *V. cholerae* isolates in this study lacked the *ctxB* gene. According to Chow et al. (2001), non-epidemic *V. cholerae* non-O1 serogroup strains that are positive for *rtxA* and *rtxC* but negative for *ctxB* only caused sporadic, milder cases of diarrhea. However, these strains should not be underestimated as RTX cytotoxins can still induce necrosis and inflammation in the gastrointestinal tract (Chow et al., 2001; Kim et al., 2012b).

The primers used for targeting the *toxR* and *toxS* genes used in this study (Table 4.1) were originally used for targeting *V. cholerae*-associated *toxR* and *toxS* genes. However, subsequent studies revealed that the *toxR* gene is also present in other *Vibrio* spp., including *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, and *V. vulnificus*, with homologous sequences observed in the internal portion of the nucleotide sequences of the *toxR* genes of at least three different *Vibrio* species (Okuda et al., 2001; Wang et al., 2015). As such, *toxR* and its integral membrane binding partner, *toxS* genes were also detected in all *V. parahaemolyticus* isolates in addition to all *V. cholerae* isolates in this study. Unlike the *tdh*, *trh*, and *tlh* gene in *V. parahaemolyticus* and *rtxA*, *rtxC*, and *ctxB* genes in *V. cholerae*, *toxR* and *toxS* are not directly related to the expression of virulence factors. Instead, they encode integral membrane proteins, ToxR and ToxS, that activate the transcription of

virulence genes in the *Vibronaceaea* family (Pfau and Taylor, 1998; Lembke et al., 2020). The *toxR* gene was initially discovered as a regulatory gene for the *ctx* gene encoding CT in *V. cholerae*. The presence of these genes in *V. parahaemolyticus* isolates suggests that they may have acquired these exogenous genes through horizontal gene transfer (Sechi et al., 2000; Baffone et al., 2006). Indeed, a study by Lin et al. (1993) showed that *V. parahaemolyticus*-associated ToxR protein is highly similar to the ToxR protein produced by *V. cholerae*.

It was later discovered that *toxR* gene plays a multifaceted role and is not only responsible to regulate the expression of virulence genes, but also in coordinating the regulation of multiple genes that can affect the survival and adaptability of *Vibrio* spp. in various environmental conditions (Okuda et al., 2001). For example, *toxR* was initially known to promote the expression of *tdh* gene with the presence of *toxS* gene in *V. parahaemolyticus* (Lin et al., 1993; Zhang et al., 2018). However, since *tdh* gene was not detected in the *V. parahaemolyticus* isolates in this study, the detection of both *toxR* and *toxS* genes in all isolates suggests that these genes play other roles that contribute to the adaptability and survival of the isolates. Similarly, the absence of *ctxB* gene in the *V. cholerae* isolates suggested that the *toxR* and *toxS* gene that were detected in them plays role other than the expression of the *ctx* virulence genes.

Contrary to the initial belief that the primary roles of the *toxR* and *toxS* genes are to stimulate the expression of virulence genes in the *Vibronaceae* family, a study by Okuda et al. (2001) demonstrated that the original function of these genes was to modulate the expression of outer membrane proteins (OMP)-encoding genes for bacterial adaptation in various environmental conditions. ToxR-modulated expression of OMPs was shown to contribute to the ability of *Vibrio* spp. to survive in the gastrointestinal tract by facilitating

colonisation, stimulating the expression of virulence factors, and conferring bile resistance (Okuda et al., 2001; Lembke et al., 2020). These findings lend support to the results obtained in this study, suggesting that the presence of *toxR* and *toxS* genes in the isolates may play a more significant role in the adaptability of the *Vibrio* spp. rather than in the expression of their virulence genes, especially considering the absence of associated virulence genes.

Moreover, several studies have indicated the involvement of the *toxR* gene in the regulation of biofilm formation. The expression of ToxR protein has been reported to be influenced by cell density and is implicated in the quorum sensing (QS) mechanism of *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum*, which can coordinate biofilm formation in response to environmental cues (Chen et al., 2018; Zhang et al., 2018; Zhang et al., 2021). A study conducted by Chen et al. (2018) suggested that ToxR plays a pivotal role in biofilm formation in *V. parahaemolyticus*. The reduced biofilm formation observed in a *toxR* mutant strain compared to the wild-type *V. parahaemolyticus* indicates the involvement of ToxR in promoting biofilm production. In addition, the restoration of biofilm formation in the complemented mutant strain further supports the role of ToxR in this process (Chen et al., 2018). Therefore, ToxR likely regulates genes or pathways that contribute to the formation of mature biofilms, thereby enhancing the adaptability and pathogenicity of *V. parahaemolyticus* (Gao et al., 2022). (Biofilm-forming ability of the *V. parahaemolyticus* isolates will be further discussed in Chapter 5).

In all *V. alginolyticus* isolates, none of the tested virulence genes were detected. Despite the *toxR* gene being commonly reported in many studies as the second most frequently detected virulence gene in *V. alginolyticus* after the *ompK* gene, no positive detections were observed in any *V. alginolyticus* isolates from the Telaga Air shrimp farm (Abd Wahid et al., 2022). Additionally, the *tlh* genes associated with *V. parahaemolyticus*,

which were reportedly found in all seven *V. alginolyticus* strains from shrimp hatcheries in Southern China as reported by Xue et al. (2022), were not detected in all *V. alginolyticus* isolates in this study (Abd Wahid et al., 2022). The absence of other virulence genes originally from various *Vibrio* spp. in all isolates suggests a low frequency of gene transfer events between *V. alginolyticus* and other *Vibrio* spp. in the shrimp farm or few strains are retaining these genes through horizontal gene transfer mechanisms (Xue et al., 2022).

In order to curtail the dissemination of MAR bacterial strains and thwart the transfer of virulence genes among bacteria within shrimp farms, employing prudent practices is imperative. Farmers must prioritize the cultivation and provision of specific pathogen-free (SPF) shrimp stocks, a measure proven to enhance shrimp production across numerous countries (Nillian et al., 2022). However, effective disease control necessitates strict management protocols encompassing physical barriers, meticulous water treatment control, carrier exclusion strategies, and vigilant feed management systems (Nillian et al., 2022). Furthermore, regular monitoring of *Vibrio* spp. prevalence within farms assumes paramount importance, serving as a pivotal precursor to informed management decisions. As underscored by Verschuere et al. (2000), proactive measures, such as disinfection protocols and controlled application of antimicrobial agents, are pivotal in pre-empting disease outbreaks and safeguarding shrimp farm integrity (Nillian et al., 2022).

4.5 Chapter Summary

This chapter has provided important insights into the antibiotic resistance profiles and the presence of virulence genes among *Vibrio* spp. isolates obtained from the Telaga Air shrimp farm. The findings indicate widespread antibiotic resistance among *Vibrio* spp. isolates, likely resulting from the extensive use of antibiotics in aquaculture settings. The presence of isolates with MAR indices higher than 0.2 suggests potential contamination from

sources with high antibiotic usage, such as from surface runoff or leaching from wastewater or nearby agricultural and aquaculture activities. Additionally, the spread of residual antibiotics can be facilitated by human and animal activities, particularly through the dispersal of contaminated faeces.

The study also sheds light on the diversity of virulence gene profiles among *Vibrio* spp. isolates. While pathogenic potential is exhibited by some isolates, others lack key virulence genes associated with pathogenicity. However, despite these observations obtained in this study, the surveillance data is only limited to the strains isolated from the Telaga Air shrimp farm, and hence, do not represent the comprehensive epidemiology in Sarawak. Moreover, given the high occurrence of horizontal gene transfer within bacterial populations, ongoing and a more comprehensive monitoring is essential to ensure that limited spread of virulence genes occurs between pathogenic and non-pathogenic strains. Additionally, monitoring antibiotic usage and bacterial resistance patterns is crucial for effective disease management and sustainable aquaculture practices.

In the subsequent chapter, the ability of bacteriophages isolated from environmental and retail shrimp samples to lyse the *V. parahaemolyticus* isolates will be explored. *Vibrio parahaemolyticus* was selected as the organism of interest because it exhibited the highest prevalence compared to the other two *Vibrio* spp. as highlighted in Chapter 3. Furthermore, the highest MAR index was demonstrated by one of the *V. parahaemolyticus* isolates, underscoring the need for alternative methods beyond solely relying on antibiotic treatment.

CHAPTER 5

ISOLATION AND CHARACTERISATION OF NOVEL *Vibrio parahaemolyticus* BACTERIOPHAGES FROM ENVIRONMENTAL AND RETAIL SHRIMP SOURCES

5.1 Overview

Ecological equilibrium in nature relies on complex interactions among diverse species populations, where each species contributes to the overall stability and functioning of the ecosystem. Taking inspiration from this natural phenomenon, biological control has emerged as an environmentally sustainable approach to managing problematic organisms within ecosystems. Biological control involves regulating species interactions by introducing or enhancing the natural enemies of the target organism (Kenis et al., 2017). One prominent example of biological control is phage therapy, which utilises bacteriophages to combat bacterial infections across various domains. Due to numerous advantages, the study of bacteriophages has gained popularity and remains an active research area, with ongoing studies exploring their potential benefits and limitations (Lin et al., 2022).

Bacteriophages, often simply called phages, are viruses that infect bacteria. They represent the most ubiquitous organisms on Earth and are found in virtually every environment containing bacteria, including soil, water, and the human body. The first precise virus counts in water samples revealed that viruses are the most abundant organisms on Earth, ranging from 10^4 to 10^8 viruses per millilitre of water, while in soil and aquatic sediments, viral counts range from 10^8 to 10^{11} viruses per gram dry weight and millilitre, respectively (Wommack et al., 2009). The discovery of bacteriophages and their capacity to lyse bacteria dates back to the early 20th century (Twort, 1915; d'Herelle, 1917). However, these studies were largely abandoned following the introduction of antibiotics (Navarro and

Muniesa, 2017). Nonetheless, the excessive use of antibiotics has led to a decline in their effectiveness against pathogenic bacteria, raising concerns as these bacteria develop resistance to antibiotics (El-Shibiny and El-Sahhar, 2017). With the emergence of antibiotic-resistant bacteria, there has been a resurgence of interest in studying and applying bacteriophages for various purposes, including applications in medicine, aquaculture, and the food industry.

One of the most significant advantages of using bacteriophages lies in their remarkable ability to selectively target and eliminate their host bacteria. In contrast to the broad-spectrum activity of antibiotics, phages discerningly lyse target bacteria while preserving non-target bacterial species, thereby mitigating disruptions to the microbiota and the associated risks of secondary infections (Patangia et al., 2022). This specificity emanates from specialised attachment structures on phage particles, which interact specifically with binding receptors on their host bacteria. The resultant phage-bacteria specificity minimises the likelihood of non-targeted organism harm, and thus preventing potential human cellular toxicity (Drulis-Kawa et al., 2012). As antibiotic-resistant bacteria continue to pose a growing concern, phage therapy emerges as a promising alternative for managing bacterial populations in a manner that supports ecosystem integrity and overall health (Lin et al., 2017).

In addition to their precision in targeting specific bacteria, another compelling feature that positions bacteriophages as a promising solution in combating bacterial infections is their rapid and cost-effective isolation and characterisation process. Unlike the lengthy process involved in developing new antibiotics, the swift identification and characterisation of phages facilitate the tailored development of phage therapies, particularly crucial in

addressing emerging bacterial strains that exhibit resistance to conventional antibiotics (El-Shibiny and El-Sahhar, 2017; Chapman, 2023). The rapid isolation and characterisation of phages entail phage screening, a method for identifying effective phage strains against target bacteria (Hyman, 2019). This screening enables the selection of phages specifically lytic to the bacterial strain causing an infection, thereby enhancing the likelihood of successful treatment. This capacity for quick isolation and characterisation not only accelerates the development of phage therapies but also provides a flexible and adaptable approach to addressing emerging bacterial challenges. As bacteria continually evolve and develop resistance to existing treatments, the rapidity of phage therapy offers a valuable tool in the ongoing battle against infectious diseases, contributing to the advancement of personalised and highly effective treatments for bacterial infections.

This study specifically focused on bacteriophages with the capacity to lyse *Vibrio parahaemolyticus* bacteria, a common causative agent of vibriosis in aquaculture farms. *Vibrio parahaemolyticus* is a Gram-negative bacterium frequently found in estuarine and coastal marine environments, including seawater (Letchumanan et al., 2014). This bacterium is also commonly detected in seafood products such as oysters, clams, and shrimp. In the context of human health, *V. parahaemolyticus* infections can result in gastroenteritis and represent a significant contributor to foodborne illness in Malaysia. Moreover, in aquatic organisms, notably shrimp, *V. parahaemolyticus* infections have been linked to substantial mortality rates, inflicting significant economic losses in the aquaculture industry (Abdullah Sani et al., 2013; Mastan and Begum, 2016). The emergence of antibiotic-resistant strains of *V. parahaemolyticus* has prompted the exploration of alternative approaches, with naturally occurring bacteriophages emerging as a potential solution. Given the ubiquity of

bacteriophages in the environment, studying phages is of paramount importance to comprehending their influence on the dynamics and evolution of bacterial populations.

To date, there is a notable scarcity of literature documenting the isolation of *Vibrio*-infecting phages in the environment and retail food products in Sarawak, especially on phages infecting *V. parahaemolyticus*. This scarcity arises due to the lack of attention and funding given to phage therapy studies in Asia, particularly in Sarawak, even when our continent provides a large biological reservoir where novel phages can be isolated and studied (Chung et al., 2023). Given the assertion that bacteriophages coexist wherever their corresponding host bacteria are present, it is reasonable to hypothesise that phages may also be present in the environment and seafood products where *V. parahaemolyticus* has been commonly detected. Furthermore, Sarawak, acknowledged for its promising potential in aquaculture development projects due to its favourable landscape and geographical resources (The Borneo Post, 2015), offers an enticing setting for exploring applications of phage therapy. With its abundant resources and the rise of antibiotic-resistant strains, it is imperative to initiate research on the prospective use of phage therapy in Sarawak. Consequently, the primary objective of this chapter is to isolate and characterise bacteriophages specific to *V. parahaemolyticus* from environmental water samples and retail shrimps in Sarawak, Malaysia

5.2 Materials and Methods

5.2.1 Bacterial Strains and Growth Conditions

In this study, eleven strains of *V. parahaemolyticus* were used. Seven isolates were previously isolated from the Telaga Air shrimp farm and four isolates including one ATCC strain were obtained from the laboratory stock. Additionally, two strains each of *V.*

alginolyticus and *V. cholerae* were used for host range analysis. The identity of the isolated strains was verified through PCR amplification utilising primer sets designed by Kim et al. (2015), targeting specific sequences in each species. *Escherichia coli*, *Bacillus cereus*, and *Pseudomonas aeruginosa* were also used for the host-specificity test. A comprehensive list of the bacterial strains used in this investigation, along with their sources, is provided in Table 5.3 in the Results section. All bacteria were cultivated in Tryptic Soy Broth (TSB) overnight at a temperature of 37 °C. In addition, all media used for the growth of *Vibrio* spp. in this study were supplemented with 2% (w/v) sodium chloride (NaCl).

5.2.2 Sample Collection

Water samples were obtained from ten locations around Kuching and Kota Samarahan areas. Each sample, totalling at least one litre, was carefully collected using sterile polypropylene bottles. Subsequently, these samples were promptly transported to the laboratory in an ice box. Meanwhile, retail shrimp samples were purchased from ten different supermarkets and wet markets in the Kuching and Kota Samarahan areas. Three different shrimp samples were acquired from each location. To ensure the integrity of the samples, all water and shrimp specimens were processed within 24 hours after their respective collections.

5.2.3 Phage Enrichment and Isolation

Prior to phage isolation, an overnight culture of the host bacteria, *V. parahaemolyticus* TA01, was cultivated in 7.5 mL of double-strength (2x) Tryptic Soy Broth (TSB) supplemented with 2% NaCl and 0.01 M Magnesium Chloride ($MgCl_2$). The culture was grown until it reached the mid-log phase (approximately OD_{600} 0.5).

The collected samples were processed within 24 hours after collection, following a method by Lal et al. (2016a) with slight modifications. The water samples were filtered using 0.45 µM filter syringes to get rid of impurities. Meanwhile, shrimp samples were homogenized in a 1:10 (w/v) ratio with Salt of Magnesium (SM) buffer (50 m Tris-HCl [pH 7.5], 100 mM NaCl, 8mM $MgSO_4.7H_2O$, and 0.01% (w/v) gelatin) using a sterilised conventional blender and allowed to settle for approximately 60 seconds.

For the enrichment of phages, equal volumes of the water samples and homogenised shrimp samples were introduced into the mid-log phase host bacterial culture respectively. The mixtures were then incubated overnight at 37 °C with agitation at 170 rpm, following the protocol by Lal et al. (2016a). After incubation, the mixtures were subjected to centrifugation at 10,000 ×g for 10 minutes to precipitate the bacterial cells. The supernatants were subsequently sterile-filtered through a 0.2 µm pore filter into new sterile centrifuge tubes. Chloroform was added to the filtered supernatant to achieve a final concentration of 40 µL/mL, and the mixture was thoroughly vortexed. Following this, the mixtures underwent a second centrifugation at 10,000 ×g for 5 minutes, and the presence of bacteriophages was assessed using the spot inoculation test, as described by Stenholm et al. (2008).

5.2.4 Phage Purification

After the presence of bacteriophages was confirmed, the phage was plated with the host bacteria using a double-layer agar assay as described by Kropinski et al. (2009). After the assay, plates with separated plaques with clear lysis appearance were selected, and a single plaque was picked using a sterile Pasteur pipette. The plaque was dispensed into 500 µL of Salt of Magnesium (SM) buffer. The phage suspension was incubated at 37 °C for one hour and then at 4 °C overnight. After incubation, the suspension was centrifuged at 10,000 ×g for 5 minutes and filter sterilized by using a 0.2 µm pore size syringe filter.

To further purify the phage, the phage is reinfected with host bacteria by adding 100 µL of phage lysate into 5mL of mid-log phase bacteria grown in TSB+2% NaCl + 0.1M $MgCl_2$. The mixture is incubated for 2 hours at 37 °C with agitation at 170rpm. After incubation, the mixture was centrifuged at 10,000 ×g for 5 minutes, and the presence of plaque was validated again by using the double-layer agar assay. The reinfection and purification process are repeated twice more to ensure complete purification of the phages (Middleboe et al., 2010). The final phage lysates in SM buffer were stored at 4 °C for subsequent analyses.

5.2.5 Observation of Phage Morphology

The morphology of the phage was examined by using a Transmission Electron Microscope (TEM). A drop of the purified phage sample was spotted on the 300-mesh carbon-coated copper grid and allowed to adsorb to it for 10 min. After 10 min, the excess liquid was removed by using sterile filter paper and the phage particles were negatively stained with Uranyl Acetate Replacement Stain (UAR-EMS) (Electron Microscopy Sciences, U.S.) for 2 min. The excess stain was removed using filter paper, and the copper grid was allowed to dry before it was examined with a JEOL JEM-1230 electron microscope (JEOL Ltd., Japan) at 80 kV. Ten different virions were measured to get the mean values for the phage sizes.

5.2.6 Bacteriophage Titre Determination

Phage titre was determined by plating the phage using the double-layer agar assay. Before plating, host bacteria were grown to the mid-log phase in TSB supplemented with 2% NaCl. Phage lysate was serially diluted 10-fold (10^{-1} – 10^{-7}) in SM buffer. In a tube containing molten top agar (TSB, 2% (w/v) NaCl, 0.01M $MgCl_2$, and 0.45% (w/v) bacteriological agar), 100 µL of the diluted phages were cultured with 100 µL of host

bacteria, and the tube was gently rolled on the palm to mix them well. Then, the top agar was poured onto a plate of solid bottom agar (Tryptic soy agar, 2% (w/v) NaCl, 0.01M $MgCl_2$) and allowed to solidify before it was incubated inversely at 37 °C for 24 hours. After overnight incubation, the dilutions with 20 – 200 plaque-forming units (PFUs) were selected and counted, and the value of phage titre was expressed as PFU/mL. This assay was done in triplicates to obtain the average value of the phage titre.

5.2.7 Host Range Specificity Test

All of *Vibrio* spp. mentioned in Table 5.3 were grown to the mid-log phase in TSB supplemented with 2% NaCl. The other non-*Vibrio* bacteria were also grown to the mid-log phase in TSB. The ability of the isolated phages to lyse them was tested by using the spot-inoculation method (Stenholm et al., 2008). The bacteria lawn was prepared by adding 100 µL of the mid-log phase bacteria culture into 3 mL molten top agar. The mixture was poured on top of the bottom agar and allowed to solidify. After the media had solidified, 10 µL of the purified phage lysate was spotted by using a pipette on the top agar and allowed to dry for 15 minutes. The plates were then incubated inversely at 37 °C for 24 hours. A plate with the bacterial lawn that is spotted with fresh TSB was included as the negative control. Lysis zones were observed and recorded the next day.

5.2.8 Determination of Optimum Multiplicity of Infection (MOI)

Overnight culture of *V. parahaemolyticus* TA01 was grown to mid-log phase (OD_{600} 0.4 – 0.6). Multiplicity of infection determination was performed by inoculating the mid-log phase bacteria with bacteriophage suspension at different ratios: 0.01, 0.1, 1.0, 10.0, and 100.0 PFU/CFU. The volume and dilution of the phage lysate were determined using the following calculations:

$$\text{Desired PFU} = \text{Colony Forming Unit (CFU)} \times \text{Desired MOI} \qquad \text{Equation 5.1}$$

$$\text{Volume of phage suspension} = \frac{\text{Desired PFU}}{\text{PFU/mL of phage suspension}} \qquad \text{Equation 5.2}$$

Note: If the volume of the phage suspension is too large, the phage lysate is diluted accordingly.

The mixture was then incubated at 37 °C for 4 hours. After incubation, phage activities were enumerated in triplicates by using the double-layer agar assay (Kropinski et al., 2009). Bacteriophage-free samples and bacteria-free samples were used as the negative controls.

5.2.9 One-step Growth

The host bacterium was grown to the mid-log phase and then infected with phage suspension at the optimum MOI (as determined in the previous section). The culture was incubated for 10 min at 37 °C with shaking at 170 rpm to allow the phages to adsorb to the bacterial cells. The culture was then centrifuged at 8,000 ×g for 5 min, and the supernatant was discarded. The resulting pellet was resuspended with an equal volume of pre-warmed TSB supplemented with 2% NaCl. One hundred microliter was withdrawn immediately to represent the sampling time at 0 min (T_0) and enumerated by using the double-layer agar assay. The suspension was then incubated with shaking at 37 °C and 170 rpm for up to 4 hours. At 15-minute intervals, samples were collected and enumerated by using the double-layer agar assay to determine phage titres. This experiment was performed in triplicates, and a one-step growth curve was generated by using GraphPad Prism, version 9.5.1 (GraphPad Software, Inc., San Diego, CA). The latent period burst size of the phage was determined from the curve.

5.2.10 Phage Adsorption Test

A mid-log phase host bacterial culture was inoculated with phage lysate at the optimal MOI. From this suspension, 100 µL was immediately extracted to determine the initial phage titre. The remaining mixture was left to incubate at room temperature without agitation. After a 10-minute interval, 1 mL of the suspension was collected and subjected to centrifugation at 16,000 ×g for 2 minutes. This step was repeated at 10-minute intervals over a total duration of 60 minutes. From the collected phage lysate, phage titre was determined at each time point using the double-layer agar assay. The percentage of unadsorbed (free) phage was calculated using the Equation 5.3 below:

$$\text{Unadsorbed free phage (\%)} = \frac{\text{Phage titre at time x } (T_X)}{\text{Initial phage titre } (T_0)} \times 100\% \qquad \text{Equation 5.3}$$

5.2.11 Temperature and pH Stability Test

The stability of phage EniLVP01 and EniLVP02 in different temperatures and pHs was tested according to the method by Phumkhachorn and Rattanachaikunsopon (2010) with slight modifications. This was done to evaluate the stability of both phages when stored in different temperatures and pHs without the presence of their host bacteria.

Prior to incubation, the bacteriophage suspension was diluted with SM buffer to a ratio of 1: 100, and the titre was determined using the double-layer agar assay. The titre of this phage suspension will be known as the initial PFU/mL. Then, 500 µL of the diluted phage was aliquoted each into microcentrifuge tubes that corresponded with the incubation temperatures 28, 37, 45, 65, and 72 °C. The tubes were incubated in a dry bath for one hour. After incubation, the tubes were placed in ice to allow them to cool down. The titre of the surviving phage was determined by using the double layer-agar assay.

For the pH stability test, SM buffers were prepared by adjusting them to different pH (pH 2, 3, 4, 5, 6, 7, 8, and 9) with 1 M hydrochloric acid. Bacteriophage suspension was diluted at a ratio of 1:100 with each of the SM buffer and then incubated at room temperature for one hour. An initial phage titre was also determined similar to the method in the temperature stability test using the normal SM buffer. After incubation, the titre of the surviving phages in each pH was determined by using the double layer-agar assay.

In both tests, each parameter was done in triplicate. The percentage of the surviving phages was calculated according to the following calculation:

$$\text{Surviving phages (\%)} = \frac{\text{Titre of surviving phages}}{\text{Initial phage titre } (T_0)} \times 100\% \qquad \text{Equation 5.4}$$

5.2.12 Phage Application on Biofilm Prevention and Destruction

For the biofilm prevention and destruction assay, the static microtitre plate assay was adapted from Gao et al. (2022) with slight modifications. To initiate biofilm prevention, an overnight culture of host bacteria was grown to mid-log phase in Tryptic Soy Broth (TSB) supplemented with 2% NaCl and 0.01 M $MgCl_2$. The phage lysate was then diluted tenfold (10^{-1} to 10^{-7}) in SM buffer. In a sterile 96-well plate, 100 µL of mid-log bacteria was incubated with 100 µL of the original phage lysate and the diluted lysates respectively. The plate was incubated at 37 °C for 24 hours.

For the biofilm destruction assay, similar steps were undertaken, but instead of growing mid-log host bacteria with the phage lysates, the bacterial culture was grown overnight in the 96-well plate at 37 °C before inoculation with the phage lysates. The next day, once the biofilm had formed, 100 µL of the original phage lysate and its dilutions were inoculated into the respective wells, followed by incubation at 37 °C for 4 hours.

After incubation, the liquid cultures in the 96-well plates were discarded, and the wells were washed thrice with 200 µL sterile phosphate-buffered saline (PBS) buffer (pH 7.4). The plates were evenly shaken to ensure the removal of planktonic cells, dried at room temperature, and stained with 200 µL of 0.2% crystal violet solution for 30 minutes. Following staining, the dye was removed, and the wells were washed again with PBS thrice to eliminate excess dye. The plates were allowed to dry at room temperature. The biofilm in each well was dissolved in 33% glacial acetic acid, and the solution was pipetted into a new 96-well plate. The absorbance of the dissolved solutions was measured at a wavelength of 600 nm. The blank control used was fresh TSB (with 2% NaCl and 0.01M $MgCl_2$), while the negative control was the bacterial culture without phage inoculation.

All samples were prepared in triplicates (Gao et al., 2022). Statistical analyses were conducted using GraphPad Prism, version 9.5.1 (GraphPad Software, Inc., San Diego, CA) to analyse the differences between the untreated and treated groups through unpaired, two-tailed Student's t-test. Significance was established with a P-value of 0.05 or less. For group comparison, two-way ANOVA test was used to compare the values at the same time point, with P-value of 0.05 or less considered as significant.

5.2.13 Phage Application on Retail Shrimps

The biocontrol effects of phages on shrimp samples were assessed using the method outlined by Waturangi et al. (2021) with modifications. This method was selected to test whether the lytic ability of the phages extends to matrices other than liquid and agar media. The shrimps were artificially infected using the host bacteria instead of utilising existing bacterial contaminants due to the narrow host range of both phages.

Shrimps were procured from supermarkets, washed with sterile distilled water three times to eliminate impurities, and approximately 1 g of shrimp samples was weighed and

sterilised using an autoclave to eliminate any natural contamination. Following sterilisation, the shrimp samples were allowed to dry. The shrimp samples were inoculated with 100 µL of the diluted host bacterial culture (approximately 10^4 CFU/mL) and left for one hour to facilitate bacterial interaction with the shrimp surface. Subsequently, 100 µL of the purified phage suspension was inoculated onto the infected shrimp samples at an MOI of 1.0. For the phage cocktail treatment, both purified phages were combined in a 1:1 ratio to a final concentration of 10^5 PFU/mL. The infected shrimps were infected with 100 µL of the phage cocktail to obtain MOI 1.0. Negative control groups were prepared by inoculating samples with the host bacteria at the same concentration, followed by inoculation with sterile SM buffer. Blank controls consisted of autoclaved shrimp samples inoculated with fresh SM buffer were also included to validate the absence of any preliminary contaminants. The samples were divided into two groups, each consisting of triplicates: one group was incubated at 4 °C, while the other group was incubated at room temperature for a total of 24 hours. At 6-hours interval, one set of samples were collected and the viable counts of bacterial cells in each sample were measured on Tryptic Soy Agar (TSA) plates (Waturangi et al., 2021).

Statistical analyses were conducted using GraphPad Prism, version 9.5.1 (GraphPad Software, Inc., San Diego, CA) to analyse the differences between the untreated and phage-treated groups through unpaired, two-tailed Student's t-test. Significance was established with a P-value of 0.05 or less.

5.3 Results

5.3.1 Isolation of Bacteriophages from Environmental and Retail Shrimp Samples

A total of two bacteriophages were successfully isolated from environmental water and retail shrimp samples collected around Kuching and Kota Samarahan area (Table 5.1 and Table 5.2).

Table 5.1: Isolation of bacteriophage from environmental water samples in Kuching and Kota Samarahan areas.

Sample types	Location	Detection of bacteriophages
Lake water	Universiti Malaysia Sarawak, Kota Samarahan	-
Pond water	Universiti Malaysia Sarawak, Kota Samarahan	-
Sewage water	Universiti Malaysia Sarawak, Kota Samarahan	-
River water	Riveria, Kota Samarahan	-
River water	Kampung Entanggor, Kota Samarahan	-
Pond water	Taman Sahabat, Kuching	-
Sewage water	Jalan Matang Kuching	-
Coastal river water	Batang Salak, Kuching	+
Sea water	Damai Beach, Kuching	-

Table 5.1 continued

River water	Maong River, Kuching	-
TOTAL	10	1/10 (10.0%)

(+): positive detection; (-): negative detection

In the examination of bacteriophage presence in various water sources across Sarawak and Kuching, only one sample out of the ten samples tested positive for the ability to lyse *V. parahaemolyticus* TA01, specifically in the coastal river water from Batang Salak, Kuching (Table 5.1). The study encompassed diverse water types, such as lake water, pond water, sewage water, river water, and seawater, gathered from different locations. The overall result highlights a 10.0% positive detection rate, underscoring the rarity of bacteriophages capable of lysing *V. parahaemolyticus* TA01 in the surveyed water samples.

Table 5.2: Isolation of bacteriophage from retail shrimp samples acquired from hypermarkets and wet markets around Kuching and Kota Samarahan areas.

Market type	Location	No. of sample	Positive detection of bacteriophages
Supermarket	Emart Summer Mall, Kota Samarahan	3	1
Wet market	Asajaya, Kota Samarahan	3	0
Supermarket	H&L Supermarket, Kota Samarahan	3	0
Wet market	Sebuyau, Kota Samarahan	3	0
Wet market	Sebangan, Kota Samarahan	3	0

Table 5.2 continued

Supermarket	Emart Matang, Kuching	3	0
Wet market	Emart Matang, Kuching	3	0
Wet market	Kubah Ria, Kuching	3	0
Wet market	Buntal Esplanade, Kuching	3	0
Supermarket	Emart Batu Kawa, Kuching	3	0
TOTAL	10	30	1/30 (3.33%)

The investigation into the presence of bacteriophages with the ability to lyse *V. parahaemolyticus* TA01 in shrimps bought from various market types across locations in Kota Samarahan and Kuching revealed a limited occurrence, with only one positive detection out of the 30 samples analysed (Table 5.2). The positive detection occurred in one shrimp sample bought from a supermarket at Emart Summer Mall, Kota Samarahan. Conversely, wet markets in Asajaya, Sebuyau, and Sebangan, as well as in H&L Supermarket, and Emart Matang supermarket, showed negative results. Additionally, wet markets in Emart Matang, Kubah Ria, Buntal Esplanade, and another supermarket at Emart Batu Kawa exhibited no detection of bacteriophages.

Overall, only two (5.0%) out of 40 samples (10 water samples and 30 shrimp samples) showed the presence of bacteriophages with the ability to form lysis on *V. parahaemolyticus* TA01. These bacteriophages from both sources would be isolated and

used for further analysis. Bacteriophage isolated from the coastal river water of Batang Salak, Kuching was designated as EniLVP01 while bacteriophage isolated from the shrimp bought from supermarket Emart Summer Mall, Kota Samarahan was designated as EniLVP02.

5.3.2 Morphological Characteristics

A bacteriophage isolate, designated EniLVP01, was successfully isolated from coastal river water, while another bacteriophage, designated EniLVP02, was isolated from shrimp obtained at a local supermarket in Kota Samarahan, Sarawak. Both phages exhibit lytic activity, forming clear plaques on *V. parahaemolyticus* TA01 bacterial lawns, as depicted in Figures 5.1(A) and Figure 5.1(C).

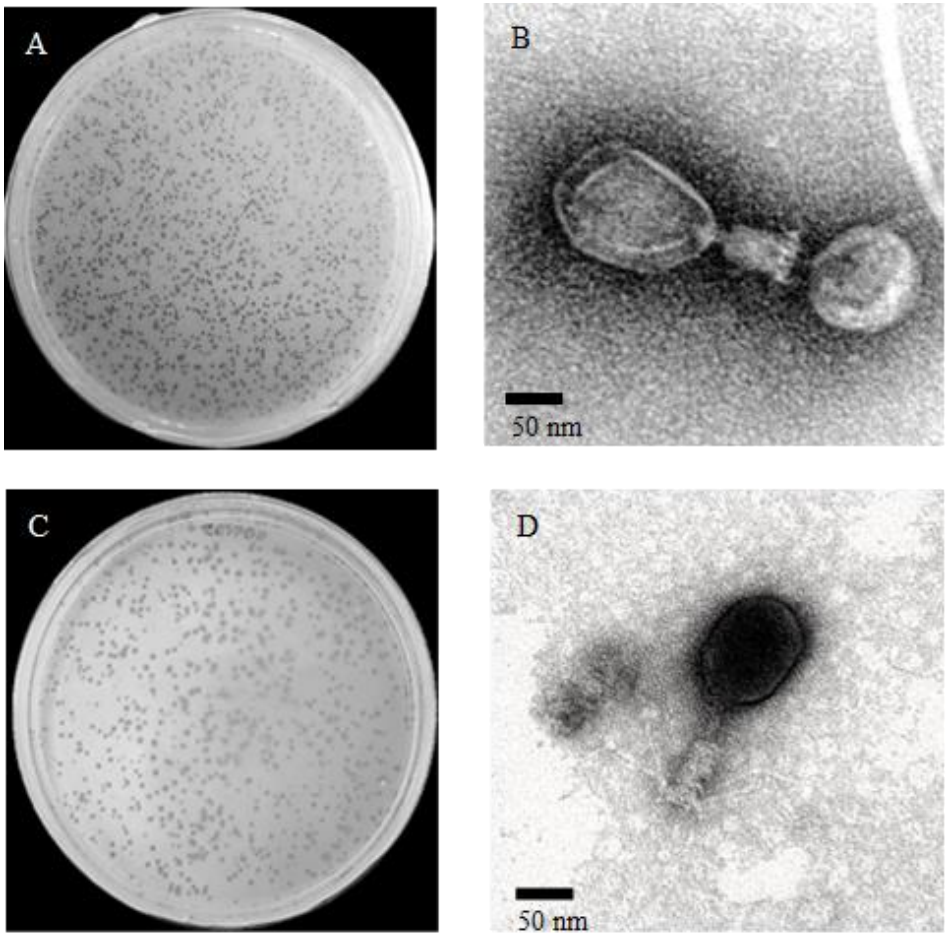


Figure 5.1: Morphological characteristics of phage EniLVP01 and EniLVP02. (A) Plaques formed by phage EniLVP01 on a lawn of host bacteria, *V. parahaemolyticus* TA01 grown on TSA + 2% NaCl plate. (B) Electron micrograph of phage EniLVP01. (C) Plaques formed by phage EniLVP02 on a lawn of host bacteria, *V. parahaemolyticus* TA01 grown on TSA + 2% NaCl plate. (B) Electron micrograph of phage EniLVP02. (Bars = 50 nm).

The lytic phage nature of EniLVP01 and EniLVP02 was indicated by the presence of clear plaques approximately 1.0 mm in size respectively (Figure 5.1A and 5.1C). Moderately elongated icosahedral heads were observed in both phages through Transmission Electron Microscopy (TEM). EniLVP01 exhibited a head length, head diameter, and tail length of 87.8±3.0 nm, 66.9±3.1 nm, and 43.1±3.7 nm, respectively (Figure 5.1B). EniLVP02 displayed an icosahedral head with a length of 82.91±2.87 nm and a diameter of 74.59±2.47 nm, along with a contractile tail measuring 69.97±2.93 nm in length (Figure

5.1D). Tail fibres and a base plate were also observed in both phages. Based on these characteristics, EniLVP01 and EniLVP02 are classified as members of the *Caudovirales* order and belong to the *Straboviridae* (formerly *Myoviridae*) family.

5.3.3 Host Specificity Test

Based on the spot-inoculation test on various bacterial species (Table 5.3), 17 different bacterial isolates from various sources were subjected to testing with phage EniLVP01 and EniLVP02. Among the 11 *V. parahaemolyticus* strains examined, only seven isolates from the shrimp farm exhibited susceptibility to lysis by both phages. Conversely, no lysis was observed with *V. parahaemolyticus* ATCC® 27969™ and *V. parahaemolyticus* isolates from retail sources. Furthermore, no lysis was evident in the case of *V. alginolyticus*, *V. cholerae*, *E. coli*, *P. aeuroginosa*, and *B. cereus* strains. These findings indicate that both bacteriophage EniLVP01 and EniLVP02 demonstrate a narrow host range.

Table 5.3: Host range of bacteriophage EniLVP01 and EniLVP02.

Bacterial spp.	**Strain**	**Source**	**Activity (±)**	
			EniLVP01	**EniLVP02**
V. parahaemolyticus	ATCC® 27969™	Laboratory stock	-	-
V. parahaemolyticus	TA01	Shrimp pond, this study	+	+
V. parahaemolyticus	TA02	Shrimp pond, this study	+	+
V. parahaemolyticus	TA03	Shrimp pond, this study	+	+
V. parahaemolyticus	TA04	Shrimp pond, this study	+	+

Table 5.3 continued

V. parahaemolyticus	TB01	Shrimp pond, this study	+	+
V. parahaemolyticus	TB02	Shrimp pond, this study	+	+
V. parahaemolyticus	TB03	Shrimp pond, this study	+	+
V. parahaemolyticus	VP01	Retail, laboratory stock	-	-
V. parahaemolyticus	VP02	Retail, laboratory stock	-	-
V. parahaemolyticus	VP03	Retail, laboratory stock	-	-
V. alginolyticus	ATCC® 17749™	Laboratory stock	-	-
V. alginolyticus	TA21	Shrimp pond, this study	-	-
V. cholerae	KCDC 13589	Laboratory stock	-	-
V. cholerae	TA31	Shrimp pond, this study	-	-
E. coli	-	Laboratory stock	-	-
P. aeuroginosa	-	Laboratory stock	-	-
B. cereus	-	Laboratory stock	-	-

(+): lysis; (-): no lysis

5.3.4 Bacteriophage Titre and Multiplicity of Infection (MOI)

The double-layer agar assay revealed that phage EniLVP01 has a titre of approximately $3.20\times10^{8} \pm 1.60$ PFU/mL, while phage EniLVP02 has a titre of $2.06\times10^{10} \pm 0.82$ PFU/mL. Meanwhile, after testing various MOIs, it was determined that the ratio that resulted in the highest titre for both phage EniLVP01 and EniLVP02 infecting *V. parahaemolyticus* TA01 was MOI 1.0. Conversely, MOI 100 produced the lowest titre among the tested ratios for both EniLVP01 and EniLVP02 (Figure 5.2). These findings establish MOI 1.0 as the optimal ratio for phage EniLVP02 to infect *V. parahaemolyticus* TA01.

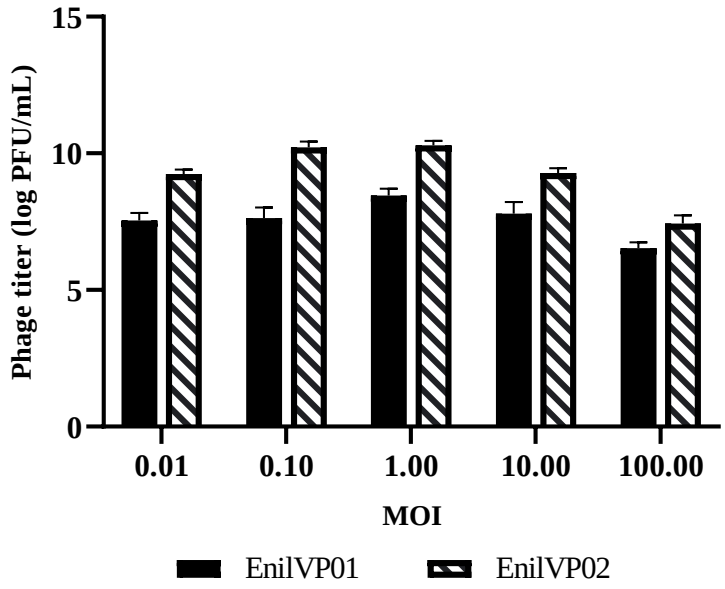


Figure 5.2: Titres of phage EniLVP01 and EniLVP02 at different multiplicity of infections (MOIs) expressed as log CFU/mL.

5.3.5 One-step Growth and Adsorption Curves

The one-step growth and adsorption curves were generated to determine the different phases of EniLVP01 and EniLVP02 infection process using *V. parahaemolyticus* TA01 as

the host (Figure 5.3 and Figure 5.4). MOI 1.0, which was the optimum MOI for the infection process of both phages, was used to generate the curves.

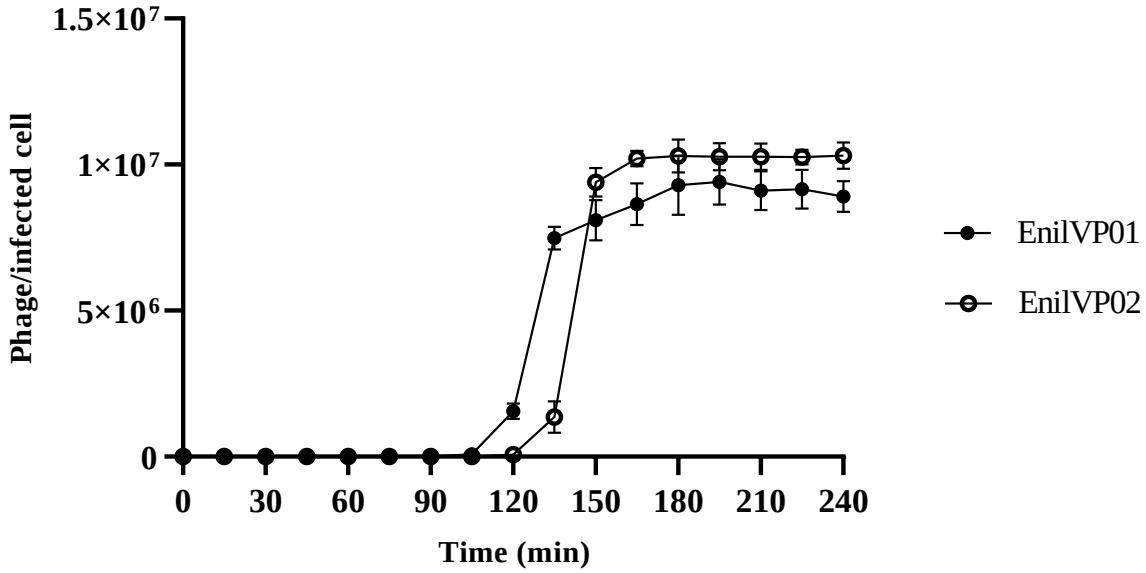


Figure 5.3: One-step growth curve of phage EniLVP01 and EniLVP02 againt *V. parahaemolyticus* TA01 at MOI 1.0.

The one-step growth curve analysis (Figure 5.3) revealed that the phage EniLVP01, propagated on *V. parahaemolyticus* TA01, exhibited a latent period (L) of approximately 105 minutes, whereas phage EniLVP02 had a latent period of approximately 120 minutes. Additionally, phage EniLVP01 and EniLVP02 demonstrated an estimated burst size (B) of 110 and 144 phages per infected cell, respectively. The rise period for phage EniLVP01 lasted approximately 75 minutes, while for phage EniLVP02, it was 45 minutes before reaching the plateau stage of infection.

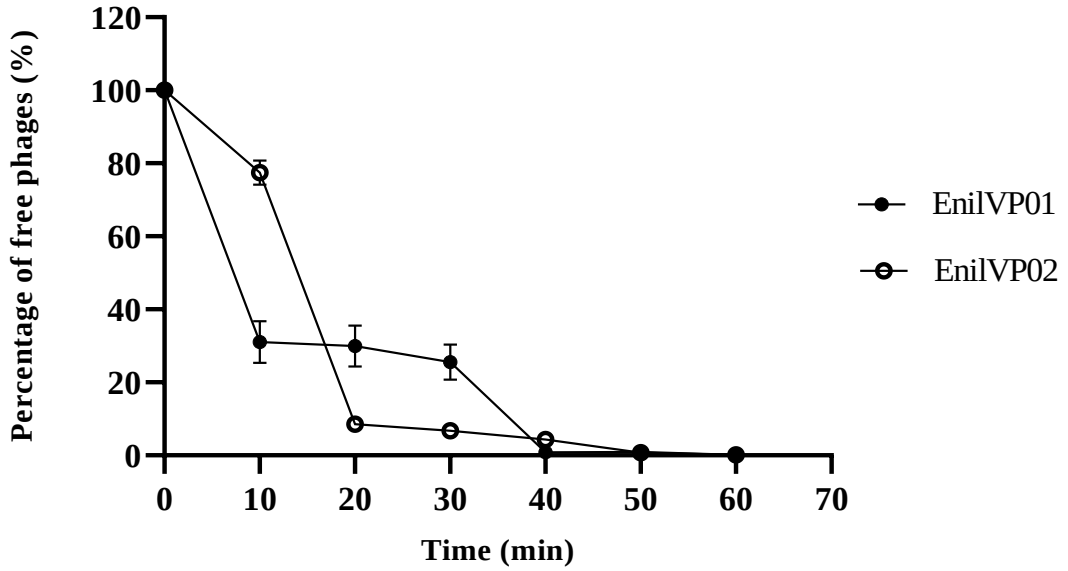


Figure 5.4: Adsorption curve of phage EniLVP01 and EniLVP02 infected with host bacteria, *V. parahaemolyticus* TA01, at MOI 1.0.

The adsorption curve analysis (Figure 5.4) revealed three phases for phage EniLVP01. The initial rapid phase occurred within the first 10 minutes, achieving approximately 70% adsorption. Subsequently, a slow phase occurred between 10 and 30 minutes of infection, with only 5% of phage adsorption. Finally, from 30 to 40 minutes, another rapid adsorption phase occurred, leading to almost 100% adsorption.

EniLVP02 exhibited rapid adsorption in the first 20 minutes of incubation, leading to a reduction in the percentage of free phages to approximately below 10%. Following this, a slow adsorption phase was observed during the 20- to 60-minute interval, with nearly 100% of the phage particles being adsorbed by the end of this period.

5.3.6 Temperature and pH Stability

The stability of phage EniLVP01 and EniLVP02 was assessed at various pH levels and temperatures, and the results are displayed in Figures 5.5 and 5.6 below.

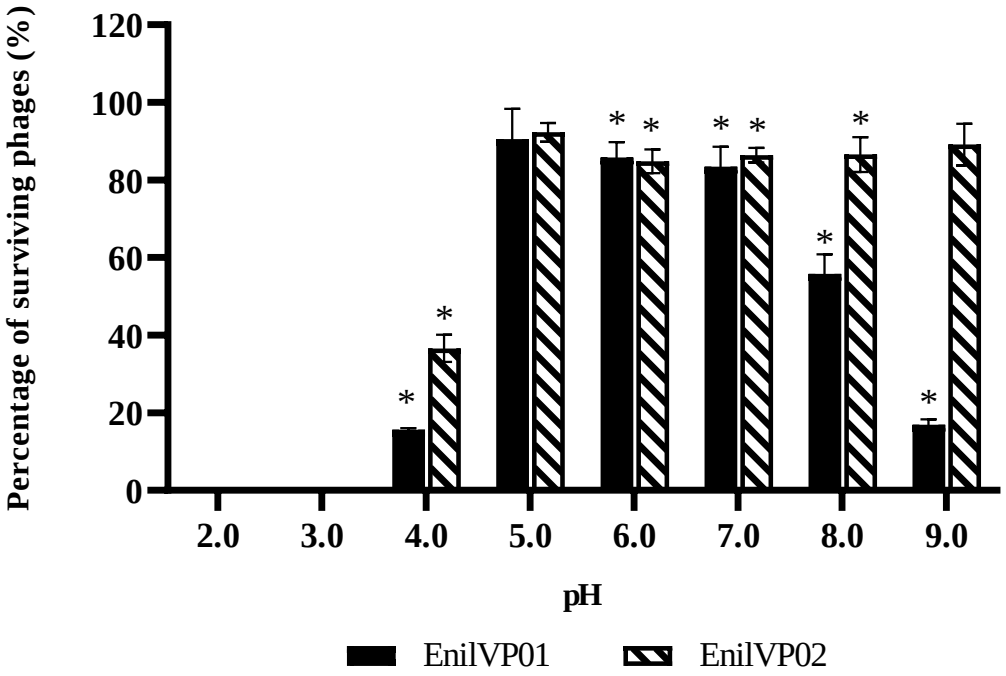


Figure 5.5: Percentage of surviving phages in SM buffer in different pHs. Assays were performed in triplicate and the percentages were expressed as the mean±standard deviation Asterisk (*) indicates a significant difference (P<0.05) compared with the initial phage titre.

The stability assessments of phages EniLVP01 and EniLVP02 across varying pH levels reveal their robustness in adverse environmental conditions (Figure 5.5). EniLVP01 exhibited optimal stability at pH 5.0 ($P > 0.05$), with notable survivability at pH 6.0 and 7.0 with the percentage of surviving phages still above 80% of the initial titre ($P < 0.05$), albeit declining significantly at pH 8.0 and beyond ($P < 0.05$). EniLVP02, on the other hand, demonstrated remarkable stability across the pH range of 5.0 to 9.0 with less than 20% fluctuations in survivability of the phages throughout the range. Furthermore, minimal fluctuations with no significant difference in the percentage of surviving phages were observed at pH 5.0 and 9.0 ($P > 0.05$). Both phages exhibited inactivation at pH 3.0 and below, suggesting their sensitivity to extreme acidity. These findings underscore the

potential of these phages for biotechnological and therapeutic applications, where pH fluctuations may be encountered, further emphasizing their versatility and resilience in diverse environmental conditions.

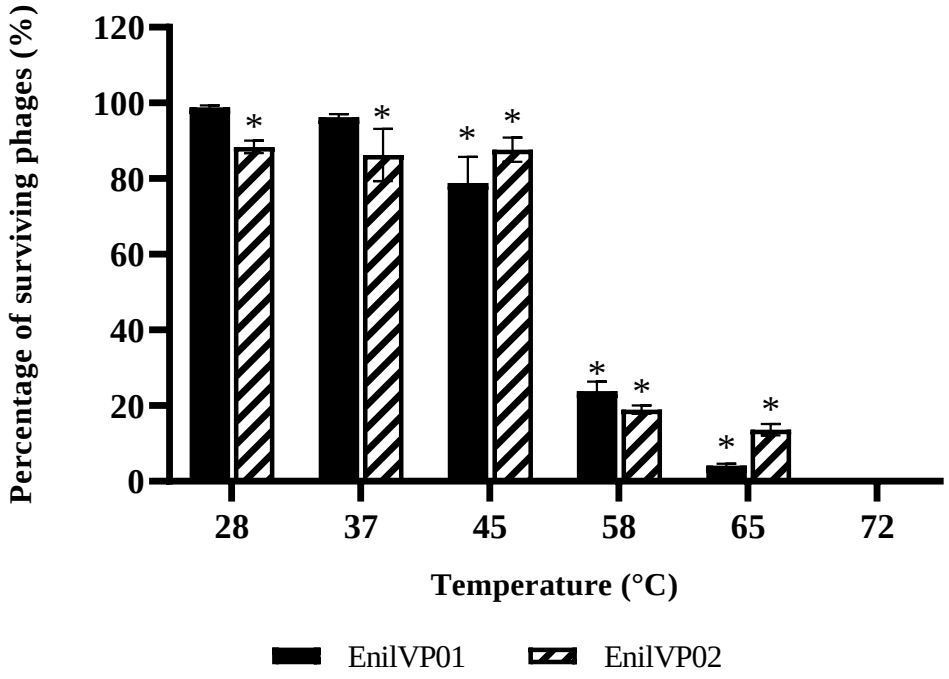


Figure 5.6: Percentage of surviving phages in SM buffer in different temperatures. Assays were performed in triplicate and the percentages were expressed as the mean±standard deviation. Asterisk (*) indicates a significant difference (P<0.05) compared with the initial phage titre.

The findings from the stability assessments of phages EniLVP01 and EniLVP02 reveal intriguing insights into their resilience under varying temperature conditions (Figure 5.6). These results indicate that phage EniLVP01 and EniLVP02 exhibits high stability across a wide range temperature condition. EniLVP01 demonstrated exceptional stability at moderate temperatures of 28 °C and 37 °C, with almost 100% of phages remaining viable ((P > 0.05). Meanwhile EniLVP02 exhibited robust activity across a broader temperature range, maintaining over 80% viability at temperatures up to 45 °C (P < 0.05). However, both phages experienced significant declines in survivability as temperatures escalated beyond

this threshold. At 58 °C and 65 °C, EniLVP01 ($P < 0.05$) and EniLVP02 ($P < 0.05$) exhibited notable decreases in viability, indicating their susceptibility to higher temperatures. Notably, at 72 °C, both phages ceased activity altogether. Overall, these findings demonstrate that phages EniLVP01 and EniLVP02 remain viable across a broad range of temperatures, even without co-culturing with their host bacterium. Understanding this aspect is essential for potential applications in different temperature environments.

5.3.7 Application on Biofilm Prevention and Destruction

The microtitre plate assays demonstrated that the host bacterium *V. parahaemolyticus* TA01 is capable of forming biofilms in TSB supplemented with 2% NaCl after an overnight incubation. Figure 5.7 below illustrates the effects of phage EniLVP02 in preventing and destroying the biofilm at various phage dilutions.

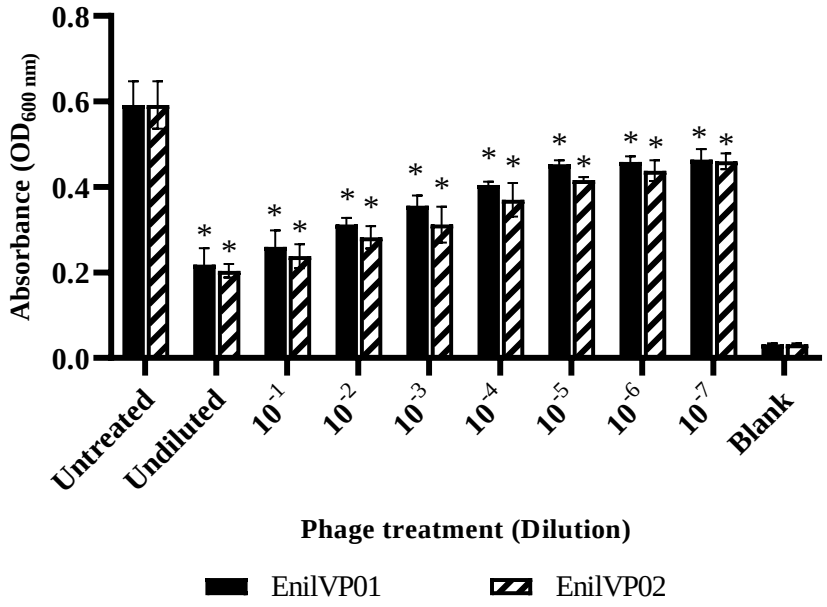


Figure 5.7: Application of phage EniLVP01 and EniLVP02 against *V. parahaemolyticus* TA01 for biofilm prevention at different dilutions. The absorbance values were expressed as the mean±standard deviation. Asterisk (*) indicates a significant difference ($P < 0.05$) compared with the untreated groups.

To evaluate the efficacy of phage EniLVP01 and EniLVP02 in preventing biofilm formation, the phages were co-cultured with host bacteria and incubated overnight. The results revealed that mixtures containing the host bacteria with the undiluted phage suspension for both phages exhibited the lowest absorbance (OD_{600}). In comparison to the bacterial culture without phage treatment, the absorbances were reduced from 0.592 ± 0.055 to 0.218 ± 0.039 for EniLVP01 ($P < 0.05$) and to 0.204 ± 0.016 for EniLVP02. It was further observed that the reduction rate of the phage treatment decreased with increasing dilution. Despite this, the absorbances of the mixture of host bacteria treated with the 10^{-7} dilution of phage EniLVP01 and EniLVP02 demonstrated significant reductions to 0.464 ± 0.024 ($P < 0.05$) and 0.460 ± 0.018 ($P < 0.05$), respectively.

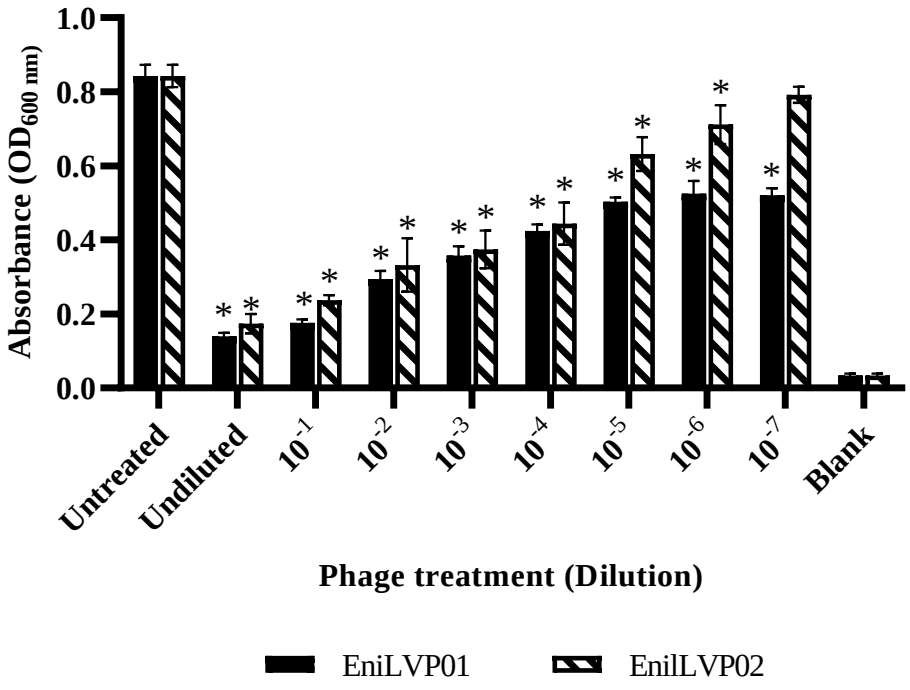


Figure 5.8: Application of phage EniLVP01 and EniLVP02 against *V. parahaemolyticus* TA01 for biofilm destruction at different dilutions. The absorbance values were expressed as the mean±standard deviation. Asterisk (*) indicates a significant difference ($P<0.05$) compared with the untreated groups.

In the biofilm destruction assay (Figure 5.8), a similar pattern to the prevention assay was observed, with the mixtures having the lowest absorbance were those where the host bacteria were incubated with the undiluted phage lysates. In comparison with the host bacteria without phage treatment, which had an absorbance of 0.843 ± 0.029, the mixture treated with the undiluted phage lysate of EniLVP01 and EniLVP02 showed an absorbance of 0.139 ± 0.009 ($P < 0.05$) and 0.174 ± 0.026 ($P < 0.05$), respectively. It was also noted that even at a dilution of 10^{-7}, phage EniLVP01 retained the ability to destroy the biofilm, resulting in a significant absorbance reduction to 0.520 ± 0.018 ($P < 0.05$) after 4 hours of incubation. On the other hand, EniLVP02 at dilution 10^{-7} were only able to cause a reduction in the absorbance to 0.792 ± 0.022 which are not significantly different compared to the untreated group ($P > 0.05$).

These results indicate that both phage EniLVP01 and EniLVP02 possesses the capability to not only reduce the formation of biofilm but also destroy existing biofilms, even at low phage concentration. EniLVP01 exhibited better ability to destroy biofilm, even at lower dilutions, compared to EnilVP02.

5.3.8 Phage Application in Shrimp Samples

The ability of phage EniLVP01 and EniLVP02 to inhibit or reduce bacterial infection in different temperatures was also accessed on shrimp samples. Shrimp samples that were previously sterilised and artificially contaminated with the host bacterium, *V. parahaemolyticus* TA01, were then treated with phage EniLVP01 and EniLVP02. The summary of results to compare the bacterial counts in the untreated and treated samples after 24 hours of incubation were shown in Table 5.4 below.

Table 5.4: The ability of phage EniLVP01 and EniLVP02 to reduce *V. parahaemolyticus* TA01 in shrimp samples at MOI 1.0 after 24 hours. The bacterial count (CFU/mL) were expressed as the mean values. Asterisk (*) represents significant difference ($P < 0.05$) between the treated and untreated groups, while superscripts ($^{a, b, A, B}$) represents significant difference ($P < 0.05$) between different treatment groups in the same culture conditions.

Phage treatment	Culture condition	Bacterial count in negative control after 24 hrs (CFU/mL)	Bacterial count with phage treatment after 24 hrs (CFU/mL)	Percentage of reduction (%)	Log of reduction (log_{10})
EniLVP01	Room temperature	1.59×10^{11}	$6.07 \times 10^{10} *^{a}$	61.8	0.41
	4 °C	6.6×10^{5}	$5.1 \times 10^{5} *^{A}$	22.7	0.11
EniLVP02	Room temperature	1.59×10^{11}	$3.93 \times 10^{10} *^{b}$	75.2	0.61
	4 °C	6.6×10^{5}	$5.53 \times 10^{5} *^{B}$	16.2	0.08
EniLVP01 + EniLVP02	Room temperature	1.59×10^{11}	$3.60 \times 10^{9} *^{ab}$	97.7	1.65
	4 °C	6.6×10^{5}	$5.67 \times 10^{5} *^{AB}$	14.1	0.07

At room temperature, the bacterial count was reduced by 61.8% or 0.41 log10 after 24 hours treatment with phage EniLVP01 ($P < 0.05$). Meanwhile, at 4 °C, equivalent to refrigerator temperature, the bacterial concentration was reduced by 22.7% or 0.11 $\log_{10}$ ($P < 0.05$). The results also indicated a 75.2% or 0.61 $\log_{10}$ reduction in bacterial concentration on shrimp samples when treated with phage EniLVP02 at room temperature relative to the untreated samples (negative control) ($P < 0.05$). Meanwhile, at 4 °C, a lower but significant ($P < 0.05$) reduction of 16.2% or 0.08 $\log_{10}$ was observed in the phage-treated samples compared to the untreated samples.

When the infected shrimps were treated with a cocktail of both phages, a reduction of 97.7% or 1.65 $\log_{10}$ ($P < 0.05$) in the bacterial count was observed at room temperature after 24 hours. However, treatment with the phage cocktail resulted in the lowest reduction, albeit still significant, at 14.1% or 0.07 $\log_{10}$ at 4 °C ($P < 0.05$). From these results, it can be summarised that the mixture of both phages produced the highest reduction in bacterial count at room temperature, while treatment with individual phage EniLVP01 showed the highest reduction at 4 °C. The blank controls did not show any presence of bacteria after the incubation (data not shown).

Based on the student's t-test, comparison between the treated and untreated groups (as indicated by *) revealed that the bacterial concentrations in all of the treated groups, regardless of the incubation temperature, were significant ($P < 0.05$), as indicated in Table 5.4 above. Meanwhile, comparison between groups incubated at room temperature and 4 °C (as indicated by the superscripts) demonstrated significant differences ($P < 0.05$) in the bacterial concentrations between samples treated with individual phages EniLVP01 and EniLVP02 and those treated with the mixture of both phages, respectively. However, no

significant difference (P > 0.05) was observed in the bacterial concentrations between samples treated with the individual phages in both incubation conditions.

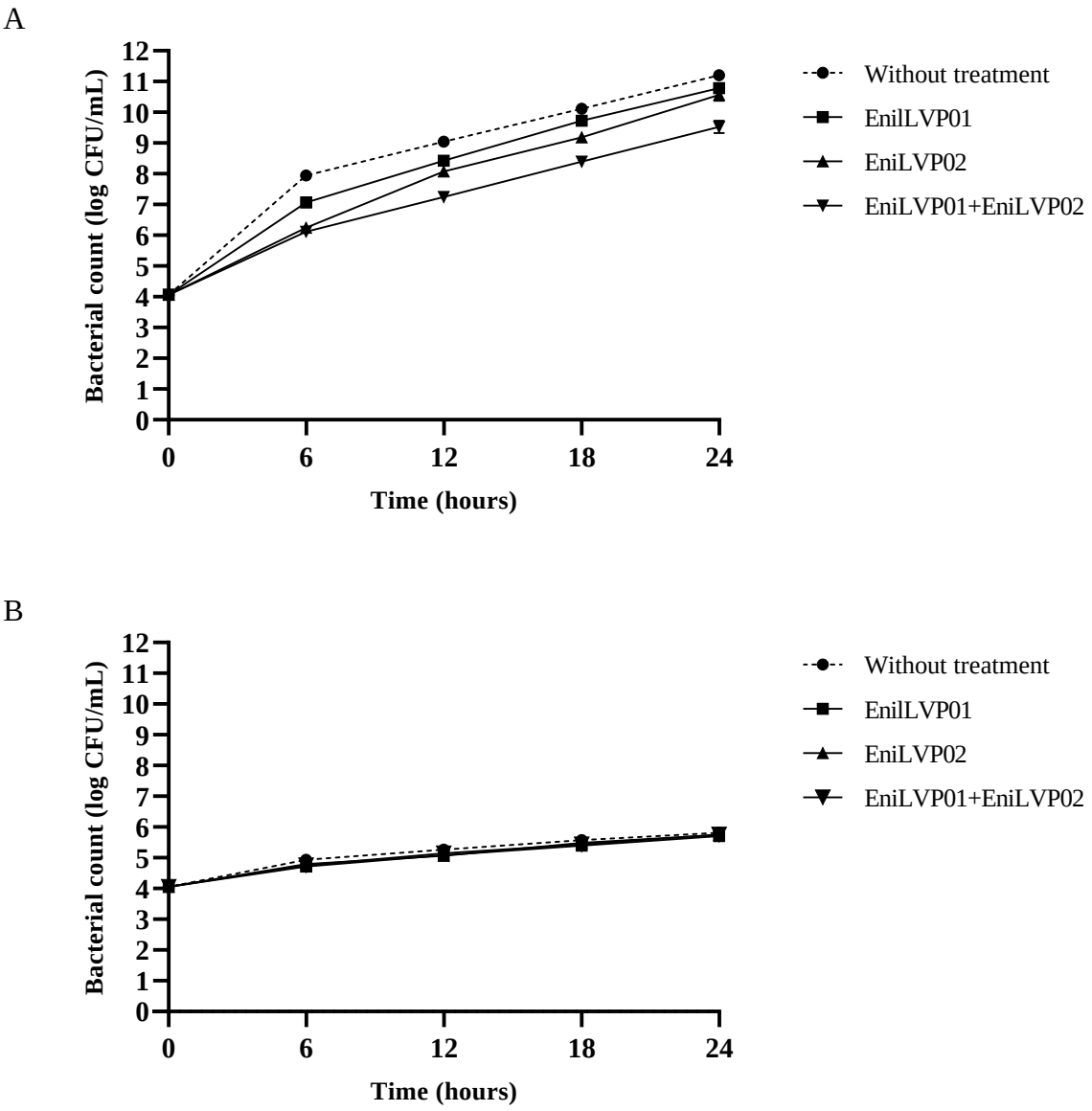


Figure 5.9: Reduction of *V. parahaemolyticus* TA01 in retail shrimps upon phage treatments at (A) room temperature and (B) 4 °C at 6-hours intervals. MOI 1.0 were used for all treatments.

Based on Figure 5.9A, a significant decrease (P < 0.05) was observed after 6 hours of treatment with individual phages EniLVP01 and EniLVP02, respectively, compared to

the untreated samples (negative controls). Substantial reductions were still evident after 6 hours, albeit to a lesser extent, continuing until the 24th hour. In contrast, the phage cocktail displayed a consistent reduction pattern at every 6-hour interval, resulting in the greatest reduction at the 24th hour compared to the treatments with the individual phages.

Meanwhile, treatments at 4 °C exhibited a similar pattern for all three treatments (Figure 5.9B), with substantial reductions ($P < 0.05$) observed after 6 hours and continuing up to the 24th hour at lower rates. However, there were minimal differences observed in the rate of bacterial reductions among the three treatments. Additionally, as highlighted in Table 5.4 and Figure 5.9, the degree and rate of bacterial reduction were significantly lower at 4 °C compared to room temperature. Notably, *V. parahaemolyticus* TA01 in the untreated sample increased rapidly at room temperature compared to at 4 °C.

5.4 Discussion

Vibrio parahaemolyticus is a Gram-negative bacterium frequently encountered in coastal marine ecosystems, encompassing environments like seawater as well as seafood such as oysters, clams, and shrimp. This bacterium has the potential to induce gastroenteritis, a condition marked by symptoms including diarrhoea, vomiting, and abdominal pain (Abdullah Sani et al., 2013). The rise of antibiotic-resistant strains of *V. parahaemolyticus* has prompted the exploration of alternative therapeutic approaches, with bacteriophages emerging as a promising solution.

In this study, bacteriophages of *V. parahaemolyticus* were successfully isolated from environmental and seafood sources, indicating their presence in locations where *V. parahaemolyticus* is commonly detected. Phage EniLVP01 was successfully isolated from an environmental water sample, particularly from a water sample originating from the coastal river area. This aligns with the preferred habitats of *V. parahaemolyticus*, which

require high-salinity environments to survive and propagate (Broberg et al., 2011). In addition to environmental samples, phage EniLVP02 was also successfully isolated from shrimp purchased at a supermarket. Besides being commonly found in water and soil samples, bacteriophages are frequently identified in various seafood products. Shrimp samples, in particular, have been documented as the most diverse source of phages targeting *V. parahaemolyticus* (Thung et al., 2017).

While many studies have traditionally focused on phages isolated from environmental samples, investigations into phage isolation from retail food products, including seafood, have only recently gained popularity. A study by Tan et al. (2021) successfully isolated six different phages of *V. parahaemolyticus* from various seafood products such as blood clam, surf clam, and shrimp. Considering the ubiquity of *V. parahaemolyticus* in high-salinity environments and seafood products, it is reasonable to hypothesize that phages, abundant as they are and possessing the ability to lyse them, are also present. This hypothesis is based on the understanding that a high concentration of host bacteria in an environment may indicate the concurrent presence of their phages (Tan et al., 2021).

The double-layer agar assay demonstrated that phage EniLVP01 and EniLVP02 possess the capability to form clear plaques on a *V. parahaemolyticus* lawn, confirming their lytic nature. In the context of phage therapy, lytic phages are typically favoured over lysogenic or temperate phages owing to their immediate bactericidal action, aligning with the primary objective of phage therapy. Conversely, lysogenic phages may raise safety concerns due to potential genetic alterations in the host bacterial genome through horizontal gene transfer (Naureen et al., 2020). The insertion of foreign genes into the bacterial genome can cause the bacteria to undergo mutations that lead to the emergence of new traits,

including antibiotic resistance and virulence (Emamalipour et al., 2020). Therefore, the preference for lytic phages stems from their ability to swiftly eliminate target bacterial cells without causing any side effects, a crucial attribute in the context of therapeutic applications.

In addition to their lytic nature, it was found that both phage EniLVP01 and EniLVP02 similarly have a narrow host range, as determined by the host specificity test. They selectively lyse a limited number of *V. parahaemolyticus* strains, particularly the strains that were isolated from the shrimp farm, with no observed impact on other non-*V. parahaemolyticus* strains. This attribute can be advantageous, as phages with specific host ranges exhibit affinity only for the target bacteria, facilitating their targeted elimination while leaving mammalian cells or other non-targeted bacteria unharmed (Drulis-Kawa et al., 2012; Knecht et al., 2020). Consequently, they are regarded as non-toxic, especially to humans, and are deemed safe for in vivo applications.

However, in addition to non-*V. parahaemolyticus* strains, phage EniLVP02 is also not infective towards *V. parahaemolyticus* ATCC® 27969^{TM} and other *V. parahaemolyticus* strains from retail sources. This implies that the phage is limited to infecting only the bacterial strains that could potentially coexist with each other (Stenholm et al., 2008). Another possible reason is that the other strains may already have resistance to phages (Lal et al., 2016a). Fortunately, bacteria that exhibit resistance to bacteriophages are frequently observed to be non-virulent, or their virulence factors may have been diminished (Laanto et al., 2012). This phenomenon can be supported by the fact that the surface structures of bacterial cells serving as receptors for bacteriophages may also function as virulence factors in the bacterial strains. Modification of these structures can lead to simultaneous resistance to phages as well as reduction in the virulence factors (León and Bastías, 2015). The specificity of phage EniLVP01 and EniLVP02 towards limited number of strains may also

be caused by their tail fibre proteins, which only encodes a single tail fibre protein (Gao et al., 2022). Our finding aligns with the results reported by Lal et al. (2016a), where they isolated a *Siphoviridae* phage, phage VpKK55, capable of lysing a narrow range of *V. parahaemolyticus* strains.

Phages are categorised into order, families and genera using specific criteria set by the International Committee of Taxonomy of Viruses (ICTV), based on visible traits of the phage particles (Ackermann, 2009a). Based on the TEM image, the structural analysis of both phage EniLVP01 and EniLVP02 categorises them within the *Straboviridae* (former *Myoviridae*) family, belonging to the *Caudovirales* order. *Myoviridae* phages are commonly reported to be larger than other groups (Ackermann, 2009a). Similar to *Escherichia coli*'s T4 phage, EniLVP01 and EniLVP02 exhibit distinctive features: an icosahedral capsid enclosing its genetic material, a contractile tail with sheath, a baseplate, and tail fibres. Among these traits, the defining characteristic distinguishing *Myoviridae* from other bacteriophage families is the presence of a contractile tail. This specific tail structure facilitates the efficient infection of host bacteria, enabling a rapid and forceful injection of phage genetic material upon attachment to the host cell (Leiman and Shneider, 2012). The phage's baseplate serves as a nerve centre, transmitting signals to the capsid upon binding to the host cell (Yap et al., 2016). In addition to sending signals, the baseplate also changes its confirmation which triggers the tail sheath to contract and to thrust the internal tail tube through the bacterial cell wall (Aksyuk et al., 2009; Hu et al., 2015). Both processes lead to the release of genetic materials through the phage's tail tube into the bacterial cytoplasm, initiating the infection process.

During the infection process, the rapid phase in the adsorption of phage EniLVP01 and EniLVP02 occurred within 10 minutes and 20 minutes, respectively, of incubation with

the host bacteria. The latent periods of phage EniLVP01 and EniLVP02 lasted for a long time at more than 100 minutes with a large burst size of 110 and 144 phages per infected cell, respectively. Phages with a burst size exceeding 100 PFU per infected cell are generally considered more effective in their lytic activities against host bacteria, making a large burst size a desirable characteristic for most phage therapy candidates (Okano et al., 2007; Lal et al., 2016a). Moreover, a large burst size implies that a lower initial concentration of the phage is required to initiate the infection (Marashi et al., 2022).

Although EniLVP01 and EniLVP02 have longer latent periods compared to other Vibriophages, such as phage VpKK5 with a 10-minute latent period and phage PW2 with a 30-minute latent period, it is comparable to phage KHP30 infecting *Helicobacter pylori*, which has a latent period of 140 minutes (Phumkhachorn and Rattanachaikunsopon, 2010; Uchiyama et al., 2013; Lal et al., 2016a). The latent period of a phage is influenced by various factors, including host physiology and quantity. It has been asserted that the latent period of a phage shortens when the density or quality of the host bacteria is high (Wang et al., 1996; Abedon et al., 2001). Thus, optimising the host bacterial culture to achieve both high quality and quantity could potentially lead to a shortened latent period in the phage replication dynamics. In addition to host attributes, the latent period is also subject to the influence of holin, a phage protein complex encoded by holin genes. This protein regulates the timing of phage-induced bacterial cell lysis by controlling the activity of cell-wall digesting endolysins. Consequently, mutations in the genes encoding this protein can impact the timing of bacterial cell lysis (Young et al., 2000; Abedon et al., 2001).

The ability of different bacteriophages to survive in different environments is highly diverse (Jończyk et al., 2011). Even though bacteriophages experiences decay in their lytic ability over time, determining the conditions in which they are the most stable can help to

extend their survivability (Rotman et al., 2020). Therefore, assessment of phage EniLVP01 and EniLVP02's stability under varying pH conditions and temperatures without the presence of the host is crucial for understanding their potential applications on food products as well as to determine conditions that are optimum for their long-term storage.

Variations in the results of these two phages within the tested pH range reveal differences in their tolerance towards different pHs. Notably, Phage EniLVP02 demonstrated stability within the pH range of 5.0 to 9.0 and still viable at pH 4.0. In contrast, EniLVP01 exhibited stability at pH 5.0 – 7.0, while also remaining viable at pH 4.0, 8.0, and 9.0. This tolerance suggests that the phages maintain their infectivity and efficacy within the slightly acidic to slightly alkaline pH conditions commonly encountered in various environments, including those relevant to potential therapeutic applications. However, a significant decrease in phage activity occurred at pH 3.0 and below, resulting in phage inactivation. These findings align with those of Lal et al. (2016a), who also reported that their isolated phage, phage VpKK5, remained stable at pH 4.0 to 9.0 but deactivated at pH 2.0 to 3.0.

It has been reported that the titre of a bacteriophage experiences a gradual decline with decreasing pH (Ly-Chatain, 2014). Studies indicate that variations in hydrogen ion concentrations, which impact the pH of the storage medium, can induce phage aggregation, resulting in a decrease in phage titre. It has been observed that when the pH is equal to or below the phage isoelectric point, phages exhibit a heightened propensity to aggregate with each other (Langlet et al., 2007; Jończyk et al., 2011). Hence, it is plausible that EniLVP02 may have a higher isoelectric point compared to EniLVP01, which could explain the former’s higher tolerance towards a wider range of pH compared to the latter.

The vulnerability of phage EniLVP01 and EniLVP02 to highly acidic conditions may prompt considerations for practical applications, especially in environments with low pH, such as specific stages of food processing or the acidic conditions of the human stomach. Fortunately, for application in seafood products, the pH levels are generally not as low or rarely reach such acidity. For instance, the pH of fresh shrimp typically falls within the range of 6.0 to 7.0, while the pH of frozen raw shrimp is slightly higher, typically ranging from 7.3 to 7.8, with values never exceeding pH 8.0 (Okpala, 2015; Condurso et al., 2016). Additionally, reports indicate that the presence of pathogenic bacteria contaminating food at pH levels less than 4.5 is also low (Ly-Chatain, 2014). Therefore, phage EniLVP01 and EniLVP02 remain viable candidates for application in frozen seafood products that do not have a pH as low as pH 3.0.

Both phages demonstrate practicality not only for application in frozen seafood products but also in shrimp farms, owing to their high stability within the pH range of 5.0 to 7.0. As previously discussed in Chapter 3, pond water in the Telaga Air shrimp farm typically maintains a slightly acidic to neutral pH range (pH 5.9 – 7.9). This range is crucial as pH 6.5 to 9.0 is optimal for shrimp growth (Nilsa-ad, 2023; Supriatna et al., 2023). Therefore, phage EniLVP01 and EniLVP02 hold potential for utilisation not only in food product applications but also in the shrimp farming contexts.

Other than pH, temperature is also one of the factors that affects the survival of bacteriophages. The temperature stability test results reveal EniLVP01 and EniLVP02 to have similar high resistance to temperature variations within certain limits. Notably, both phages showed high tolerance at the tested temperatures of 28 °C, 37 °C, and 45 °C. This high thermostability at physiologically relevant temperatures, including those resembling ambient and human body temperatures, is promising for potential therapeutic applications,

where maintaining phage activity under these conditions is crucial (Malik et al., 2017). As the temperature increased beyond these physiological ranges, specifically at 58 °C and 65 °C, the percentage of surviving phages decreased, but still indicating substantial stability. This decline suggests a temperature-dependent reduction in phage stability, where high temperatures can affect the integrity of the phage's capsid and genetic material (Jończyk et al., 2011; Liu et al., 2023). However, at 72 °C, no phage activity was detected for both phages, indicating complete inactivation.

This finding aligns with the known susceptibility of many phages to high temperatures, reflecting the denaturation of their structural proteins, loss of lipid, and structural changes in their genetic materials (Jończyk et al., 2011; Malik et al., 2017) Furthermore, in contrast to phage VpKK55, which exhibits measurable lytic activity only up to 50 °C, EniLVP01 and EniLVP02 demonstrates higher tolerance, enduring temperatures as high as 65 °C (Lal et al., 2016a). This high thermostability positions phage EniLVP01 and EniLVP02 as a potential candidate for application in diverse conditions with varying temperature ranges.

The stability of phages EniLVP01 and EniLVP02 across diverse environmental conditions may be attributed to their specific structural features. It has been reported that tailed phages, such as those belonging to the *Caudovirales* order, to which both phages belong, exhibit high stability under adverse conditions compared to other phage types (Ackermann et al., 2009b; Jończyk et al., 2011). Interestingly, according to Ackermann et al. (2009b), tailed phages stored at 4 °C can maintain their viability even after 10 to 12 years in storage. Additionally, studies suggest that phages with larger capsids, approximately 100 nm in diameter, tend to display greater resilience than those with smaller capsids, typically around 60 nm in diameter (Jończyk et al., 2011). Although the capsid diameters of phages

EniLVP01 and EniLVP02 are approximately 67.0 nm and 75.0 nm, respectively, which is below the reported 100 nm threshold, they are still larger than the less stable counterparts. Moreover, the higher stability of EniLVP02 compared to EniLVP01 may also be explained by the larger size of the former's capsid compared to the latter's. Consequentially, the substantial capsid size of EniLVP01 and EniLVP02 could potentially contribute to their ability to endure a broad spectrum of external factors, thereby enhancing their overall stability.

In addition to capsid size, the characteristics of a phage's tail have been proposed to play a role in determining its thermal stability (Jończyk et al., 2011). For instance, the *Siphoviridae* phage TSP4, isolated from a hot spring, features a remarkably long and flexible tail, measuring approximately 785 nm in length and 10 nm in width. This phage exhibits high thermostability, with an optimum temperature recorded at 65 °C (Lin et al., 2010). Furthermore, when comparing the stability of both phages, phage EniLVP02, which has higher pH and thermal stability than phage EniLVP01, was also found to possess a tail length of approximately 70.0 nm, while phage EniLVP01 has a shorter tail measuring approximately 43.0 nm in length. Therefore, it is plausible that the phage's structural characteristics can contribute to their overall stability. It is also important to note that while most phages are tolerant towards freezing and thawing, repeating these processes in short intervals may lead to the loss of phage stability (Jończyk et al., 2011).

The study also investigated the ability of phage EniLVP01 and EniLVP02 to prevent and destroy biofilm formation. *V. parahaemolyticus* possesses the capability to form biofilms, which are among the key factors contributing to its pathogenicity (Gao et al., 2022). *Vibrio parahaemolyticus* employs two distinct flagellar systems, depending on environmental conditions, to adapt to changes. Polar flagella enable bacterial swimming,

while lateral flagella are activated when motile cells transform into swarmer cells, subsequently facilitating biofilm formation (Wang et al., 2015). Bacterial biofilm formation causes many complications including difficulties for treatment drugs to penetrate to reach bacterial cells, as well as compromising the host's immune response. Additionally, the accumulation of many bioactive compounds such as enzymes in the biofilm can affect host physiology and alters ambient pH levels which subsequently affects various biological functions (Topka-Bielecka et al., 2021). Recent studies have reported that biofilms play roles in contributing to antibiotic resistance in bacteria, potentially owing to high cell density, close proximity between cells, and the accumulation of mobile genetic elements (MGEs) carrying antimicrobial resistance genes (AGRs). Both phages demonstrated a significant capacity to control biofilm formation and dismantle mature biofilms produced by *V. parahaemolyticus*, whether in undiluted or diluted lysate form. These characteristic positions phage EniLVP01 and EniLVP02 as potential candidates for biocontrol, whether applied individually or as part of a phage cocktail (Gao et al., 2022).

One of the mechanisms contributing to the phages ability to eradicate biofilms involves their capacity to encode enzymes known as depolymerases (which can be divided into hydrolases and lyases) and lysins (or endolysins), capable of degrading biofilms (Liu et al., 2022). These enzymes participate in the degradation of capsular polysaccharides and peptidoglycan within bacterial cells, leading to the death of the host bacteria and, consequently, impeding biofilm formation. Furthermore, these enzymes facilitate phage penetration, enabling them to eradicate existing biofilms by degrading the bacterial polysaccharides, and as a result dismantle the bacterial defence barrier (Chang et al., 2022; Dewanggana et al., 2022). This explanation finds further support in the discovery of open

reading frames (ORFs) encoding putative hydrolase and tail lysozyme (a type of endolysin) enzymes within the genomes of both phages, as detailed in Chapter 6.

Based on the results of the application of phage EniLVP01 and EniLVP02 to treat *Vibrio*-infected shrimps, significant reductions were observed when comparing the negative control groups with all treated groups at both room temperature and 4 °C. This demonstrates that phage EniLVP01 and EniLVP02 can effectively lyse the targeted bacterial cells not only in media but also in shrimp matrices. It was observed that EniLVP01 and EniLVP02 were more effective in reducing bacterial concentration at room temperature compared to at 4 °C. Similar pattern was also observed with the phage cocktail, where higher bacterial reduction was observed at room temperature than at 4 °C. Several factors may contribute to this, with one of them being the impact of temperature on the growth of the host bacteria. The bacterial count in the shrimp samples incubated overnight at 4 °C was notably lower compared to the samples incubated at room temperature. For most bacteria, temperatures lower than 12 °C often render the cells inviable. Since phages are host-dependent for their self-replication, fewer viable cells mean fewer cells available for infection (Ly-Chatain, 2014; Waturangi et al., 2021).

Furthermore, it was observed that samples treated with the combination of both phages yield greater reduction in the bacterial load compared to the individual treatments. For narrow host range phages like phage EniLVP01 and EniLVP02, combining them with other phages that have different hosts can broaden the host range of the treatment by increasing the spectrum of action toward other strains or even species. Furthermore, development of phage resistance by bacteria, which can quickly develop with one phage application, can be prevented or delayed when phages are combined in a cocktail (Haines et al., 2021; Ribeiro et al., 2023).

In phage therapy, the administration of multiple phages in the form of a cocktail is more common than using a single phage for treatment. When multiple phages are administered in a cocktail to treat a host bacterium, the effect can either be additive (synergy), where the lytic activity is increased compared to when a single phage is used, or subtractive (interference), where the combination of multiple phages causes the bacterial lysis rate to be reduced (Schmerer et al., 2014). In our study, although both phages have the same narrow host range, their combination in a phage cocktail was tested on the infected shrimps to assess their compatibility and effectiveness. It was found that the cocktail of phages EniLVP01 and EniLVP02 was able to reduce bacterial count more effectively than when each phage was used individually. This shows that the combination of both phages leads to synergistic effects, where the combined action of the phages enhances their overall efficacy compared to individual phages alone. This could be due to complementary mechanisms of action wherein the growth of one phage augments the infection ability of the other phage (Schmerer et al., 2014; Naghizadeh et al., 2019). A study by Malik et al. (2021) showed that combination of four phages in the form of a cocktail resulted in 86.7% lysis of the clinical isolates of *Escherichia coli*, while treatments using each phage resulted in only 50% to 66% lysis. From a phage therapy standpoint, synergy between phages targeting either the same host bacterium or multiple bacteria are an advantage that can enhance treatment efficiency.

5.5 Chapter Summary

In this chapter, two *Straboviridae* (formerly *Myoviridae*) phages capable of lysing *V. parahaemolyticus* were successfully isolated from environmental and retail sources, respectively. This finding underscores the presence of phages in the environmental and retail samples around Kuching, Sarawak. This chapter also highlights the promising attributes that

position both phages as viable candidates for phage therapy applications, as well as offering valuable insights into their potential application against bacterial infections, particularly in seafood products, and against bacterial biofilms. Our results reveal that phages EniLVP01 and EniLVP02 not only target the host bacterial biofilm but also exhibit antagonistic activity against bacteria in shrimp matrices. Further investigations involving other food matrices will be crucial in determining whether these phages could serve as potential candidates for utilisation as food preservatives.

Additionally, it was observed that the combination of both phages at room temperature leads to enhanced lytic ability in infected shrimps. Therefore, investigating the compatibility of both phages with other phages targeting diverse host bacteria within a phage cocktail is crucial to expanding the range of targeted bacteria and to determine their compatibility. This study will evaluate the stability of the phage cocktail and elucidate whether their collective actions result in additive or subtractive effects. In the next chapter, genome characterisation of phages EniLVP01 and EniLVP02 was performed to understand their genetic makeup further and to validate whether both phages are safe and practical for phage therapy and biotechnological applications.

CHAPTER 6

GENOME CHARACTERISATION OF VIBRIOPHAGES EniLVP01 AND EniLVP02 SPECIFIC TO SHRIMP STRAIN OF *Vibrio parahaemolyticus*

6.1 Overview

Phages are highly genetically diverse and due to horizontal gene transfer with other organisms such as bacteria and other phages, their genome are characteristically mosaic and complex (Mavrish and Hatfall, 2017). Although mosaicism in not unique to phages only as horizontal gene transfer also happened among bacteria, it was reported that the extent of this event is greater in bacteriophages (Hatfull and Hendrix, 2011). With the emergence of numerous novel bacteriophages, genomic characterisation becomes a crucial step for gaining a deeper understanding of these phages. In the context of phages, the characterisation of their genomic DNA not only provides information about their mechanisms and reproduction but also sheds light on their interactions with bacterial communities, particularly their hosts. Phages have been reported to play a significant role in host genome evolution, including their ability to encode various toxins and gene products that can either positively or negatively impact bacterial cells, especially their hosts with which they maintain a lysogenic relationship (Clokie and Kropinski, 2009).

Another intriguing aspect regarding phage genomic characteristics is the high genetic diversity even among phages that infect the same host bacteria (Clokie and Kropinski, 2009). This diversity underscores the complexity of phage-host interactions and suggests an intricate relationship between phage genetics and bacterial physiology. Understanding this genetic diversity is crucial, particularly in the context of phage therapy, where the safe and

effective application of phages relies on a comprehensive grasp of their genomic variability (Clokie and Kropinski, 2009).

The process of characterising the genome of phages is often more complex compared to bacteria due to the absence of universally conserved region. In bacteria, for example, 16S rRNA gene is often used as the marker for the identification of bacteria (Tran et al., 2017). However, due to their diverse and mosaic genome, phages lack the conserved markers, and only minor regions of the genome are used as family specific markers (Rogovski et al., 2021). For example, the gene that encode viral capsid g20 is often used to identify phages that belongs to the T4 bacteriophages family (Sullivan et al., 2008; Rogovski et al., 2021).

Within the realm of phage-encoded protein exploration, Whole Genome Sequencing (WGS) stands as an important application, renowned for its comprehensive approach in elucidating the intricate complexities inherent in these phage genomes (Clokie and Kropinski, 2009). The advantages of WGS method for viral genome sequencing include the ability to provide details of the complete genome which includes both coding and non-coding regions, ability to detect significant genetic variations that could escape detection through targeted methods, as well as the ability to identify new and potentially important genetic variations that are suitable for further investigation. Furthermore, WGS has the ability to generate substantial quantities of data rapidly, which can streamline the process of novel genome assembly at a relatively low cost (Hyman, 2019; DeWitt, 2024).

In this chapter, the molecular characterisation of the previously isolated EniLVP01 and EniLVP02 was undertaken using Next Generation Sequencing (NGS), also known as second-generation sequencing, a high-throughput sequencing technology that enables rapid sequencing of DNA or RNA samples (Slatko et al., 2018). Based on the sequencing data, bioinformatic analysis was conducted to assemble and annotate the genomes, predict protein

functions, and determine the phylogenetic and genetic relationships with other phages in existing databases. Through this process, a better understanding of the genetic makeup of the phages and their interactions with their hosts can be gained, ensuring their safety and suitability for phage therapy applications.

6.2 Materials and Methods

6.2.1 Polyethylene Glycol (PEG) Phage Precipitation

Prior to DNA extraction, the phage lysate underwent concentration using the Polyethylene glycol (PEG) precipitation method as devised by Antibody Design Lab, San Diego, CA (2015). Subsequently, purified phages from the previous chapter (Chapter 5) were concentrated through the addition of 5x PEG/NaCl solution, comprising 20% (w/v) PEG-8000 (Sigma-Aldrich, St. Louis, USA) and 2.5M NaCl, to 1,200 µL of the phage lysate. The resulting mixture was gently inverted several times to ensure thorough mixing and was then incubated at 4 °C overnight to facilitate virion precipitation.

On the subsequent day, the precipitated virions were pelleted via centrifugation at 13,000 ×g for 3 minutes. The supernatant was carefully removed without disturbing the pellet. After complete removal of the supernatant, the pellet was resuspended in 120 µL of SM buffer by vigorous vortexing. Subsequently, the suspension was incubated on ice for one hour to facilitate the complete diffusion of virions into the buffer. Following the incubation, the phage solution was vortexed once more and then subjected to centrifugation for 1 minute at 13,000 ×g to clarify the solution. The resulting supernatant was transferred to a fresh sterile microcentrifuge tube and stored at 4 °C for subsequent analysis (Antibody Design Lab, 2015).

6.2.2 Genomic DNA Extraction

Phage genomic DNA extraction was performed using the phenol-chloroform extraction method as described by Ceelen (2019) with slight modifications. Upon concentration of the phages through the PEG precipitation method, 1 mL of the phage lysate was combined with 12.5 µL of 1M MgCl2. The resulting phage-$MgCl_2$ mixture was then treated with 1.25 µL DNAse I (1U/µL) and 6.25 µL RNase A (4 mg/mL) and incubated statically at 37 °C for one hour. Following the incubation, 20 µL of 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0, 1.25 µL of Proteinase K (20 mg/mL), and 25 µL of 10% sodium dodecyl sulfate (SDS) were added to the mixture, and then incubated at 60 °C for another one hour. An equal volume of phenol: chloroform (1:1) was added to the cooled mixture and thoroughly mixed by inverting the tube several times. The mixture was then centrifuged at 13,000 rpm for 5 minutes at room temperature, and the supernatant was transferred to a new microcentrifuge tube. These extraction steps were repeated once more using phenol: chloroform, followed by another repetition with an equal volume of chloroform.

After centrifugation, 1/10 volume of 3 M sodium acetate (NaOAc; pH 7.5) and 2.5 volumes of ice-cold 100% ethanol were added to the resulting supernatant. The mixture was incubated overnight at -20 °C. The following day, the mixture was centrifuged at 13,000 rpm for 20 minutes at 4 °C. The resulting pellet was washed twice with 70% ethanol before the tube was air-dried for approximately 15 to 30 minutes. The washed pellet was then resuspended in 100 µL TE buffer (pH 7.6). The presence of genomic DNA was confirmed by using 1% agarose gel electrophoresis (100 V, 60 min). The DNA was stored at -20 °C until further analysis.

6.2.3 DNA Sequencing and Analysis

Before sequencing, the extracted genomic DNA underwent assessment for purity and concentration using the DeNovix DS-11 UV-vis spectrophotometer (Labgene Scientific SA, Switzerland). This step was crucial to ascertain the quality and adequacy of the DNA for subsequent sequencing processes.

The extracted phage DNA was sequenced by using the Next Gene Sequencing (NGS). Illumina NovaSeq 150 PE run whole-genome sequencing was carried out by Apical Scientific Sdn. Bhd. (Selangor, Malaysia). The quality-controlled reads were subsampled at random using seqtk_sample and then assembled *de novo* using Shovill-SPAdes v1.1.0. Phage genomes were annotated using Pharokka v1.3.0 (Bouras et al., 2022) followed by manual curation. Coding sequences (CDS) were predicted with PHANOTATE v1.5.1 (McNair et al., 2019) and tRNAs were predicted with tRNAscan-SE v2.0.11 (Chan and Lowe, 2019). The genome maps were constructed using Proksee (Grant et al., 2023).

6.2.4 Construction of Phylogenetic Trees

Phylogenetic trees were constructed based on the putative terminase large subunit and DNA polymerase amino acid sequences. Phylogenetic affiliations with each gene of interest were obtained from GenBank database by using the basic local alignment search nucleotide tool (BLASTP). The sequences were then aligned using MUSCLE and the phylogenetic relationships were constructed using the neighbour-joining method with bootstrap value of 1000 resamplings using MEGA5.2 software (Kumar et al., 2018).

6.2.5 Nucleotide-based Intergenomic Similarity Analysis

Intergenomic similarity analysis was performed on phage EniLVP01, EniLVP02 and 15 other related *Vibrio* phages that were also used in the phylogenetic tress by using the

Virus Intergenomic Distance Calculator (VIRIDIC), which are available on the web server https://rhea.icbm.uni-oldenburg.de/viridic/ (Moraru et al., 2020). The whole genome sequences of the phages were obtained from the publicly available Genbank database by using the basic local alignment search nucleotide tool (BLASTN).

6.3 Results

6.3.1 Genome Sequence Analyses

Prior to sequencing, the presence of the extracted DNA was validated through 1% gel electrophoresis to ensure sufficient DNA was available. A single band was observed for each phage, with a size exceeding 10,000 bp (Figure 6.1).

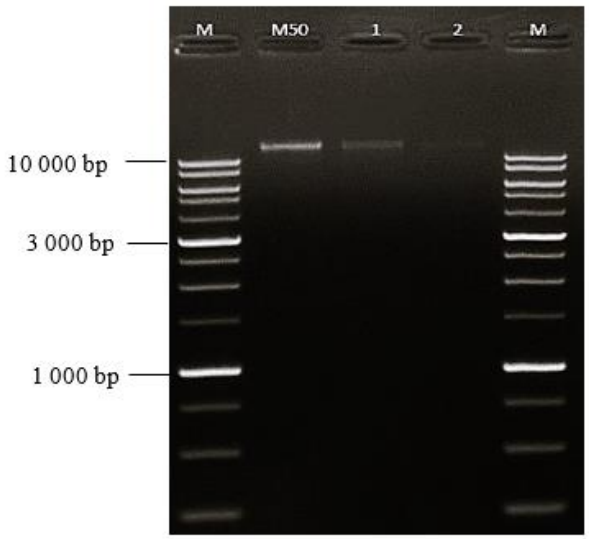


Figure 6.1: Gel electrophoresis of the extracted genomic DNA (1µL) of the phage lysates. Lane M: 10 000 bp DNA ladder; Lane M50: positive control (bacterial genomic DNA template); Lane 1: genomic DNA of EniLVP01; Lane 2: genomic DNA of EniLVP02.

The characteristics of genome sequences of EniLVP01 and EniLVP02 are summarised in Table 6.1 below. Both phages EniLVP01 and EniLVP02 genomes were consisted of double-stranded DNA (dsDNA) with both of their sizes estimated to be 186,715 base pairs with G+C contents of 48.35% and 48.34%, respectively. Annotation analysis of

the sequences predicted 268 open reading frames (ORFs) for phage EniLVP01 and 269 ORFs for EniLVP02. The predicted coding sequences (CDS) of EniLVP01 and EniLVP02 accounting for 94.34% and 94.44% of the total genomes, respectively. Based on the annotation of the putative functional proteins in EniLVP01 genome, 101 CDSs had significant homology to the sequences in the database (Table 6.2), while 167 CDSs were predicted as hypothetical protein (data not shown). Meanwhile for phage EniLVP02 genome, 102 CDSs had significant homology to the sequences in the database (Table 6.3)., and 167 CDSs are designated as putative hypothetical proteins (data not shown).

Table 6.1: Summary of characteristics of genome sequences of phage EniLVP01 and EniLVP02.

Phage	**EniLVP01**	**EniLVP02**
Origin	Water sample	Shrimp sample
Total genome (bp)	186,715	186,715
G+C contents	90,280 (48.35%)	90,267 (48.34%)
Total ORFs	268 (94.34%)	269 (94.44%)
Rightward (+) strand	130	139
Leftward (-) strand	138	130

Figure 6.2 below illustrates the genome maps of phages EniLVP01 and EniLVP02, showing the predicted molecular functions of identified genes. The annotation of the coding sequences (CDSs) in both phages categorizes the predicted proteins into six functional groups related to tail and connector assembly, head and packaging, host lysis and lysis inhibition, DNA and RNA nucleotide metabolism, tRNAs, and others, such as post-translational modifications.

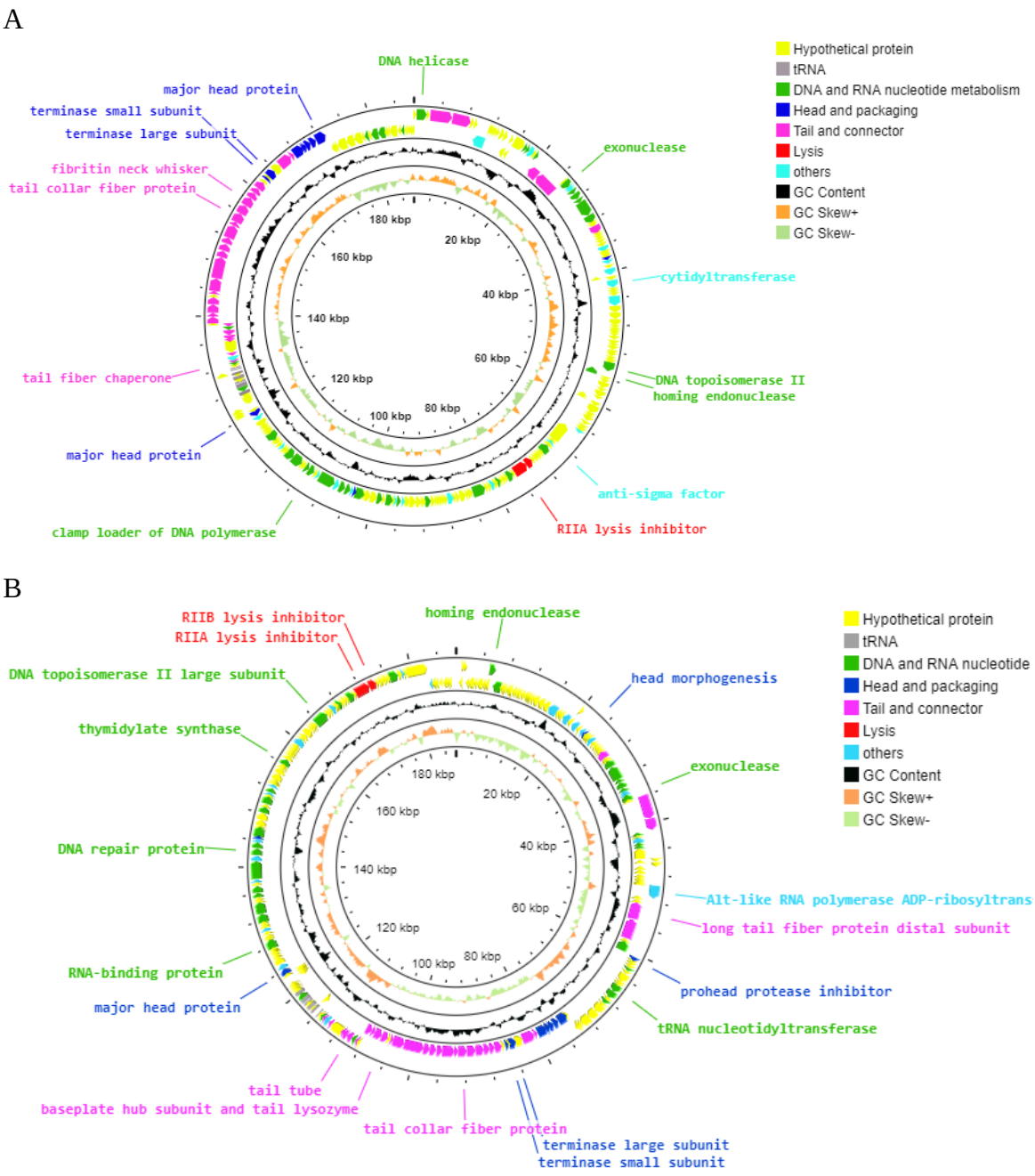


Figure 6.2: Genome maps of (A) EniLVP01 and (B) EniLVP02.

The predicted coding sequences (CDSs) involved in DNA, RNA, and nucleotide metabolism amounted to 36 CDSs in both phages, while those involved in head and packaging roles were 11 and 12 CDSs in EniLVP01 and EniLVP02, respectively. Interestingly, 28 CDSs were involved in the tail functioning of both bacteriophages. Out of

the annotated CDSs of both phages, 21 CDSs encoding tRNAs were also predicted, and notably, no virulence factors or antimicrobial resistance genes were detected. Additionally, no lysogeny and integrases were detected in the genomes of both phages. Based on their genomic characteristics, phage EniLVP01 and EniLVP02 both belong to the *Caudoviricetes* class, *Straboviridae* family, and unclassified *Straboviridae* (formerly *Myoviridae*).

Table 6.2: Annotation of EniLVP01 and ORFs putative functions using PSI-BLAST. ORFs encoding for hypthetical proteins and unknown functions are not included.

ORF	Strand	Start	End	Nucleotide length (bp)	Score	E-value	Identity (%)	Annotation
ORF002	+	388	1905	1518	304	5.66E-95	53	DNA helicase
ORF004	+	2433	5648	3216	102	1.57E-23	17	Long tail fibre protein distal subunit
ORF005	+	5734	8463	2730	174	2.01E-45	45	Long tail fibre protein distal subunit
ORF008	-	11424	9541	1884	61	1.53E-11	23	Alt-like RNA polymerase ADP-ribosyltransferase
ORF021	+	17227	17661	435	38	3.89E-06	40	UvsY-like recombination mediator
ORF022	+	17667	18101	435	38	9.45E-06	30	tRna amidotransferase
ORF023	+	18101	18949	849	167	1.65E-48	42	DNA methyltransferase
ORF025	+	19246	19713	468	148	8.25E-44	57	Endonuclease V N-glycosylase UV repair enzyme

Table 6.2 continued

ORF026	-	21439	19754	1686	122	5.89E-30	23	Hinge connector of long tail fibre protein distal connector
ORF027	-	24984	21460	3525	103	1.60E-24	22	Tail fibre protein proximal subunit
ORF029	+	25088	26086	999	94	3.99E-23	31	Exonuclease
ORF030	+	26094	26369	276	57	3.26E-13	39	Transcriptional regulator
ORF031	+	26385	26699	315	46	5.51E-09	38	Late promoter transcriptional regulator
ORF032	+	26710	27315	606	104	1.36E-27	35	DNA helicase loader
ORF033	+	27367	28263	897	287	4.32E-89	65	Single strand DNA binding protein
ORF034	+	28331	28897	567	104	1.67E-27	38	Dihydrofolate reductase
ORF035	+	28881	31115	2235	520	4.82E-164	47	Ribonucleotide reductase large subunit
ORF036	+	31187	32326	1140	235	9.28E-70	39	Ribonucleoside diphosphate reductase small subunit

Table 6.2 continued

ORF039	+	32930	34111	1182	267	2.13E-80	41	RNA ligase and tail fibre protein attachment catalyst
ORF045	+	36203	36976	774	68	3.49E-16	30	Phosphatase
ORF050	+	37871	38314	444	81	4.53E-21	51	Head morphogenesis
ORF051	+	38318	38896	579	215	3.38E-66	59	Mom-like DNA modification protein
ORF053	+	39626	40549	924	49	1.10E-08	21	Lipoprotein
ORF056	+	41365	42456	1092	205	3.78E-59	34	Cytidyltransferase
ORF060	+	43720	45150	1431	403	9.46E-126	50	Nicotinamide phosphoribosyl transferase
ORF079	+	53593	54927	1335	214	2.91E-59	34	DNA topoisomerase II
ORF080	-	55836	54910	927	132	5.23E-35	54	Homing endonuclease
ORF099	+	64589	65035	447	57	2.95E-12	49	Starvation-inducible transcriptional regulator
ORF102	-	68954	68595	360	72	1.75E-18	53	Anti-sigma factor
ORF104	-	70866	69517	1350	155	1.31E-42	36	ATP-dependent DNA ligase

Table 6.2 continued

ORF110	-	74112	72982	1131	197	1.94E-56	45	RIIB lysis inhibitor
ORF111	-	76369	74114	2256	219	3.31E-61	39	RIIA lysis inhibitor
ORF113	-	77779	76763	1017	119	2.63E-31	28	RNA ligase
ORF117	-	79642	78977	666	49	8.61E-09	27	DNA primase
ORF120	-	80735	80019	717	80	6.36E-19	26	Metallo-phosphoesterase
ORF123	-	83777	81717	2061	458	4.80E-142	41	DNA topoisomerase II large subunit
ORF130	-	87989	86982	1008	198	2.90E-58	37	Polynucleotide kinase
ORF138	-	91594	90674	921	124	8.59E-35	35	Thymidylate synthase
ORF146	-	95294	94824	471	56	8.67E-12	33	Hydrolase
ORF147	-	96115	95453	663	156	4.35E-45	43	Exonuclease
ORF148	-	96492	96112	381	83	8.05E-22	42	MazG-like pyrophosphatase
ORF150	-	97917	96748	1170	97	6.29E-24	24	DNA primase
ORF152	-	99316	98429	888	70	1.60E-15	65	DNA helicase
ORF156	-	102936	101440	1497	173	6.52E-48	29	DnaB-like replicative helicase

Table 6.2 continued

ORF157	-	103163	102870	294	49	7.28E-10	42	Head vertex assembly chaperone
ORF158	-	103759	103220	540	261	8.74E-82	76	Recombinase
ORF159	-	104610	103756	855	87	8.47E-22	47	Homing endonuclease
ORF160	-	105653	104673	981	339	5.76E-107	58	DNA repair protein
ORF161	-	106494	105745	750	91	4.34E-22	25	DNA methyltransferase
ORF162	-	109202	106515	2688	738	2.70E-235	47	DNA polymerase
ORF163	-	109694	109299	396	181	2.62E-55	74	Translation repressor
ORF165	-	111236	110256	981	171	9.81E-49	39	Clamp loader of DNA polymerase
ORF166	-	111933	111256	678	112	8.57E-30	33	DNA polymerase processivity factor
ORF169	-	114392	112680	1713	128	2.84E-32	36	SbcC-like subunit of palindrome specific endonuclease

Table 6.2 continued

ORF170	-	115709	114396	1314	168	1.45E-46	34	SbcD-like subunit of palindrome specific endonuclease
ORF173	-	117090	116560	531	85	6.55E-22	36	5'-3' deoxyribonucleotidase
ORF177	-	119812	118415	1398	412	3.84E-129	49	RNA-binding protein
ORF184	-	123161	122472	690	109	6.83E-30	42	Late sigma transcription factor
ORF185	-	124129	123230	900	93	7.79E-22	21	Major head protein
ORF199	-	128447	127653	795	114	2.66E-30	48	Homing endonuclease
ORF206	-	132600	132109	492	98	3.75E-26	43	RNA ligase
ORF207	-	132849	132607	243	37	3.60E-06	33	Tail fibre chaperone
ORF208	-	133562	132828	735	91	4.59E-23	29	Deoxynucleoside monophosphate kinase
ORF209	-	134164	133631	534	84	1.27E-19	32	Tail tube
ORF211	-	136219	136046	174	40	3.18E-08	52	Baseplate hub assembly catalyst
ORF212	-	136944	136219	726	83	2.66E-20	29	Baseplate hub

Table 6.2 continued

ORF213	-	137770	136958	813	114	1.15E-29	28	Tail tube
ORF214	-	138376	137777	600	201	8.36E-59	56	DNA end protector
ORF215	-	138842	138387	456	159	3.32E-48	68	Head closure
ORF217	+	138933	139964	1032	109	1.80E-27	30	Baseplate tail tube cap
ORF218	+	139961	140524	564	126	4.70E-35	39	Baseplate wedge subunit
ORF219	+	140521	141765	1245	108	5.99E-27	22	Tail protein
ORF220	+	141762	142811	1050	243	1.02E-73	51	Baseplate hub subunit and tail lysozyme
ORF222	+	143312	143734	423	131	6.76E-38	58	Baseplate wedge subunit
ORF223	+	143734	145629	1896	443	1.85E-136	44	Baseplate wedge subunit
ORF224	+	145631	148813	3183	507	1.78E-156	41	Baseplate wedge subunit
ORF225	+	148819	149832	1014	461	9.40E-149	68	Baseplate wedge subunit
ORF226	+	149850	150878	1029	81	1.11E-18	25	Baseplate wedge tail fibre protein connector
ORF227	+	150962	151873	912	109	6.90E-28	27	Baseplate wedge tail fibre protein connector

Table 6.2 continued

ORF228	+	151873	153834	1962	277	7.80E-83	41	Baseplate wedge subunit
ORF229	+	153831	154445	615	135	5.83E-37	37	Baseplate wedge subunit
ORF230	+	154452	155870	1419	135	2.21E-35	30	Tail collar fibre protein
ORF231	+	155867	157264	1398	211	7.73E-61	41	Tail collar fibre protein
ORF232	+	157286	158608	1323	108	4.07E-26	28	Fibritin neck whisker
ORF233	+	158610	159536	927	136	8.57E-38	31	Head-tail adaptor Ad2
ORF234	+	159544	160374	831	184	4.10E-55	59	Head closure Hc2
ORF235	+	160387	161544	1158	220	4.45E-66	58	Tail sheath stabilizer
ORF237	+	162134	162691	558	116	3.01E-32	42	Terminase small subunit
ORF239	+	162774	163916	1143	223	4.41E-68	60	Terminase large subunit
ORF241	+	165081	167090	2010	233	8.30E-70	41	Tail sheath
ORF242	+	167147	167662	516	132	1.67E-35	46	Tail tube
ORF243	+	167741	169327	1587	476	1.82E-149	50	Portal protein
ORF244	+	169344	169556	213	36	2.50E-06	39	Prohead
ORF245	+	169540	169803	264	85	7.59E-22	55	Head scaffolding protein

Table 6.2 continued

ORF246	+	169803	170624	822	226	1.09E-69	73	Head maturation protease
ORF247	+	170629	171525	897	120	2.24E-31	35	Head scaffolding protein
ORF248	+	171590	173158	1569	444	1.65E-140	50	Major head protein
ORF256	-	179353	178715	639	112	1.79E-30	37	DprA-like DNA recombination-mediator protein
ORF258	-	180934	179801	1134	76	1.21E-16	25	tRNA nucleotidyltransferase
ORF259	-	182081	180948	1134	136	6.25E-36	43	tRNA nucleotidyltransferase
ORF264	-	185107	184655	453	60	3.51E-13	28	RNase H

Table 6.3: Annotation of EniLVp02 ORFs putative functions using PSI-BLAST. ORFs encoding for hypothetical proteins and unknown functions are not included.

ORF	Strand	Start	End	Nucleotide length (bp)	Score	E-value	Identity (%)	Annotation
ORF012	+	5032	5958	927	132	5.23E-35	54	Homing endonuclease
ORF013	-	7275	5941	1335	214	2.92E-59	34	DNA topoisomerase II
ORF032	-	17148	15718	1431	403	9.472E-126	50	Nicotinamide phosphoribosyl transferase
ORF036	-	19503	18412	1092	205	3.788E-59	34	Cytidyltransferase
ORF039	-	21242	20319	924	49	1.10E-08	21	Lipoprotein
ORF041	-	22550	21972	579	215	3.38E-66	59	Mom-like DNA modification protein
ORF042	-	22997	22554	444	81	4.54E-21	51	Head morphogenesis
ORF047	-	24665	23892	774	68	3.50E-16	30	Phosphatase
ORF053	-	27938	26757	1182	267	2.14E-80	41	RNA ligase and tail fibre protein attachment catalyst

Table 6.3 continued

ORF056	-	29681	28542	1140	235	9.293E-70	39	Ribonucleoside diphosphate reductase small subunit
ORF057	-	31987	29753	2235	520	4.829E-164	47	Ribonucleotide reductase large subunit
ORF058	-	32537	31971	567	104	1.67E-27	38	Dihydrofolate reductase
ORF059	-	33501	32605	897	287	4.33E-89	65	Single strand DNA binding protein
ORF060	-	34158	33553	606	104	1.36E-27	35	DNA helicase loader
ORF061	-	34483	34169	315	46	5.51E-09	38	Late promoter transcriptional regulator
ORF062	-	34774	34499	276	57	3.27E-13	39	Transcriptional regulator
ORF063	-	35780	34782	999	94	4.00E-23	31	Exonuclease
ORF065	+	35884	39408	3525	103	1.60E-24	22	Tail fibre protein proximal subunit
ORF066	+	39429	41114	1686	122	5.89E-30	23	Hinge connector of long tail fibre protein distal connector

Table 6.3 continued

ORF067	-	41622	41155	468	148	8.26E-44	57	Endonuclease V N-glycosylase UV repair enzyme
ORF069	-	42767	41919	849	167	1.65E-48	42	DNA methyltransferase
ORF070	-	43201	42767	435	38	9.46E-06	30	tRNA amidotransferase
ORF071	-	43641	43207	435	38	3.90E-06	40	UvsY-like recombination mediator
ORF084	+	49444	51327	1884	61	1.53E-11	23	Alt-like RNA polymerase ADP-ribosyltransferase
ORF087	-	55095	52405	2691	152	7.73E-39	42	Long tail fibre protein distal subunit
ORF088	-	58396	55220	3177	102	1.57E-23	17	Long tail fibre protein distal subunit
ORF090	-	60480	58963	1518	304	5.67E-95	53	DNA helicase
ORF091	+	60552	61139	588	61	3.14E-13	24	Prohead protease inhibitor
ORF095	+	62365	62817	453	60	3.51E-13	28	RNase H

Table 6.3 continued

ORF100	+	65391	66524	1134	136	6.26E-36	43	tRNA nucleotidyltransferase
ORF101	+	66538	67671	1134	76	1.21E-16	25	tRNA nucleotidyltransferase
ORF103	+	68119	68757	639	112	1.80E-30	37	DprA-like DNA recombination-mediator protein
ORF112	-	75882	74314	1569	441	2.03E-139	49	Major head protein
ORF113	-	76843	75947	897	120	2.24E-31	35	Head scaffolding protein
ORF114	-	77669	76848	822	226	1.09E-69	73	Head maturation protease
ORF115	-	77932	77669	264	85	7.60E-22	55	Head scaffolding protein
ORF116	-	78128	77916	213	36	2.50E-06	39	Prohead
ORF117	-	79731	78145	1587	476	1.82E-149	50	Portal protein
ORF118	-	80325	79810	516	132	1.67E-35	46	Tail tube
ORF119	-	82391	80382	2010	233	8.31E-70	41	Tail sheath
ORF121	-	84698	83556	1143	223	4.41E-68	60	Terminase large subunit
ORF123	-	85338	84781	558	116	3.02E-32	42	Terminase small subunit

Table 6.3 continued

ORF125	-	87085	85928	1158	220	4.45E-66	58	Tail sheath stabilizer
ORF126	-	87928	87098	831	184	4.10E-55	59	Head closure Hc2
ORF127	-	88862	87936	927	136	8.58E-38	31	Head-tail adaptor Ad2
ORF128	-	90186	88864	1323	108	4.08E-26	28	Fibritin neck whisker
ORF129	-	91605	90208	1398	211	7.74E-61	41	Tail collar fibre protein
ORF130	-	93020	91602	1419	135	2.21E-35	30	Tail collar fibre protein
ORF131	-	93656	93027	630	135	5.84E-37	37	Baseplate wedge subunit
ORF132	-	95599	93638	1962	277	7.81E-83	41	Baseplate wedge subunit
ORF133	-	96510	95599	912	109	6.91E-28	27	Baseplate wedge tail fibre protein connector
ORF134	-	97622	96594	1029	81	1.12E-18	25	Baseplate wedge tail fibre protein connector
ORF135	-	98653	97640	1014	461	9.41E-149	68	Baseplate wedge subunit
ORF136	-	101841	98659	3183	507	1.78E-156	41	Baseplate wedge subunit
ORF137	-	103738	101843	1896	443	1.85E-136	44	Baseplate wedge subunit

Table 6.3 continued

ORF138	-	104160	103738	423	131	6.77E-38	58	Baseplate wedge subunit
ORF140	-	105710	104661	1050	243	1.02E-73	51	Baseplate hub subunit and tail lysozyme
ORF141	-	106951	105707	1245	108	5.99E-27	22	Tail protein
ORF142	-	107511	106948	564	126	4.70E-35	39	Baseplate wedge subunit
ORF143	-	108479	107508	972	109	1.80E-27	30	Baseplate tail tube cap
ORF145	+	108630	109085	456	159	3.33E-48	68	Head closure
ORF146	+	109096	109695	600	201	8.37E-59	56	DNA end protector
ORF147	+	109702	110514	813	114	1.15E-29	28	Tail tube
ORF148	+	110528	111253	726	83	2.67E-20	29	Baseplate hub
ORF149	+	111253	111426	174	40	3.18E-08	52	Baseplate hub assembly catalyst
ORF151	+	113308	113841	534	84	1.27E-19	32	Tail tube
ORF152	+	113910	114644	735	91	4.59E-23	29	Deoxynucleoside monophosphate kinase
ORF153	+	114623	114865	243	37	3.60E-06	33	Tail fibre chaperone

Table 6.3 continued

ORF154	+	114872	115363	492	98	3.75E-26	43	RNA ligase
ORF161	+	119025	119819	795	114	2.67E-30	48	Homing endonuclease
ORF175	+	123343	124242	900	93	7.80E-22	21	Major head protein
ORF176	+	124311	125000	690	109	6.84E-30	42	Late sigma transcription factor
ORF183	+	127660	129057	1398	412	3.85E-129	49	RNA-binding protein
ORF187	+	130382	130912	531	85	6.56E-22	36	5'-3' deoxyribonucleotidase
ORF190	+	131763	133076	1314	168	1.45E-46	34	SbcD-like subunit of palindrome specific endonuclease
ORF191	+	133080	134792	1713	128	2.84E-32	36	SbcC-like subunit of palindrome specific endonuclease
ORF194	+	135539	136216	678	112	8.58E-30	33	DNA polymerase processivity factor
ORF195	+	136236	137216	981	171	9.82E-49	39	Clamp loader of DNA polymerase

Table 6.3 continued

ORF197	+	137778	138173	396	181	2.62E-55	74	Translation repressor
ORF198	+	138270	140957	2688	738	2.70E-235	47	DNA polymerase
ORF199	+	140978	141727	750	91	4.35E-22	25	DNA methyltransferase
ORF200	+	141819	142799	981	339	5.77E-107	58	DNA repair protein
ORF201	+	142862	143716	855	87	8.48E-22	47	Homing endonuclease
ORF202	+	143713	144252	540	261	8.75E-82	76	Recombinase
ORF203	+	144309	144602	294	49	7.28E-10	42	Head vertex assembly chaperone
ORF204	+	144536	146032	1497	173	6.53E-48	29	DnaB-like replicative helicase
ORF208	+	148156	149043	888	70	1.60E-15	65	DNA helicase
ORF210	+	149555	150724	1170	97	6.30E-24	24	DNA primase
ORF212	+	150902	151360	459	83	8.06E-22	42	MazG-like pyrophosphatase
ORF213	+	151357	152019	663	156	4.35E-45	43	Exonuclease
ORF214	+	152178	152648	471	56	8.68E-12	33	Hydrolase
ORF222	+	155878	156798	921	124	8.60E-35	35	Thymidylate synthase

Table 6.3 continued

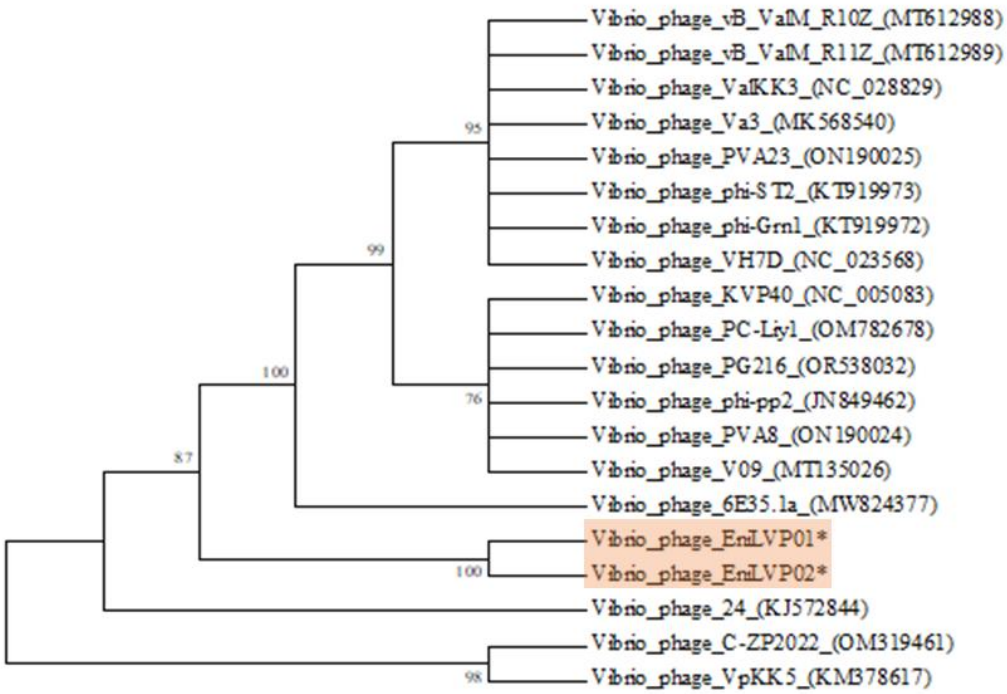
ORF230	+	159483	160490	1008	198	2.91E-58	37	Polynucleotide kinase
ORF237	+	163695	165755	2061	458	4.81E-142	41	DNA topoisomerase II large subunit
ORF240	+	166737	167453	717	80	6.36E-19	26	Metallo-phosphoesterase
ORF243	+	167830	168495	666	49	8.62E-09	27	DNA primase
ORF247	+	169693	170709	1017	119	2.63E-31	28	RNA ligase
ORF249	+	171097	173358	2262	222	3.05E-62	39	RIIA lysis inhibitor
ORF250	+	173360	174490	1131	197	1.94E-56	45	RIIB lysis inhibitor
ORF256	+	176606	177955	1350	155	1.31E-42	36	ATP-dependent DNA ligase
ORF258	+	178518	178877	360	72	1.75E-18	53	Anti-sigma factor
ORF261	-	182883	182437	447	57	2.96E-12	49	Starvation-inducible transcriptional regulator

6.3.2 Phylogenetic Trees

The phylogenetic trees based on terminase large subunit (Figure 6.3A) and DNA polymerase (Figure 6.3B) showed the same patterns. In both trees it was observed that both phage EniLVP01 and EniLVP02 were clustered together in the same clade. Additionally, in the terminase large subunit tree, both phages belong to a superclade that includes 15 other *Vibrio* phages as seen in Figure 6.3A. This similar pattern with the same 15 *Vibrio* phages were also observed in the DNA polymerase tree as shown in Figure 6.3B.

From these results, it can be inferred that both phage EniLVP01 and EniLVP02 are closely related to each other and shared the most recent ancestors. Additionally, both phages showed distant phylogenetic relationship with 15 other *Vibrio* phages, indicating a common ancestor farther back in their evolutionary history. Notably, both phages do not share common ancestors with phage VpKK5 (KM378617), C-ZP2022 (OM319461), and phage 24 (KJ572844) in both phylogenetic trees.

A



B

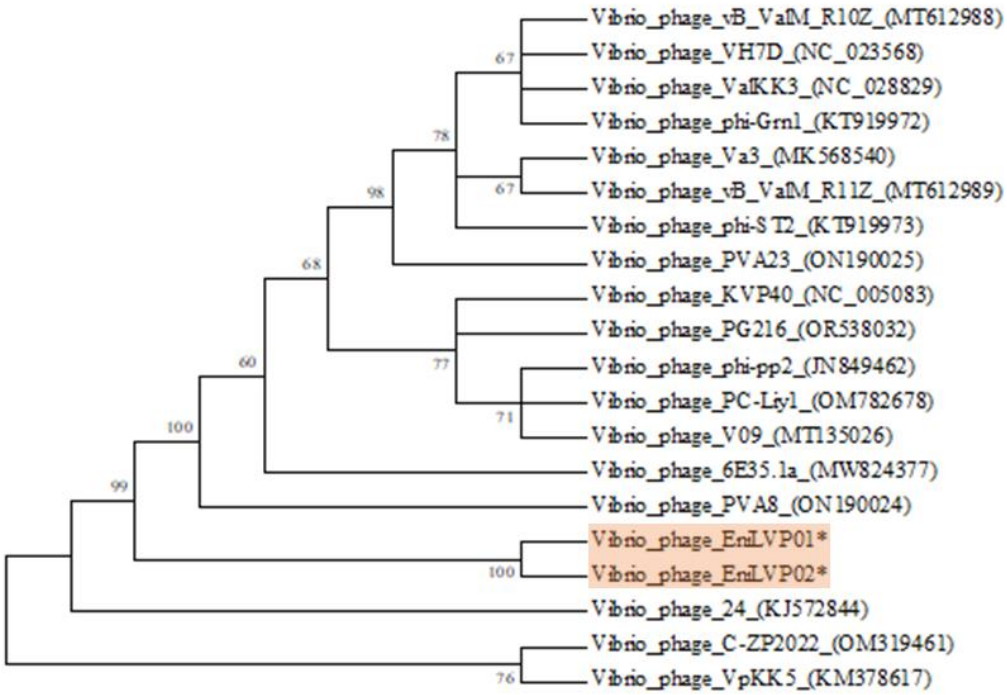


Figure 6.3: Phylogenetic tree based on (A) large terminase subunit and (B) DNA polymerase sequence showing the position of phage EniLVP01* and EniLVP02*. The alignments of the sequences were performed by using MUSCLE. The evolutionary relationships were predicted using the Neighbor-Joining method and the distances were computed using the Jukes-Cantor method. Bootstrap values were based on 1000 resamplings. Only values greater than 50 are shown in the trees.

6.3.3 Nucleotide-based Intergenomic Similarity Analysis

From the results of the phylogenetic trees, 15 phages that shared distant common ancestors with phage EniLVP01 and EniLVP02 were selected for the nucleotide-based intergenomic similarity analysis. A heatmap showing the percentage of similarities between the phages were illustrated in Figure 6.4 below. The VIRIDIC calculation unveiled that phage EniLVP01 and EniLVP02 exhibited striking genetic resemblance with 100% similarity in their genomes. Furthermore, upon comparison with the other publicly available phages, it became evident that both EniLVP01 and EniLVP02 shared minimal nucleotide

sequence similarities with them, accounting for merely 5.3% to 6.1% of intergenomic similarities.

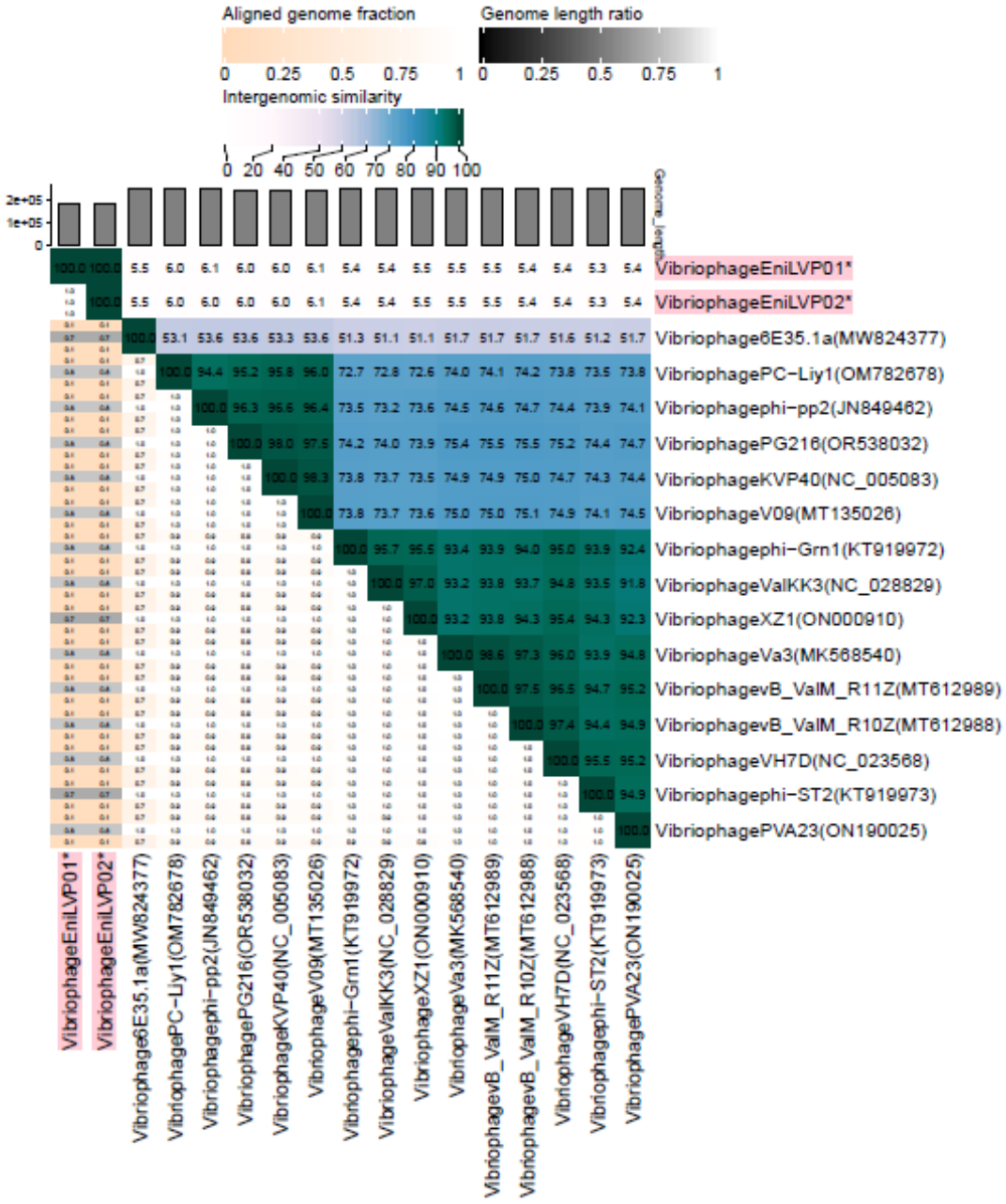


Figure 6.4: Heatmap of nucleotide-based intergenomic similarities of phage EniLVP01, EniLVP02 (highlighted), and a selection of related phages within the family *Straboviridae*, using VIRIDIC. A heatmap of hierarchical clustering of the intergenomic similarity values (%) was generated (right half). Species and genus thresholds were set at 95% and 70% respectively (Moraru et al., 2020).

6.4 Discussion

Phages in the *Caudovirales* order typically possess genomes with sizes ranging from 15 to 500 kilobase pairs (kbp) (Fokine and Rossmann, 2014). Phage EniLVP01 and EniLVP02 possesses a large genome size, a characteristic commonly shared with many phages that were formerly classified under the *Myoviridae* family, which can fall within the range of approximately 170 kbp and above, although they are other *Myoviridae* phages with smaller genome of less than 100 kbp (Comeau et al, 2012; Rybicki, 2023).

Based on their genome characterisation, the genomes of both phages possess the same size and the annotation of their ORFs are highly similar despite the difference in the organizations. This may indicate that phage EniLVP01 and EniLVP02 are genetically related even though they originated from different sources. This finding is similar to the findings of Al-Fendi et al. (2013) who isolated three *V. cholerae* phages portraying similar morphologies and identical genetic characteristics. Notably, all three phages were isolated from different water sources in different locations (Al-Fendi et al., 2013). It has been reported that bacteriophages may exhibited specific biogeographical patterns in accordance to their hosts' geographical patterns (Spencer, 1960; Kalatzis et al., 2017). Phages with genetic similarities often exhibit common features in their genomes, such as conserved genes, genomic organization, or functional elements, as demonstrated by the annotated ORFs of both phage EniLVP01 and EniLVP02. These shared genetic characteristics may be the result of vertical inheritance, horizontal gene transfer, or coevolution with the hosts (Hendrix et al., 1999; Rohwer and Edwards, 2002; Chénard et al., 2016). While studies in local settings often reveal high phage diversity, a broader global perspective can show a relatively limited diversity within the phage community due to their ability to traverse different biomes (Kellogg et al., 1995; Breitbart and Rohwer, 2005; Kalatzis et al., 2017).

This suggests that despite originating from different sources, bacteriophages infecting the same bacterial host may exhibit similarities in their genomes because they may previously originate from the same source.

The exploration of bacteriophage phylogeny stands as compelling research due to their rapid evolution and genetic diversity. Even phages that have the same host boast distinctive genome sequences, an example to their diversity generally caused by both vertical and horizontal gene transfers (Kropinski and Clokie, 2009; Asthana et al., 2021). Understanding the evolutionary relationships among phages enhances our comprehension of their virulence and reproduction (Moura de Sousa et al., 2021). In constructing phylogenetic trees for phages, single-gene analyses were employed, utilising commonly conserved regions, including DNA and RNA polymerases, DNA primase, as well as terminase gene sequences, as the common candidates. These sequences are known to be conserved in the phage genome, providing a robust approach to address the challenges posed by the mosaic nature of bacteriophage genomes (Casjens, 2005; Lal et al., 2016a).

For our investigation, the terminase large subunit and DNA polymerase genes were specifically chosen for constructing the phylogenetic trees. The terminase large subunit is essential for the assembly and translocation of viral DNA during the packaging process, ensuring the correct encapsulation of viral genetic material into the capsid. Meanwhile, DNA polymerase is responsible for viral DNA replication, catalysing the synthesis of new DNA strands based on existing templates (Lin et al., 2022; Morcinek-Orlowska et al., 2022). Additionally, these conserved genes were compared at their amino acid sequences level to construct the phylogenetic trees instead of using their nucleotide sequences is mainly due to the mosaicism in the phage genome. This is because at the amino acid level, the sequences of important functional proteins are often more conserved compared to their nucleotide

sequences that may have diverged sufficiently that their similarity is not recognizable anymore (Hatfull and Hendrix, 2011). Therefore, phylogenetic trees based on amino acid sequences can better represent evolutionary relationship between phages, especially over a long-time scale.

In the study, phage EniLVP01 and EniLVP02 appears to occupy a similar position in the evolutionary landscape based on the terminase large subunit and DNA polymerase phylogenetic tree. The terminase large subunit phylogenetic tree illustrates the close phylogeny between phage EniLVP01 and EniLVP02, thus showing their relatedness (Yuan et al., 2012). Meanwhile both phages also showed distant phylogenies with several other *Vibrio* phages, suggesting potential genetic divergence in the large terminase subunit and DNA polymerase region at some point in their evolutionary history (Lin et al., 2022). Hence, the phylogenetic relationships among these phages and the relatedness between phage EniLVP01 and EniLVP02 depending on the conserved gene regions analysed, suggest a complex evolutionary relationship and genetic similarities, potentially from a common distant ancestor (Yuan et al., 2012).

Notably, both phages do not share a common evolutionary history with phage VpKK5, phage C-ZP2022, and phage 24 in both phylogenetic trees. Phage VpKK5 is a *Vibrio* siphophage (former *Siphoviridae*) isolated from coastal sand sediment in Kota Kinabalu, Sabah, Malaysia targeting *V. parahaemolyticus* from a disease farmed hybrid grouper (Lal et al., 2016a). Meanwhile both phage C-ZP2022, and phage 24 are unpublished phages of *V. parahaemolyticus* and *V. cholerae*, respectively, that were obtained from the GenBank database. Therefore, it is inferred that these phages are not closely related to phage EniLVP01 and EniLVP02 and do not share common ancestor.

Additionally, according to the VIRIDIC analysis, it was found that phage EniLVP01 and EniLVP02 shared 100% intergenomic similarity, suggesting that they may be the same species or highly genetically related despite their different origins and distinct genome organisations. According to the International Committee of Taxonomy of Viruses (ICTV) standard, the species threshold is set at 95% of sequence similarity (Moraru et al., 2020). This can further prove that the same phages can be found in many places given the presence of the same host bacteria, as they have the ability to traverse between biomes (Kalatzis et al., 2017). Interestingly, phage EniLVP01 and EniLVP02 shared very low similarities with the other known Vibriophages, indicating that they belong to a different cluster from them. The low homology also suggested that both phages are novel. According to the International Committee of Taxonomy of Viruses (ICTV), phages will be classified under the same genus when their nucleotide sequences identities are higher than 70% (Moraru et al., 2020). Therefore, it is safe to consider that phage EniLVP01 and EniLVP02 are likely to represent a new family under the *Caudoviricetes* class.

The absence of lysogeny related genes and integrases in the genomes of EniLVp01 and EniLVp02 indicates that they are strictly lytic. In the context of phage therapy, lytic phages are favoured for therapeutic applications due to their ability to selectively target and eliminate their host bacteria, thereby reducing the risk of lateral gene transfer. This stands in contrast to lysogenic or temperate phages, which have the potential to integrate their DNA into the host genome and facilitate gene transfer between bacteria (Ramisetty and Sudhakari, 2019; Strathdee et al., 2023). In many instances, acquisition of virulence genes by *Vibrio* spp. is linked to prophage-encoded genes through lysogenic conversion. For instance, cholera toxin (CT) and zonula occludens toxins (Zot) produced by *V. cholerae* are attributed to prophage-generated virulence (Fasano et al., 1991; Waldor and Mekalanos, 1996; Kalatzis

et al., 2017). Moreover, phage-derived fitness factors and bacterial immunity have been identified in environmental *Vibrio* populations, underscoring the significant roles of lysogenic phages in contributing to bacterial functional and genetic diversity (Kalatzis et al., 2017). However, this presents risks in terms of controlling pathogenic bacteria, as lysogenic phages may confer virulence and resistance potential instead of eradicating the population (Altamirano and Barr, 2021). Therefore, the absence of these characteristics in phage EniLVP01 and EniLVP02 means that they are safe to be used in phage therapy as they can effectively serve the intended purpose of controlling and reducing the number of target bacteria without side effects.

6.5 Chapter Summary

This chapter has elucidated the intriguing genetic and evolutionary landscape of phages EniLVP01 and EniLVP02 by using technologies like Next Gene Sequencing (NGS), showcasing their distinctiveness and potential significance in phage therapy. Notably, both phages exhibit strict lytic characteristics, devoid of lysogeny-related genes, which makes them promising candidates for therapeutic applications due to their selective bactericidal activity.

Their 100% intergenomic similarity suggests that they may represent the same species, and low similarity with other known phages suggest that they represent a new family under the *Caudoviricetes* class. Based on the overall genomic characterisation of both phages, phage EniLVP01 and EniLVP02 are novel bacteriophages of *V. parahaemolyticus* as they shared very low similarities with other reported phages in the publicly available databases. Moreover, this study has also highlighted the potential of EniLVP01 and EniLVP02 as safe and effective agents in phage therapy strategies.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

The impact of *Vibrio* species extends beyond immediate health risks to humans consuming contaminated shrimp; it also poses substantial economic threats to shrimp farming operations. The ability to promptly detect and address *Vibrio* infections is crucial for maintaining farm productivity and reducing the economic losses associated with sick or deceased shrimp that are unfit for sale. Therefore, this study aimed to determine the prevalence of *Vibrio* spp. in a shrimp farm, identify antibiotic resistance and virulence gene profiles of *Vibrio* spp. isolated from the farm, isolate bacteriophages with the ability to lyse *V. parahaemolyticus*, and study their genomic characteristics. At the end of this study, all objectives have been successfully achieved.

The study successfully identified and quantified *Vibrio* species - *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* - in various samples from two ponds throughout the shrimp farming cycle in a local shrimp farm using the MPN-mPCR method. Notably, *V. parahaemolyticus* emerged as the predominant species, indicating its superior ability to exploit resources in the shrimp farm compared to *V. cholerae* and *V. alginolyticus*. This dominance raises concerns due to *V. parahaemolyticus* association with seafood-related illnesses, highlighting potential risks to food safety within shrimp aquaculture.

Moreover, the MPN-mPCR approach has also successfully enhanced the process of detecting virulence genes in the *Vibrio* isolates, thereby enriching understanding of their pathogenicity profiles. It was observed that all V. *parahaemolyticus* and *V. cholerae* isolates possess virulence genes associated with their pathogenicity. However, no virulence genes

were detected in *V. alginolyticus*. Nonetheless, the common occurrence of horizontal gene transfers, especially in aquatic environments, suggests that the acquisition of virulence genes from other bacteria or the environment is a potential event.

Additionally, the growing concern over antimicrobial-resistant (AMR) strains of *Vibrio* spp., particularly in Malaysia and specifically in Sarawak, adds complexity to control efforts and highlights the potential for severe public health and economic repercussions. This study reveals that all 30 Vibrio isolates from the Telaga Air shrimp farm exhibited resistance to at least two commonly used antibiotics, with their MAR indices ranging from 0.11 to 0.39. These findings suggest an urgent need for alternative treatment strategies to control bacterial populations while simultaneously preventing or reducing the spread of AMR strains. One promising approach is phage therapy.

This study successfully isolated two myophages, namely phage EniLVP01 and EniLVP02, with antagonistic activities against *V. parahaemolyticus* from the Telaga Air shrimp farm, thus showcasing significant potential for phage therapy applications. The phages demonstrated many characteristics ideal for therapeutic use, such as large burst sizes, strictly lytic nature, and stability across a wide range of temperatures and pH levels. They also exhibited the capacity to disrupt and inhibit bacterial biofilm formation to some extent. Importantly, these phages maintained their functionality not only in liquid media but also on shrimp matrices at both room temperature and 4 °C, with their combined use resulting in a significant enhancement in activity.

Through Whole Genome Sequencing (WGS) approach, the genomes of phage EniLVP01 and EniLVP02 have been successfully characterised. Genome sequence analysis revealed high genomic similarities between the two phages, suggesting that they are either the same species or very closely related despite originating from different sources. However,

low similarities with other publicly available phages indicate that phage EniLVP01 and EniLVP02 are novel phages. Additionally, the absence of lysogens and lysogeny-related genes, such as integrases and transposases, alongside their lack of antibiotic resistance and virulence genes, confirms their obligately lytic behaviour and underscores their safety for use in food or clinical settings. These findings position these bacteriophages as promising agents for developing phage-based alternatives to combat bacterial infections in aquaculture and the food industry.

7.2 Recommendations

From this study, it has been highlighted that constant monitoring and surveillance of bacterial population, especially *Vibrio* spp., in aquaculture farms are imperative as the first step in preventing disease outbreaks. By implementing effective detection and control measures at the farming stage, it is possible to mitigate the spread of infections and their subsequent escalation into larger outbreaks, which are more complex and costly to manage. Consequently, proactive management of *Vibrio* spp. in shrimp farms is not only essential for protecting public health but also for ensuring the economic viability and sustainability of these aquaculture operations. Thus, integrating standard detection and preventive strategies against *Vibrio* spp., as well as providing platforms to gather recent disease occurrence and management data should be prioritised by both public health officials and the aquaculture industry to maintain a safe food supply and to secure the economic stability of shrimp farming.

Additionally, it is evident from this study that the presence of phages in various environmental and food resources in Sarawak presents an intriguing avenue for exploration. Further research is essential to optimise and expand the application of phage therapy using the bacteriophages isolated in this study. To enhance the effectiveness of these phages, future

studies should conduct scaled-up trials to validate the efficacy and safety of these phages in *in vivo* applications, such as by using live shrimps in real aquaculture operations, potentially including different species and environmental conditions. Additionally, optimising phage therapy for *V. parahaemolyticus* infections and developing phage-based products suitable for use in food production and resource-limited settings should be prioritised. Investigating the preparation of dry cultures for phage application could facilitate their widespread use and can potentially be commercialised for public use.

Another promising avenue for research with these two phages involves investigating methods to broaden their host ranges. The intriguing concept of phage training, wherein phages are cultivated alongside bacteria to enhance their efficacy against diverse strains and to expand their host ranges, offers a tailored therapeutic strategy worth exploring. Moreover, delving into the synergistic relationship of phage-antibiotic combinations holds considerable potential for reducing reliance on antibiotics, thus addressing the pressing issue of antibiotic resistance. Lastly, studying the genetic adaptability of these phages in response to bacterial resistance mechanisms would further support their standing as viable alternatives to conventional antibiotics in combating bacterial infections in aquaculture, food industry, and potentially in healthcare.

The exploration of phage application is far from over as numerous potential avenues remain to be studied and explored. These forthcoming investigations hold the promise of advancing our comprehension of phage therapy and its role in combating bacterial infections. In doing so, they offer innovative solutions to control the spread of bacteria particularly in aquaculture settings and address the pressing issue of antibiotic resistance.

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APPENDICES

Appendix A: Journal Publications

1. **Dalene Lesen**, Elexson Nillian, Dayang Najwa Awang Baki and Tunung Robin. (2024). Prevalence of *Vibrio parahaemolyticus*, *Vibrio cholerae*, and *Vibrio alginolyticus* in a White-leg Shrimp (*Litopenaeus vannamei*) Farm in Sarawak. Pertanika Journal of Science & Technology, 32(5), 2233 – 2257. https://doi.org/10.47836/pjst.32.5.17
2. Dayang Najwa, A. B., Elexson, N., **Dalene, L**., and Teng, S. T. (2024). *Vibrio* Species and cyanobacteria: Understanding their association in local shrimp farm using canonical correspondence analysis (CCA). *Microbial Ecology*, *87*(1), 51. https://doi.org/10.1007/s00248-024-02356-5
3. Lambuk, F., Mazlan, N., **Lesen, D.**, Nillian, E., and Thung, T. Y. (2024). *Staphylococcus aureus* lytic bacteriophage: Isolation and application evaluation. *Journal of Consumer Protection and Food Safety*. https://doi.org/10.1007/s00003-024-01479-8
4. Elexson, N., Buyong, N. L., **Lesen, D.**, Basiron, M. M., Ahad, N., Wan Jesfrydi, W. N. Q., Jamaludin, M. H., Fadilah, M. F. A., Azra, T., and R., T. (2023). A survey study on the assessment of food handler's compliance to personal hygiene practices regulation in selected Malaysia food outlets. *Food Research*, *7*(1), 64-75. https://doi.org/10.26656/fr.2017.7(1).620
5. DAYANG NAJWA AWG BAKI, ELEXSON NILLIAN, and **DALENE LESEN**. (2023). The prevalence of *Vibrio cholera* and *Vibrio parahaemolyticus* virulence genes and multiple antibiotics resistant (mar) assessment from local shrimp farm in Sarawak.

Borneo Journal of Resource Science and Technology, 13(1), 93-107. https://doi.org/10.33736/bjrst.5375.2023

6. Elexson, N., Eddy, M. B., Wong, J. C. J., **Lesen, D**., Ransangan, J., and Thung, T. Y. (2023). Prevalence of multi-antibiotics resistant (MAR) *Vibrio parahaemolyticus* in shrimp farms in Sarawak, Malaysia. *Food Research, 7*(4), 127-136. https://doi.org/10.26656/fr.2017.7(4).320
7. Nillian, E., Zakaria, N. D., **Lesen, D.,** Yusoff, N. H., Ismail, N. S., Tung, T. S., and Bilung, L. (2022). Comparison distribution of *Vibrio* species in stocking to harvesting process of shrimp at commercialize shrimp farm. *International Journal of Biology and Biomedical Engineering*, *16,* 168-174. https://doi.org/10.46300/91011.2022.16.22
8. Elexson, N., Sabrina, H., **Dalene, L**., Eddy, B., Nurul, F. R., Nasra, P., Grace, B., Amirah, Z. J., nUR, D. Z., Dayang, N. A. B., Manju, S., and Tunung, R. (2022). Assessment of *Pseudomonas aeruginosa* biofilm-forming capacities from drinking water in water vending machine. *Food Research, 6*(3), 76-83. https://doi.org/10.26656/fr.2017.6(3).324
9. Elexson, Nillian, Ja'afar, A. Z., Joel, W., Laurence Buyong, N., **Dalene, L**., and Tze, T. Y. (2023). Random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) PCR of *Vibrio cholera* from a foodborne outbreak in Limbang, Sarawak. *International Food Research Journal, 30*(3), 591-600. https://doi.org/10.47836/ifrj.30.3.04

Appendix B: Patent

1. "ENILVP02 Vibrio Phage as Biocontrol of *Vibrio parahaemolyticus*"



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REPORT

PATENT SEARCH

& PATENTABILITY OPINION

Particulars of Project

Client	:	**Universiti Malaysia Sarawak (UNIMAS)**
Title of Invention	:	**ENILVP02 Vibrio Phage as Biocontrol of *Vibrio parahaemolyticus***
PW's Ref	:	UN109/91297-PT
Client's Ref	:	please advise
Period of Search	:	6 – 20 March 2024
Date of this Report	:	20 March 2024
Date of Revision (if any)	:	3 May 2024

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Figure 2: Patent for phage EniLVP02

Appendix C: Recipe for solutions and media

1. **Salt of Magnesium (SM) buffer (per 1000 mL)**

   - 5.8 g of $NaCl_2$
   - 2.0g of $MgSO_4.7H_2O$
   - 50 mL of 1M Tris-HCl (pH 7.5)
   - 5 mL of 2% gelatin
   - Distilled water

   (Autoclave the buffer and store at RT)

2. **Bottom agar (for Double-layer agar assay) (per 400 mL)**

   - Tryptic soy agar (prepare according to the manufacturer's instructions)
   - 8 g of $NaCl_2$
   - 4 mL of 1M $MgCl_2$ (Add after autoclaving the media)

   (Pour the media into petri dishes and allowed to solidify before use. Store at 4 °C)

3. **Top agar (for Double-layer agar assay) (per 100 mL)**

   - Tryptic soy broth (prepare according to the manufacturer's instructions)
   - 0.45 g of Bacteriological agar
   - 2 g of $NaCl_2$
   - 1 mL of 1M $MgCl_2$ (Add after autoclaving the media)

   (Aliquot 4 mL of media into sterile test tubes and store at 4 °C. Remelt and equilibrate to 50 - 52 °C before use)

Appendix D: Sampling activities



Figure 3: Photos taken during sampling at Persatuan Nelayan Kawasan (PNK) Satang Biru, Lembaga Kemajuan Ikan Malaysia (LKIM), Telaga Air, Sarawak: (A) Stocking of Whiteleg shrimp (*Penaeus vannamei*) in pond, (B) Measuring environmental and chemical parameters during sampling, (C) Post-larvae (PL) of *P. vannamei* (D) Adult *P. Vannamei* suitable for harvest.