FACULTY OF BIOSCIENCE ENGINEERING

ISOLATION AND CHARACTERIZATION OF MOTILE AEROMONAD PHAGES

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Master's Dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in Aquaculture
Academic Year: 2019 - 2020

UNIVERSITEIT GENT

NITTE (Deemed to be University) | NITTE UNIVERSITY CENTRE FOR SCIENCE EDUCATION & RESEARCH

Acknowledgements

I thank the almighty God for the gift of life and having protected me especially in the challenging Covid-19 times.

I sincerely appreciate Prof. Dr. Indrani Karunasagar and the staff of Nitte University Center for Science Education and Research (NUCSER) for the technical, social and moral support during my stay at the institution.

Uniquely, I thank Prof. Juliet Roshini Mohan Raj for her technical mentorship she offered me throughout my research.

I would wish to appreciate research scholars Apoorva R Kenjar and Pallavi Bhat A, for all the guidance and support of all kinds that enabled me successfully conduct my research.

To my funders, VLIR – UOS (Vlaamse Interuniversitaire Raad – The Flemish Interuniversity Council), without your financial support, I wouldn't have made it.

I extend my earnest appreciation to Prof. Dr.ir. Peter Bossier and Artemia Reference Center, Gent University, Belgium, for all the coordination and technical guidance that catalyzed the overall success of this work.

I am very grateful to the National Agricultural Research Organization (NARO) of the government of Uganda for the study leave offered to me to upgrade my knowledge and build networks and collaborations that matter.

Finally, to my family and friends, for the patience, moral and spiritual support throughout my program.

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Preamble

Initially, the scope of this study entailed isolation of Aeromonad hosts, biochemically and molecularly characterizing them, carrying out an antibiotic susceptibility assay, and virulence factor examination. This was to be followed by isolation of bacteriophages, phenotypically, and molecularly characterizing them, including growth kinetics studies. Parameters that we had planned to study included: morphological examination under a transmission electron microscope (TEM); a host range check with the isolated Aeromonads and other taxonomically distant bacterial species and or strains, one-step phage growth curves to obtain information about the latent period, burst size, adsorption rates; an in vitro one-phage and cocktail-phage inactivation assay of the Aeromonad isolates using different multiplicities of infection (MOIs); phage stability at different temperatures, pH and salinities; prophage induction screening; and an RFLP to classify and compare the phage lysates with the already isolated Aeromonas isolates by bioinformatics. We had also planned to repeat some experiments to check on the reproducibility and consistency of the results. However, due to the Covid_19 disturbance that led to multiple lockdowns, time was lost and we were unable to do some activities. Morphological examination of the phage lysates in a TEM, phage stability tests, prophage induction screening, phage cocktail trials, host range tests with bacteria isolates unrelated to *Aeromonas sp*, and molecular characterization of both phage and bacteria host isolates were not don

Abstract

Multidrug resistance within motile Aeromonads is still one of the prime challenges affecting global aquaculture hence phage therapy is among the proposed alternatives to antibiotics. In this study, 24 motile Aeromonads were isolated from ornamental fish and water samples, used as hosts in isolation of three lytic bacteriophages 2, 3 and 10 from the same samples. Phage lysates 2, 3 and 10 had adsorption efficiencies of (89%), (42%) and (51%). Phage lysate 2 and 3 had a relatively broader host range than 10 by lysing four out of the 24 Aeromonad isolates whereas lysate 10 only lysed one. Phage lysate 10 had the shortest latent period (40 minutes) as compared to the 60 minutes of phage lysates 2 and 3, and highest burst size (271 PFU/cell) as phage lysates 3 and 2 had burst sizes of 187 PFU/cell and 56 PFU/cell. A six-hour in vitro CFU reduction assay revealed that all phage lysates were capable of significantly inactivating the proliferation of the Aeromonad isolate used in this study at all multiplicities of infection following the order of MOI (10) > (1) > (0.1) and (0.01) for phage lysates 10 and 3 as opposed to 2, where MOI (0.1) > (1) > (10) > (0.01). Following 11 hours of phage infection, resistant viable colonies were observed from MOI (10) and (1) but not (0.1) and (0.01) for phage lysates 10 and 2. Survivors of phage lysate 10 were then resistant to phage lysate 10 but sensitive to 2 and 3, while phage lysate 2 survivors were still sensitive to all phage lysates. However, morphological examination, molecular characterization, prophage induction screening and phage stability under different conditions should be done to aid further understanding of the isolated motile Aeromonad phages.

1.0 Introduction

Aquaculture which is the rearing of aquatic plants and animals in fresh, brackish and or marine aquatic environments (Prado et al., 2020; Stickney, 2001), is globally considered the fastest growing food-producing sector (Garlock et al., 2020). According to FAO, 2019; aquaculture has become a major contributor to food security and socio-economic development in many parts of the world by supplementing capture fisheries production, offering employment opportunities and income to many players. The increasing demand for fish to feed the steadily increasing global population, and the stagnant capture fisheries production despite increasing fishing effort (Ertör-Akyazi, 2020), have led to among other phenomena, a shift from extensive to intensive aquaculture production systems that maximize yield (Kobayashi et al., 2015; Muktar & Tesfaye, 2016). Intensive systems are characterized by monocultures of high stocking densities of high-value fish species and high protein-containing artificial feeds, which increase the chances of pathogen-host and substrate contact, and poor water quality with abrupt fluctuations (Muktar & Tesfaye, 2016). This has aggravated the occurrence of host-specific density-dependent infectious diseases, dominated by bacterial infections caused by *Aeromonas sp*, *Edwardsiella sp*, *Flavobacterium sp*, *Vibrio sp*, and *Pseudomonas sp*, which are responsible for most fish and shellfish morbidity and mortality (Lafferty et al., 2015; Mzula et al., 2019; Nayak, 2020). Columnaris, Vibriosis, Edwarsiellosis and Aeromoniasis are some of the key bacterial infections in aquaculture (Gui & Zhang, 2018). Aeromoniasis is caused by motile Aeromonads, a highly ubiquitous group of Gram-negative, facultatively anaerobic, oxidase and catalase positive bacteria (Jones & Wilcox, 1995; Nayak, 2020). It's composed of opportunistic pathogens to stressed and immunocompromised animals such as reptiles, fish, crustaceans, amphibians, and humans (Khushiramani et al., 2007; Nayak, 2020), which can as well act as isolation sources (Gonçalves Pessoa et al., 2019). Motile Aeromonads have very diverse and unique biochemical and serological characteristics between species and strains (Pękala-Safińska, 2018), with pathological complications such as dropsy, haemorrhagic septicaemia, epizootic ulcerative syndrome, red mouth disease, exophthalmia, and mass mortalities to key aquaculture animals including Common carp, Tilapia and Catfishes, whose contribution to global freshwater aquaculture finfish production is significant (Dash et al., 2014; Nayak, 2020). Antibiotics and other chemotherapeutics, regardless of their partial efficacy in bacterial disease

treatment, difficulty in administration at a commercial level, and associated side effects and risks, are indiscriminately used to treat Aeromoniasis (W. Wang et al., 2017).

The widespread of antibiotic usage has led to the emergence of antibiotic resistance (Huddleston, 2014) within and between bacteria of veterinary and public health concern including motile Aeromonads. Furthermore, accumulation of antibiotic residues in the environment, aquaculture produce, and human consumers has been reported, making their use a global safety and sustainability concern (Aly & Albutti, 2014). Evolution of strict regulations especially to aquaculture producers and exporters governing antibiotic usage and residues was unavoidable. This was mainly to safeguard the integrity of the natural aquatic ecosystems and the health of human consumers of aquatic produce. Therefore, to sustainably minimize disease occurrence in aquaculture farms and associated negative effects of antibiotics and chemotherapeutics, comprehensive, effective, environmentally friendly and safe prophylactic and or therapeutic measures need to be developed (Okocha et al., 2018). Phage therapy is the use of bacteriophages as therapeutic and or prophylactic agents and has been reported to be safe, eco-friendly and scientifically demonstratable in controlling a some bacteria of aquaculture importance such as *Aeromonas sp*., *Flavobacterium sp*., *Pseudomonas sp*., *Edwardsiella sp*., *Lactococcus sp*., and *Streptococcus sp*. (Gon Choudhury et al., 2017; R Gudding et al., 2014; Richards, 2014). Apart from being naturally occurring and abundant, self-replicating, self-limiting, host-specific, resistant to harsh host immune defence mechanisms, phages employ completely different bacteriolysis strategies as opposed to antibiotics and have been reported to inactivate multi-drug resistant bacterial species and strains. Additionally, phages do not cause dysbiosis as opposed to antibiotics (Cao et al., 2019, 2020).

Therefore, the goal of this study was to isolate and characterize novel motile Aeromonad bacteriophages as potential biological control agents of virulent *Aeromonas* sp., the causative agent of Aeromoniasis. Parameters such as host range, growth kinetics (one-step growth curve, burst size, adsorption rate and latent period), in vitro bacterial colony forming units (CFUs) reduction efficacy and bacteria isolate phage resistance potential were evaluated.

2.0 Literature review

2.1 Aquaculture

Aquaculture is the rearing of aquatic plants and animals in controlled and or semi-controlled fresh, marine or brackish aquatic environments (Prado et al., 2020). Food, income, employment, and biodiversity conservation are among the benefits of aquaculture (Stickney, 2001). Farmed species by 2016 totalled to over 277, including finfish (171), molluscs (59), crustaceans (27), and plants (20) (Figure 1). Currently, aquaculture is the fastest-growing food-producing sector as compared to the stagnant capture fisheries production and other livestock enterprises (Garlock et al., 2020). Earthen ponds, tanks, raceways, cages, pens, rafts and longlines (FAO, 2019; Mao, 2016) have mainly been responsible for the realized growth. An increase in per capita food fish consumption from 9.0Kg (1961) to 20.2Kg (2015) was realized, and its attributed to not only an increase in fish production but also advancement in preservation and value addition technologies that led to improved post-harvest handling and marketing of aquaculture products (FAO, 2019).

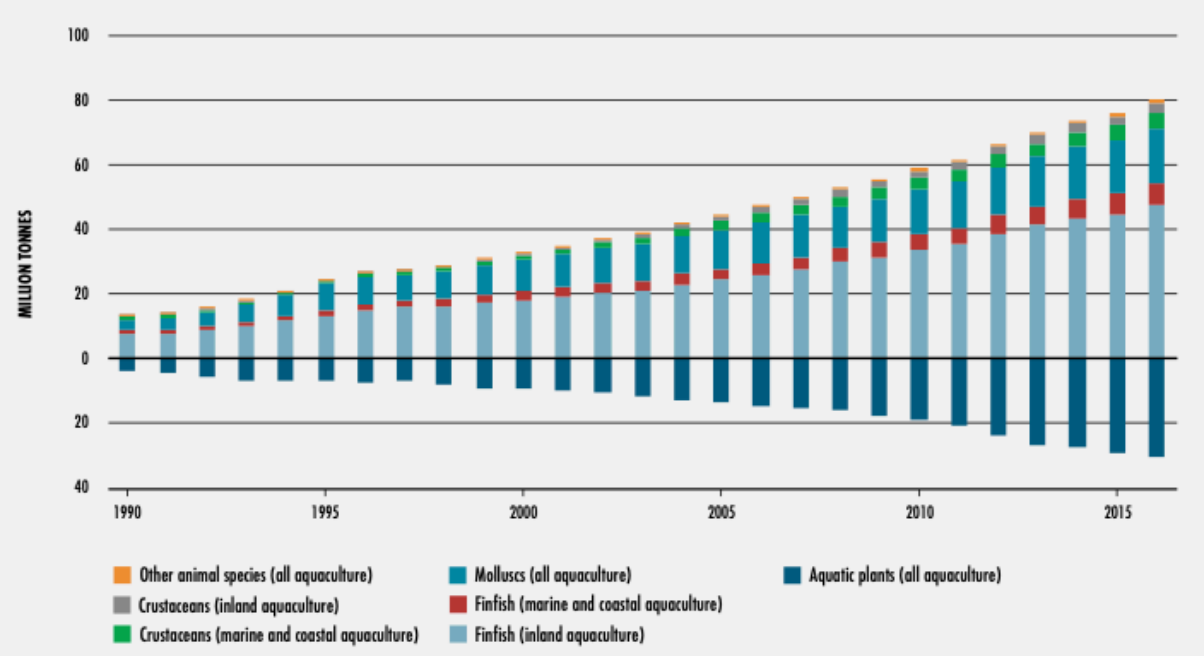

Figure 1: Global trends of aquaculture production of different categories of products from 1990 to 2016, (FAO, 2019)

2.2 Diseases in aquaculture

Disease occurrence is not just as a result of the presence of pathogens, but as a result of complex interactions between pathogens, hosts and the environment (Figure 2), prevailing climate conditions and status of fish immunity (Defoirdt et al., 2011; Pękala-Safińska, 2018). It remains one of the most fundamental constraints to aquaculture development due to morbidity, mortality and enormous economic losses which have been estimated at USD 9 billion and USD 120 million globally and in China respectively (Novriadi, 2016). As a consequence, the growth of aquaculture production is stalled by global aquaculture disease outbreaks, which jeopardize enterprise profitability, productivity and sustainability (Defoirdt et al., 2011; Roar Gudding & Van Muiswinkel, 2013).

Most aquaculture diseases are bacterial, composed of obligate and or opportunistic pathogens (Gui & Zhang, 2018). Viral diseases (Spring viremia of Carp disease & White spot syndrome disease), fungal infections (Saprolegniasis, Branchiomycosis & Epizootic ulcerative syndrome) and parasitic infestations (Icthyopthiriasis, Trichodiniasis, Dactylogyrusis, Gyrodactylosis, Argulosis and Myxosporidiasis) are also among the causative agents of morbidity and mortality in aquaculture (Gui & Zhang, 2018; Secombes & Belmonte, 2016; SS, 2017).

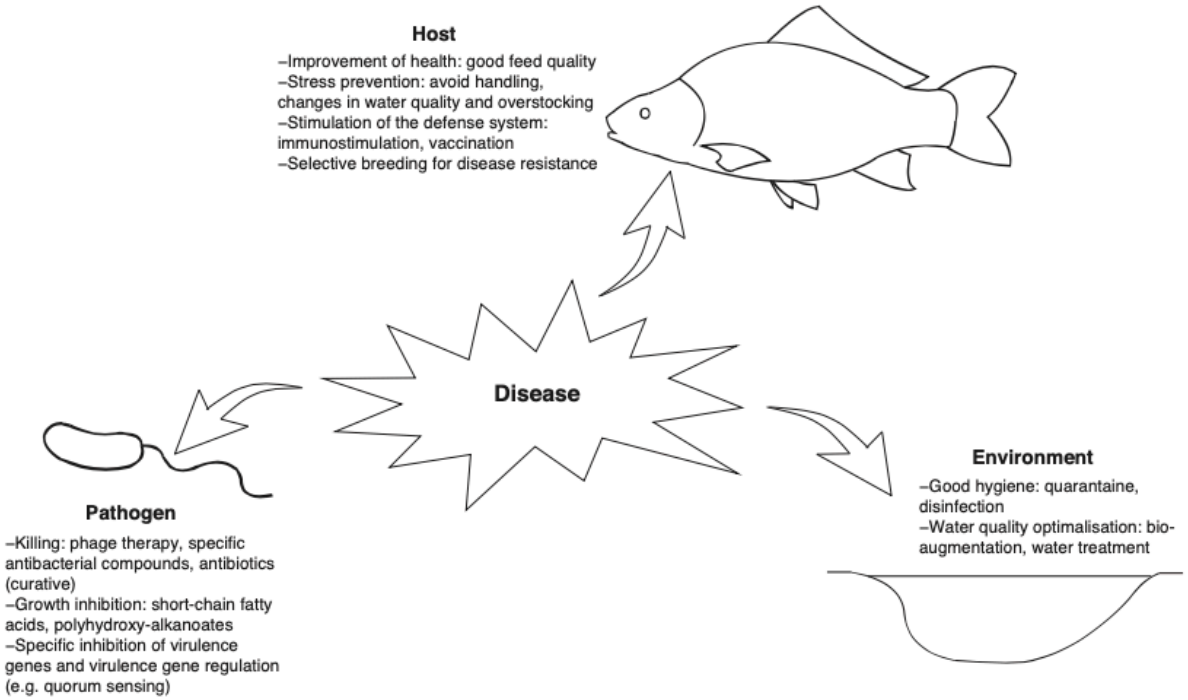

Figure 2: The infectious cycle and proposed disease control measures in aquaculture, (Defoirdt et al., 2011)

2.3 Bacterial diseases of fish

As compared to other infectious disease causative agents, bacteria have been reported to cause more infectious diseases in aquaculture and at all stages of growth, right from eggs to adults (SS, 2017). This can be attributed to expansion and intensification of aquaculture, diversified fish culture techniques and species, as well as improved diagnostic tools (Muktar & Tesfaye, 2016). As some bacteria are obligately pathogenic, others are opportunistic and can sustain themselves independent of the hosts, and are capable of causing diseases in immunocompromised fish as a result of stress, injury, and other primary infections (Plumb, 1999). These diseases cost aquaculture a lot of economic losses approximated in million dollars annually from mortalities, therapeutics, vaccination and manpower expenses (Novriadi, 2016; Plumb, 1999). Although most bacterial diseases are caused by Gram-negative bacteria as seen in (Table 1), there are also some diseases caused by Gram-positive bacteria, for example, Streptococcosis, bacterial kidney disease and Staphylococcosis in fish species of economic importance like trout (Pękala-Safińska, 2018; Plumb, 1999).

Table 1: Common key Gram-negative pathogenic bacteria of aquaculture importance and their hosts, (Gui & Zhang, 2018)

Causative agent	Host	Disease
Aeromonas hydrophila	Tilapia, Carp, Trout, Catfish, Sturgeon, Eel & Bass	Motile aeromonads septicemia
Aeromonas salmonicida	Tilapia, Trout, Carp, Salmon, Turbot & Flounder	Furunculosis
Edwardsiella ictaluri	Catfish	Enteric septicemia of Catfish
Edwarsiella tarda	Carp, Catfish, Tilapia, Flounder, Turbot and Eel	Edwardsiellosis
Flavobacterium columnare	Carp, Trout, Perch, Catfish, Tilapia and Salmon	Columnaris
Vibrio sp.	Puffer, Croaker, Seabream, Turbot, Sole & Salmon	Vibriosis
Yersinia ruckeri	Trout, Tilapia & Salmon	Enteric redmouth disease

2.4 Aeromoniasis

Aeromonas is a highly ubiquitous genus comprising of Gram-negative, rod-shaped, non-spore-forming, facultative anaerobes, which are catalase and oxidase positive as well as chemoorganotrophic (Fe et al., 2017; Nayak, 2020). It is divided into mesophilic and motile species (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas veronii biovar sobria*) which can grow optimally at 35-37 ^{0}C and the psychrophilic non-motile species (*Aeromonas*

salmonicida subsp. salmonicida, *Aeromonas salmonicida subsp. masoucida* and *Aeromonas salmonicida subsp. smithia*) which can grow optimally at 22 - 28^0 C (Fe et al., 2017). Taxonomically, they belong to class – Gammaproteobacteria, order Aeromonadales and family Aeromonadaceae and have been isolated from both aquatic and terrestrial environments with optimum pH levels of 5.5-9 and 0-4% salinity (Fe et al., 2017). Motile Aeromonads, microbes of interest in this study can phenotypically be identified by the presence of Gram-negative, motile fermentative rods, which produce catalase, oxidase and arginine dihydrolase enzymes but not lysine or ornithine decarboxylase (Austin & Allen-Austin, 1985). Members of this group are known to cause Aeromoniasis, with various pathological manifestations such as tail and fin rot, epizootic ulcerative syndrome, dropsy, exophthalmia, and red mouth disease in mainly freshwater and to a lesser extent, marine aquatic vertebrates and crustaceans (Austin & Allen-Austin, 1985; Pękala-Safińska, 2018). Among the general symptoms of Aeromoniasis are: surface lesions with loss of scales and erosion of fins; gill and vent haemorrhaging; abscesses and ulcerations; abdominal distensions; anaemia; ascites fluid accumulation; exophthalmia; damage to internal organs and musculature with generalized liquefaction and mass mortalities (Pękala-Safińska, 2018; Richards, 2014). Motile Aeromonads are opportunistic pathogens, with a couple of virulence factors that contribute to pathogenicity at different stages of the infectious cycle (Fortier & Sekulovic, 2013). For example, flagella, pili and adhesins, facilitate approach, establishment and survival within the host, production of enterotoxins, proteases, lipases, phospholipases and haemolysins enhances tissue damage and disease propagation, and biofilm formation, which plays a key role in resistance to disinfectants and overall microbial survival (Das et al., 2020). Routine maintenance of good water quality, good quality feeds and feeding, the appropriate stocking density of fish and shellfish, and other good management practices that minimize stress in the rearing systems is very important in lessening the effect of Aeromonads (Thurlow et al., 2019).

2.5 Bacterial disease control and prevention

Since prevention is better than cure and putting in mind the comprehensive interaction between the host, pathogen and the environment, several preventive measures to disease occurrence have been applied in aquaculture (Defoirdt et al., 2011) (Figure 2). Broadly, prevention and control of bacterial diseases in aquaculture is mainly through microbial management, which relies on an interaction between three key parameters (Figure 3), especially at early stages of fish development that are more vulnerable to disease infections (Vadstein et al., 1993).

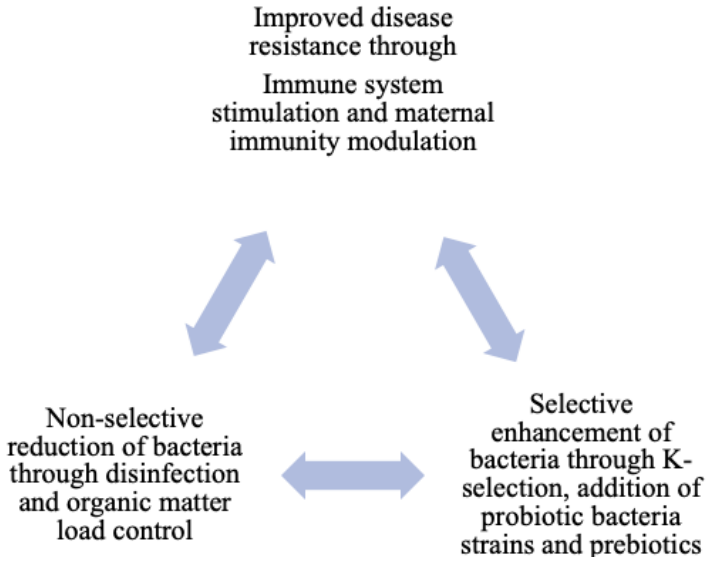

Figure 3: Holistic microbial management in an aquaculture system for control and management of infectious diseases (Vadstein et al., 1993)

2.5.1 Antibiotics

The antibiotic discovery was a very important step in the treatment of animal and human bacterial diseases even though bacteria that were initially sensitive to commercial antibiotics have recently become resistant (Odeyemi & Ahmad, 2017). Among the commonly used therapeutic compounds in aquaculture to minimize the rampant economic losses associated with infectious disease occurrences, antibiotics are at a forefront (Chuah et al., 2016). They are usually added in feed or directly in water to control bacterial populations (Scarano et al., 2018). The nature, quantity, and frequency of application in different countries is dependent on the indigenous legislations, controls and inspections by relevant authorities (Defoirdt et al., 2011). Furthermore, Defoirdt et al., 2011 reported that control of antibiotic usage is variable within and between countries and that the observed increase in antibiotic quantities used in aquaculture match with the intensity at which aquaculture is switching from being extensive to intensive. According to (Pruden et al., 2013), most key global aquaculture producing countries have limited enforcement and compliance with regulations governing antibiotic usage. Therefore, the extensive use of antibiotics, as a result, has led to the emergence of antibiotic resistance in both veterinary and public health pathogens as well as opportunistic pathogens (Defoirdt et al., 2011; Odeyemi & Ahmad, 2017). This has led to reduced efficacy of most commercial antibiotics in treating bacterial infectious diseases (Huddleston, 2014; Novriadi, 2016). Some antibiotics are nonbiodegradable and bioaccumulate within the food chain,

leading to human consumption of residues, resulting into allergies, toxic effects, changes in gut microbiota, and antibiotic resistance among veterinary and public health bacterial pathogens (Vignesh et al., 2011). Several studies have reported multidrug resistance of *Aeromonas sp*. strains to commonly used antibiotics in aquaculture, partially attributed to the selective pressure in intensive aqua-farms (Scarano et al., 2018). For example, (Odeyemi & Ahmad, 2017) reported that most Aeromonad isolates are completely resistant to ampicillin, novobiocin, trimethoprim and sulphamethoxazole but sensitive to chloramphenicol, tetracycline, oxytetracycline, kanamycin and gentamycin. The variation in antibiotic resistance could be due to the variation in the mechanisms of resistance possessed by the different species or strains, from naturally intrinsic to acquired resistance by horizontal and or vertical transfer (Aich et al., 2018). Intrinsic resistance is by naturally occurring mechanisms without prior antibiotic exposure, whereas acquired resistance is from exposure to antibiotics and mobile resistance genes and compounds from resistant strains (Hancock, 1998). Microbial antibiotic resistance can be in the form of selective uptake and efflux of drugs due to presence of selectively permeable envelopes and efflux pumps common in Gram-negative bacteria, drug modification and inactivation by enzymes like beta-lactamases, and changes in targets due to mutations (Lambert, 2002; Naas et al., 2017).

2.5.2 Vaccination

To overcome the negative ecological effect of antibiotics, prophylaxis through vaccination has become an integral part of management, and is among the most effective, already established, proven and cost-effective methods of disease prevention and control of infectious diseases in aquaculture (Sommerset et al., 2005). It targets a comprehensive and complex interaction between the host, pathogen and the environment, aimed at stimulating the immune system of fish and overall disease resistance (Roar Gudding & Van Muiswinkel, 2013). Whole organism vaccines such as killed and or attenuated vaccines are the most commonly used and have shown great potential in controlling and preventing infectious bacterial diseases according to (Mzula et al., 2019). Due to advancements and a better understanding of fish and shellfish immunology, genetics, biotechnology, and molecular biology, novel polyvalent vaccines such as plasmid DNA, Subunit and recombinant live vector and protein vaccines have been developed (Bedekar et al., 2020; Mzula et al., 2019). Most DNA vaccines are highly adopted and more effective for viral as opposed to bacterial infections (Pridgeon & Klesius, 2013). According to (Mzula et al., 2019), recombinant live vector vaccines can lead to introduction and accumulation of recombinant bacteria in the environment which are classified as

genetically modified organisms (GMOs) by European Union (EU) and other legislative bodies, limiting their utilization. This leaves recombinant protein vaccines with great potential as polyvalent vaccines (Nascimento & Leite, 2012) as a more environmentally friendly, safer and more appropriate method of vaccine production and delivery to hosts (Dalmo, 2018). However, developing a relatively inexpensive vaccine, without residual pathogenicity, administered in a less stressful manner, less time consuming to prepare, polyvalent, effective and offering long-lasting immunity to heterologous bacterial infections of aquaculture importance remains a challenge (Maiti et al., 2011; Sommerset et al., 2005). On top of that, the limited knowledge about the immune system of many fish species, absence of adaptive immune systems in crustaceans and molluscs, presence of a variety of pathogens with unique host-pathogen, and susceptibility, limited effectiveness of vaccines especially at early development stages of fish, complex pre-commercialization requirements resulting into presence of very many unlicensed vaccines on markets, as a result of localized infectious diseases to specific areas and species of fish and shellfish and not others, are also limitations to vaccine development and application in aquaculture (Muktar & Tesfaye, 2016; Mzula et al., 2019; Nakai & Park, 2002; Pérez-Sánchez et al., 2018). Nevertheless, pathogens like *Aeromonas sp,* which are highly biochemically and serologically diverse, make development of polyvalent vaccines for their control problematic (Le et al., 2018).

2.5.3 Biological control

Given the above, several biological control measures have been brought on board to minimize disease occurrence and treatment. Among these include, probiotics, prebiotics, symbiotics and postbiotics, phage therapy and phytobiotics (Table 2) (Pérez-Sánchez et al., 2018). Probiotics are live microbial feed supplements orally administered to confer health benefits to the host by creating hostile environments for pathogenic bacteria through: producing antimicrobial compounds, out-competition for space and nutrients and minimization of virulence factor expression by quorum sensing interreference (Newaj-Fyzul et al., 2014; Pérez-Sánchez et al., 2018). Prebiotics are indigestible fibres fermented by gut enzymes and commensal bacteria, whose beneficial effects are due to the by-products generated from fermentation that selectively stimulate the growth and or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health (Carbone & Faggio, 2016; Pérez-Sánchez et al., 2018). Symbiotics exploits synergistic efficacy of a combination of probiotics and prebiotics whereas postbiotics are nonviable bacterial products or metabolic by-products from probiotic microorganisms that have a biological protective activity in the host (Pérez-Sánchez et al., 2018). Phytobiotics are

plant-derived natural bioactive compounds which are added to the diet to improve nutrition and health in farm animals (Carbone & Faggio, 2016). Phage therapy, the main subject of interest of this study, involves using bacteriophages, which are viruses that infect bacteria in the environment and within hosts (Richards, 2014).

Table 2: Biological control measures to pathogens in aquaculture with their strengths and limitations (Pérez-Sánchez et al., 2018)

Technique	Advantage	Limitations
Probiotics, Prebiotics, Symbiotics and Postbiotics	Improve growth performance and health. Initiate and modulate immune responses. Prevent pathogen colonization and infection.	Limited protection with some pathogens. Variable synergistic effects. Marketing authorisation is complex.
Phytobiotics	Antimicrobial, antiparasitic, anti-inflammatory, and antioxidative activities Increase host survival.	Some constituents are unstable for example; they are photo- and thermo-labile. Interactions with host microbiota are unknown.
Phage therapy	Target specific, whereby avoiding damage to host microbiota. Self-sustained till the host is present in high numbers in the environment. Phage cocktails can reduce resistance development and be more effective than single phages.	Potential for transfer of virulence and/or antibiotic-resistance genes. Potential for resistance development.

2.5.3.1 Phage therapy

The use of bacteriophages, the naturally occurring and abundant viruses which infect specific bacterial species and or strains, is one of the eco-friendly, safe, and cost-effective infectious bacterial disease control measures that have been proposed as an ideal alternative candidate to antibiotics and other chemotherapeutics (Henein, 2013; Richards, 2014). Unlike other biological control measures, the self-perpetuating nature in presence of appropriate hosts, and the causal effect of phages can easily be scientifically demonstrated in successful treatment by the presence of large numbers of phage particles and reduction in specific bacterial loads in survivors (Nakai & Park, 2002). It has also been reported that phage therapy doesn't trigger the production of neutralizing antibodies in fish before administration and that they are also stable against internal hostile conditions such as the low gut pH, explaining their stability in vivo (Nakai & Park, 2002).

Re-emergence of phage therapy was mainly due to occurrence of multidrug-resistant bacterial strains, the regulatory approval already availed for use in meat, poultry and agricultural products, and the high effectiveness demonstrated in controlling bacterial diseases in human, crop and livestock diseases (Monk et al., 2010; Richards, 2014). As opposed to antibiotics that are nonspecific, phages have high species and or strain specificity, minimizing the effect on commensal and other beneficial bacteria in the gut and other organs (Pérez-Sánchez et al., 2018). Trials of phage therapy against Vibriosis in an aquaculture setting have been reported by several authors to have had a high degree of success, both at short and long terms for enhanced and significant survival rates of fish and shellfish challenged with the disease (Kalatzis et al., 2018). A logarithmic and significant reduction in *Vibrio sp.* colony forming units (CFU/ml) as compared to control treatments has also been reported (Kalatzis et al., 2018). Broad host range lytic bacteriophages of *Vibrio harveyi* were able to control the bacterial proliferation and also generated higher and significant survival rates as compared to controls at a low multiplicity of infection (MOI = 1) in *P. monodon*, *H. laevigata, P. ornatus and O. plicaltula* (Karunasagar et al., 2007; Stalin & Srinivasan, 2017; Y. Wang et al., 2017). The virulence of *Vibrio parahaemolyticus* in *L. vannamei*, *V.alginolyticus* in *A. japonicus*, *V. splendidus* in *A. japonicus*, *V. cyclitrophicus* in *S. salar, V. anguilarum* in *D. rerio* and *V. coralliilyticus* in *A. millepora* has been lessened by lytic phages with varying efficacy (Cohen et al., 2013; Higuera et al., 2013; Li et al., 2016; Rong et al., 2014; Silva et al., 2014). One of the key highlights of phage therapy was the capability of a bacteriophage "CHOED" to give 100% protection to the Atlantic salmon (*Salmo salar*) against pathogenic *Vibrio anguillarum*,

unlike the untreated group that had 90% mortality (Higuera et al., 2013). Like in most phage therapy trials, cocktail combinations were still more effective than sole treatments in Vibrio control trials (Kalatzis et al., 2018).

2.5.3.1.1 Phage structure

Bacteriophages are diverse in structure, biological and physicochemical attributes, hence polyphyletic in origin (Kutter & Guttman, 2004). They are generally tailed, polyhedral, filamentous and pleomorphic with majority consisting of double stranded (ds) DNA while smaller groups with single stranded (ss) DNA, ssRNA and ds RNA also exist (Paul & Sullivan, 2005). The basic Phage structure can be of an Icosahedral head with a tail, Icosahedral head without a tail or filamentous (Madhusudana Rao & Lalitha, 2015). The most commonly isolated phages in the aquatic environment belong to Myoviridae, Siphoviridae and Podoviridae families (Madhusudana Rao & Lalitha, 2015). Myoviridae phages are characterized with dsDNA, an icosahedral symmetrical head and a helical contractile tail separated by a neck, Siphoviridae phages have dsDNA, an icosahedral capsid and a filamentous non-contractile tail, whereas Podoviridae phages have dsDNA, icosahedral symmetrical head, and a very short non-contractile tail (Kutter & Guttman, 2004; Madhusudana Rao & Lalitha, 2015).

2.5.3.1.2 Types of bacteriophages

There are two general types of bacteriophages; Lytic and temperate phages with lytic and lysogenic cycles respectively (Gon Choudhury et al., 2017). In the lytic cycle, phages invade bacterial cells and use the host DNA and replication machinery to replicate and synthesize large numbers of new viruses, which after the incubation period lyse the hosts, releasing new phages, ready to attack and infect fresh hosts (Gon Choudhury et al., 2017). The lytic cycle is characterized by stages including (Figure 4): Attachment to bacterial host surface, injection of phage genome into host, cessation in synthesis of host components, host mediated replication and synthesis of phage components including capsid proteins and nucleic acids, assembly of new phage particles, lysis of hosts and release of progeny phages (Richards, 2014). Through a process known as lysogenisation, temperate phages can integrate their DNA into host chromosomes, followed by normal replication of the incorporated phage DNA together with that of the host, leading to formation of Prophages for a couple of generations without lysis (Fortier & Sekulovic, 2013; Richards, 2014). However, through induction by chemicals, antibiotics and radiations, lysogenized host cells may spontaneously undergo DNA excision,

synthesis of new phage particles with lysis of the host, releasing more lysogenic viruses in the environment (Fortier & Sekulovic, 2013). The process of phage DNA incorporation into bacterial chromosomes results in viral DNA being part of the bacterial genome, estimated to reach as high as 20% of the overall genome (Vincent et al., 2019). This can result into enhanced virulence, for example Shiga toxin production by a Prophage of *E. coli* O157:H7, increased overall bacterial fitness and resistance to phages within lysogenized hosts (Figure 4) (Fortier & Sekulovic, 2013; Munro et al., 2003; Vincent et al., 2019). During the Prophage DNA excision from the host chromosomes, some host DNA may become incorporated in the phage DNA, facilitating horizontal gene transfer from one bacterium to another, resulting in enhanced virulence (Richards, 2014). Furthermore, some phages leak through host cells without lysis and are called filamentous phages (Marvin et al., 2014). According to (Oliveira et al., 2012; Vincent et al., 2019), some prophages can undergo extra chromosomal replication like plasmids and others can undergo an intracellularly dormant phase, where their genome is not incorporated in the host genome called pseudolysogenic.

Since lytic phages do not incorporate their DNA into the host chromosomes and do not enhance virulence, as well as their capability of rapid and quick replication, host killing and lysis, they are more ideal in phage therapy and bacterial reduction in fragile and perishable foods than temperate phages (Fortier & Sekulovic, 2013; Gon Choudhury et al., 2017; Richards, 2014). In situations where it is hard to isolate lytic phages for the target bacteria, naturally present or genetically engineered temperate phages can be singly or in combination with other lytic phages used (Nale et al., 2016; Vincent et al., 2019).

2.5.3.1.3 Prerequisites for phage therapy

Firstly, establishing the causative agent of a given disease is key to the success of phage therapy. Therefore, quick, sensitive, practical and pathogen-specific detection and diagnostic tools are essential for the success of all therapeutic measures to disease control in aquaculture, including phage therapy (Oliveira et al., 2012; Richards, 2014). After phage isolation from the environment, phage titre enrichment, purification of phage stocks, phenotypic and genotypic characterization, host range, specificity and lytic potential testing, in vitro and in vivo therapeutic efficacy testing, identification of virulence genes and associated toxin factors in case present, examining phage resistance mechanisms in resistant mutants and large scale application of isolated phages with culture and preservation facilities after conforming with relevant legislations follow (Chan et al., 2013; Nakai & Park, 2002; Oliveira et al., 2012). Inhibition of phage adsorption by loss or modification of cell surface receptors and blockage

of receptors by the extracellular matrix, inhibition of phage genetic material entry, enzymatic degradation of phage nucleic acids and host death are some of the phage resistance mechanisms studied (Moreirinha et al., 2018). Although the natural evolutionary dynamism and cocktail phage therapy can in combination minimize the phenomenon of phage resistant bacteria, there is need to minimize the number of individual phages in the cocktail to minimize chances of antagonistic behaviour and recombination (Chan et al., 2013; Madhusudana Rao & Lalitha, 2015). Transformation of previously nonvirulent bacteria hosts into virulent strains after phage therapy may arise especially if the prophages are not screened out (Vincent et al., 2019). Nevertheless, when lytic phages are used, there is a possibility of yielding non-pathogenic phage resistant bacterial strains whose virulence factors are selected against during phage resistance selection (Oliveira et al., 2012).

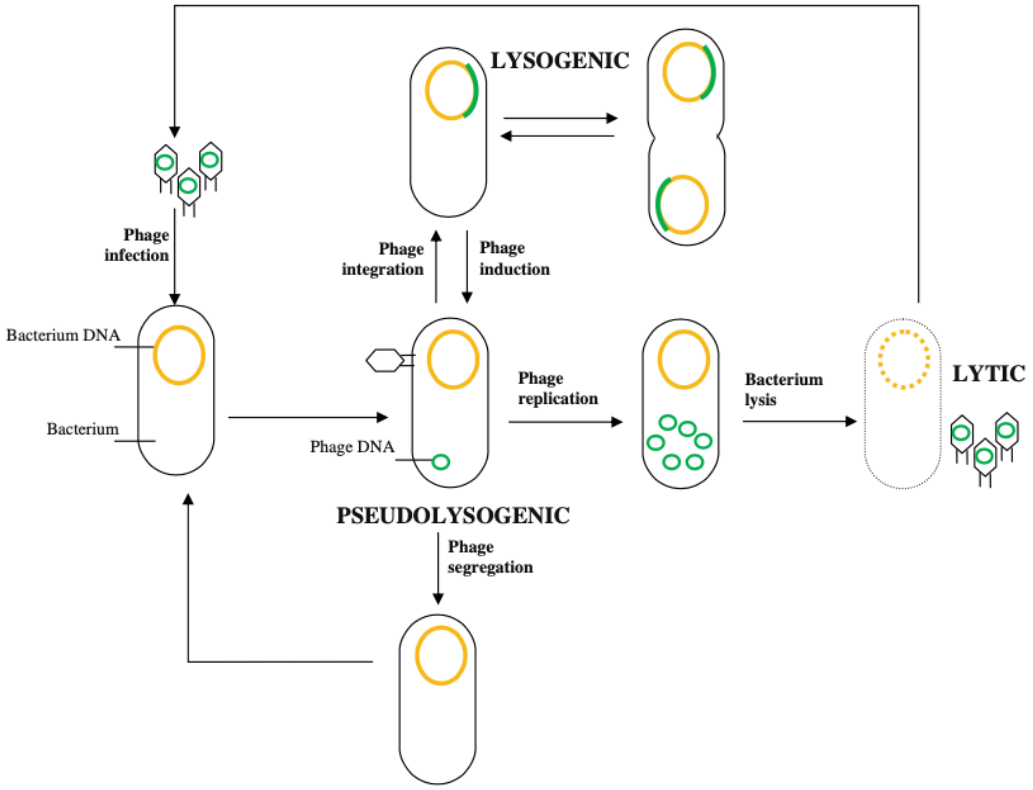

Figure 4: Bacteriophage lytic and lysogenic cycles: In the lytic cycle, the phage replicates and lyses the host cell. In the lysogenic cycle, phage DNA is incorporated into the host genome and passed on to subsequent generations. Environmental stressors may trigger prophage excision and entry into the lytic cycle by the temperate phage (Oliveira et al., 2012)

2.5.3.1.4 Techniques of bacteriophage isolation

Apart from being able to kill bacterial pathogens of interest, phages isolated from the environment should preferably have a relatively broader host range, not carry toxin genes and not capable of forming lysogens (Gon Choudhury et al., 2017). Generally, phage isolation follows a basic principle of mixing a potential phage containing environmental sample with host bacteria, followed by centrifugation and or filtration to remove the bacterial debris after 24 hours of incubation (Gill & Hyman, 2010). The functionality of bacteriophages in phage therapy is largely based on its host range (Richards, 2014). That is, narrower host range, infecting only a few strains, broader host range, infecting many strains of the same species and those infecting more than one species with a polyvalent host range(Gill & Hyman, 2010; Gon Choudhury et al., 2017).

2.5.3.1.4.1 Enrichment method

It remains the basic method of phage isolation involving an overnight incubation of a bacteria sample with an environmental sample (Clokie & Kropinski, 2009; Hyman, 2019). This is followed by filtration through a 0.22-micron filter and centrifugation to remove the bacterial debris, while the supernatant is assayed for the presence of phages. As most environmental samples do not contain the desired bacterial host phage concentrations, the enrichment method is commonly used.

2.5.3.1.4.2 Direct plating

This involves directly plating the environmental sample with the isolated bacterial hosts, plaque formation or clearing is visible in case lytic phages are present (Atterbury et al., 2003; Jäckel et al., 2019). This method requires higher phage concentrations as it's only a limited volume of sample that can be used on a Petri plate. Errors and biases due to sample processing can be avoided with this technique and also can be used to isolate phages that cannot grow under standard conditions by making some adjustments. This method is better suited for purposes of viral community studies as opposed to isolations for phage therapy. The description is according to (Gill & Hyman, 2010; Hyman, 2019) with some modifications.

2.5.3.1.4.3 Others

Nevertheless, there are variations in the basic methods above depending on the nature of the sample, pre-infection processing involved, choice of isolation hosts and how they are combined with the processed sample, post-infection processing and detection of the phage (Hyman, 2019).

2.5.3.1.5 Environmental sample collection and processing

Bacteriophages are found where their hosts are found (Richards, 2014). Therefore, to isolate bacteriophages of farmed fish pathogenic bacteria, samples should be collected from aquaculture systems (Duffy & Hay, 1998; Stenholm et al., 2008). However, finding a phage that is specific for a host of interest is not straightforward, hence requiring analysis of many environmental samples to increase the chances of coming across the phage of interest (Richards, 2014; Stenholm et al., 2008). After collection, samples are processed to obtain phages at the right titre using the right media for efficient infection of bacterial hosts used in isolation (Hyman, 2019; Suttle et al., 1991). Different sample types may require different processing techniques which might involve a combination of a couple of them in samples from for example seawater which are known to contain very low phage concentrations (Ghugare et al., 2017; Hyman, 2019). For example, precipitation, filtration or a combination of the two is mainly used during preparatory concentration of the samples (Gill & Hyman, 2010; Hyman, 2019; Suttle et al., 1991). According to Czajkowski et al., 2016; Hyman, 2019; Jäckel et al., 2019, zinc chloride can be used to precipitate phages from water, plant and or soil environmental extracts to facilitate direct detection of phages without enrichment. Flocculation is another technique for phages in highly dilute samples by forming small insoluble aggregates that drop out of suspension even when the phage concentration is low (Hyman, 2019). Adherence to, and washing from bituminous coal, can also be used to concentrate phages (Dafale et al., 2008). For soil particles, thorough washing is required to ensure that phages adhering to the soil particles are released whereas sewage samples required the least of processing since filtration can lead to immediate clogging of filters, thus leaving one option of centrifugation to remove the solid debris (Hyman, 2019; Williamson et al., 2013).

A filtration step is important for removing endogenous bacteria by using 0.45μm and or 0.22μm depending on the size of targeted bacteriophages at a compromise of removing all bacterial debris (Hyman, 2019).

2.5.3.1.6 Choice of isolation hosts

The most important step in phage isolation is the choice of the isolation hosts because they help in restricting the phages isolated basing on their host range (de Jonge et al., 2019). Phages infecting Gram-negative bacteria use Gram-negative isolation hosts even though some can infect hosts different from those used during isolation (Hyman, 2019). This is governed by the complementarity in receptors for phages on their target hosts and this has led to receptor

specificity manipulation to enhance the efficacy of phages (Hassan et al., 2018; Lee et al., 2011). Practical considerations such as ease of culture in the lab and pathogenicity of target hosts are key and hosts that are responsive to routine lab culture in a short time and less virulent are usually of affirmative choice (Hatfull, 2012). For environmental isolates of host bacteria, as opposed to already established laboratory strains or species in case used, they should be tested for the presence of inducible prophages through exposing the bacteria to DNA damaging agents such as UV or Mitomycin C, followed by plating to look for plaques or sequencing the bacterial genome to look for prophage sequences using a couple of already established software tools (Nirmal Kumar et al., 2012). As a general rule, temperate phages are not ideal for phage therapy and its thus important not to use hosts that might be induced to release temperate phages as enrichment strains (Henein, 2013).

2.5.3.1.7 Routes of administration

As seen in Figure 5, oral administration by impregnation in feed, immersion/direct release in water, injection (intraperitoneal/intramuscular) and anal intubation are some of the modes of administration of phages during phage therapy (Gon Choudhury et al., 2017; Silva et al., 2016). A chosen method depends on the isolated phage properties, nature of the environment and bacterial infection and the route of infection (Nakai & Park, 2002). No single mode of administration is efficient to control bacterial infections due to presence of a wide range of pathogenic mutant bacterial strains in addition to the high strain or species specificity of phages (Silva et al., 2016). Therefore, using multiple administration methods concurrently as well as phage therapy in combination with other therapeutic and prophylactic measures of bacterial control like antibiotics, phytobiotics, pre and probiotics has been reported by (Chan et al., 2013) is preferable. Furthermore, using phage cocktails with varying host specificities and range can enhance the efficiency of phage therapy and minimizes emergence of phage-resistant bacterial strains (Chan et al., 2013). However, the number and multiplicity phage infection used in a cocktail should be as minimal as possible since it is directly proportional to the cost implications (Madhusudana Rao & Lalitha, 2015). Putting into consideration the different applicability scenarios of the different modes and associated advantages, the oral method, which is ideal for large numbers of fish irrespective of their size or developmental stage, given the highly resistant nature of phages to low and high pH, a characteristic of the gastrointestinal tract (GIT) (Nakai, 2016; Nakai & Park, 2002), is a more applicable route of administration than the rest. Even though phages have been found to enter fish bodies via the skin and gills, and the large scale coverage of immersion/direct release in water as an administration method,

the requirement of large phage titres for larger volume production systems may make it ineffective (Richards, 2014). For the injection method, the stress associated with it together with the labour-intensive nature for large fish numbers and limitation to small fish limits its application (Gon Choudhury et al., 2017).

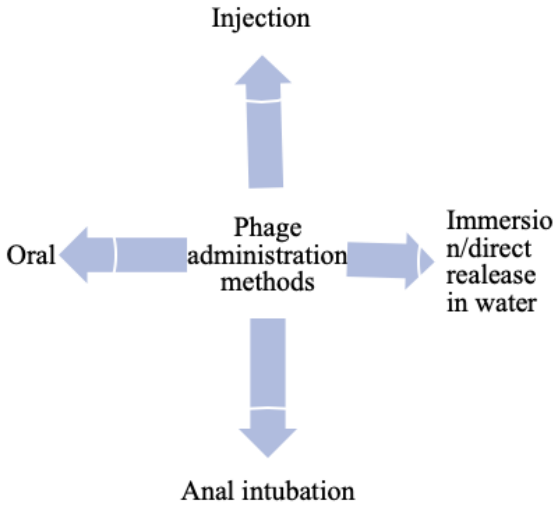

Figure 5: Routes of in vivo phage administration for the control of infectious bacterial diseases (Gon Choudhury et al., 2017)

2.5.3.1.8 Advantages and limitations of using Bacteriophages

2.5.3.1.8.1 Advantages

According to (Gon Choudhury et al., 2017), (Henein, 2013), and (Nakai & Park, 2002), bacteriophages have shown the following advantages.

- Considered as natural and hence organic, making the attainment of regulatory licences easier as compared to antibiotics and chemotherapeutics.
- Easy to isolate and propagate as they are self-replicating and self-limiting in response to bacterial numbers, thus limited environmental impact.
- Can be used either on Gram-positive or Gram-negative bacteria.
- Specific pathogen killers, do not affect normal flora whereas broad-spectrum antibiotics destroy all bacterial cells.
- Phages are easy to apply as sprays or by direct mixing with water.
- Synergistic in a cocktail and with antibiotics, preservatives and disinfectants.
- Bacteriophages are compatible with food.
- They can be applied for a therapeutic or bio-sanitization purpose.

- Phages are omnipresent, giving a large pool for the initial isolation, and presumed to be safe as undesirable effects have not been reported.
- Relatively inexpensive, fast and flexible as the host, pathogen and phages live within the same aqueous environment.
- Less severe problems of resistance as compared to antibiotics as it is easy to isolate lytic phages to the mutants which are in most cases non-virulent.

2.5.3.1.8.2 Limitations

- Phage application requires the exact identity of bacterial species/strains that cause a given infection.
- Regulatory approvals are needed before application.
- Phage resistance emergence.
- Rapid occurrence of phage-resistant variants.
- Consumer acceptance needs to be assessed.
- Gene transfer to the host bacterium through transduction and or phage conversion resulting in enhanced virulence of bacteria in the aquaculture system.
- Phage neutralizing antibodies produced after oral and or parenteral administration of phages affect the treatment of recurrent infections by similar pathogens.
- The high specificity of phages is a limitation especially during treatment of highly diverse species of pathogenic bacteria.
- High diversity of phages and bacteria, calling for comprehensive studies to understand their heterogeneity and ecology at both phenotypic and genotypic levels.
- Some modes of administration like oral and injections may not be ideal as diseased fish do not feed properly and impractical at large scale respectively.

3.0 Methods and materials

3.1 Study area

This study was conducted at Nitte University Centre for Science Education and Research (NUCSER), Paneer Campus, Deralakatte, Mangalore, Karnataka 575081, India.

3.2 Research design

The experiments were designed following standard operating procedures and protocols with some modifications. Aseptic conditions were maintained by operating in sterile laminar airflow hoods and using sterile equipment.

3.2.1 Bacteria strain isolation, purification and growth

Samples were collected into sterile 50ml falcon tubes from Aquatic Biosystems ornamental farm in Mangalore, India from rearing tanks, biological filters, moribund fish (skin and gill swabs and tissue suspensions), and other home ornamental aquaria. Rimler-Shotts (RS) Medium Base was used for selective isolation of *Aeromonas sp.* from the collected water and fish samples. Tryptic Soya Broth/Agar (TSB/A) (Himedia) were used for the growth of the isolated bacteria.

3.2.1.1 Media composition and preparation

Table 3: Rimler-Shotts (RS) Medium Base (Himedia Laboratories, PVT. Ltd., Mumbai India, M576-500G)

Ingredients	gL^{-1}
Yeast extract	3.00
Maltose	3.50
L-Cysteine hydrochloride	0.30
L-Lysine hydrochloride	5.00
L-Ornithine hydrochloride	6.50
Sodium thiosulphate	6.80
Ferric ammonium citrate	0.80
Sodium deoxycholate	1.00
Sodium chloride	5.00
Bromothymol blue	0.03
Agar	13.50
Final pH (at 25°C)	7.00

One thousand (1000) ml of distilled water which was autoclaved at 121^0C for 15 minutes was used to dissolve 45.9g of dehydrated RS media. The mixture was heated without boiling to dissolve the media. It was allowed to cool down up to a temperature of 50^0C, after which 1 vial of Novobiocin supplement (Himedia) that was dissolved in 2ml of sterile water was added and mixed well to dissolve. Media was allowed to cool for a few minutes and later gently poured onto autoclaved and pre-labelled Petri dishes where it was allowed to cool and solidify.

Table 4: Tryptone Soya Broth (TSB), Granulated (Himedia Laboratories PVT. Ltd., Mumbai, India, GM011-500G)

Ingredients	gL^{-1}
Tryptone	17.0
Soya peptone	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Dextrose (Glucose)	2.5
Final pH after sterilization (at 25°C)	7.5

Thirty (30)g of TSB was suspended in 1000ml of distilled, mixed well with heating to dissolve. Media was distributed into 15ml glass test tubes (5ml each), capped, and autoclaved at 121°C for 15 minutes.

Table 5 Tryptone Soya Agar (TSA, Himedia Laboratories, PVT. Ltd., Mumbai India, M290-500G)

Ingredients	gL^{-1}
Tryptone	15.0
Soya peptone	5.0
Sodium chloride	5.0
Agar	15.0
Final pH after sterilization (at 25°C)	7.4

Forty (40)g of TSA was suspended in 1000ml of distilled water, heated to boiling to dissolve. It was autoclaved at 121°C for 15 minutes and then poured onto sterile Petri dishes to cool and solidify under a sterile laminar airflow hood.

3.2.1.2 Inoculation

Two hundred (200) µl from the collected water samples were spread plated on RS media plates and incubated (NB-205QMC) at 30^0C for 24 hours. After incubation, the plates were retrieved and single yellowish colonies were picked from RS media plates using a sterile wire loop, streaked on labelled TSA plates, and incubated (NB-205QMC) at 30^0C for 24 hours. The biochemical screening was carried out to biochemically characterize the different cultures obtained on TSA plates.

3.2.1.3 Biochemical tests

All biochemical tests were carried out according to established methodologies with some modifications (Janda, 1985).

3.2.1.3.1 Gram-staining

The procedure followed five steps; fixation, crystal violet, iodine treatment, decolorization and counterstaining with safranin. Sterile wire loops were used to transfer a colony from each petri-dish onto labelled glass slides to make flood-air dried and heat-fixed smears. The smears were

flooded with crystal violet for 1 minute, washed with gently flowing tap water for 5 seconds, flooded with Gram's Iodine for another one minute, washed under a stream of slowly flowing tap water for 5 seconds, decolourized with 95% ethanol for 5 seconds and washed off until the slide got clear of the decolorizing agent. It was counterstained by safranin for 1 minute, after which it was washed off under a gentle stream of tap water. The slides were dry blotted with tissue paper and a dryer, and individually observed under oil immersion through the 100X magnification objective lens of a bright field microscope. Gram-negative isolates showed a pinkish colour whereas Gram-positive ones retained the purple colour of crystal violet.

3.2.1.3.3 Catalase test

A sterile loop was used to transfer a colony from each Petri fish to a clean glass slide surface with a drop of 3% hydrogen peroxide solution. The rapid development of effervescence indicated a positive result while the absence of effervescence was an indication of a negative result.

3.2.1.3.2 Oxidase test

Oxidase discs (DD018-1VL) were used to carry out the test. A single colony was picked using a pipette tip from the different Petri dishes of the bacterial cultures and smeared on individual discs while observing the colour change for 5-10 seconds. Discs whose colour turned purple were oxidase-positive while those for which there was no colour change were oxidase-negative.

The rest of the biochemical tests (Arginine dihydrolase, Ornithine decarboxylase, Glucose fermentation and Salt tolerance) were conducted on isolates that were Gram-negative rods, oxidase and catalase-positive. *Vibrio harveyi* BB120 and *Pseudomonas aeruginosa* ATCC 27853 (available at NUCSER), as well as media controls, were included in the tests to screen out any other contamination.

3.2.1.3.5 Glucose fermentation

This tests for the capability of bacteria to ferment glucose with production of gas by using Phenol Red Broth Base (Himedia).

Table 6: Phenol Red Broth Base (Himedia Laboratories, PVT. Ltd., Mumbai India, M054-100G)

Ingredient	gL^{-1}
Proteose peptone	10.0
Sodium Chloride (NaCl)	5.0
Meat extract B	1.0
Phenol red	0.018
Final pH (at 25°C)	7.5

Phenol Red Broth Base (16.02g) and 1% glucose was suspended in 1000ml of distilled water and suspension heated to dissolve completely. Durham's tubes were inverted within 10ml test tubes, followed by pouring 5ml of media and capped. They were then autoclaved at 110^0C for 15 minutes, allowed to cool down and then inoculated with single colonies from the TSA plates of the different isolates. Tubes were then incubated (NB-205QMC) for 24 hours at 30^0C. Isolates that gave a positive result produced a colour change of the medium from red to yellow and presence of an air bubble within the inverted Durham's tubes whereas those that were negative had no bubble without or without a change in the medium colour.

3.2.1.3.4 Arginine dihydrolase and Ornithine decarboxylase tests

These tests were used examine the capability of bacteria to utilize Arginine and Ornithine by using Arginine dihydrolase and Ornithine decarboxylase enzymes.

3.2.1.3.4.1 Media

Table 7: Moeller Decarboxylase Broth Base (Himedia Laboratories, PVT. Ltd., Mumbai India, M393-100G)

Ingredient	gL^{-1}
Peptone	5.000
Meat extract B	5.000
Dextrose (glucose)	0.500
Bromocresol purple	0.010
Cresol red	0.005
Pyridoxal hydrochloride	0.005
Final pH (at 25°C)	6.1

This media was used for Arginine dihydrolase and Ornithine decarboxylase tests with the addition of L-Arginine or L-Ornithine respectively. To 1000ml of distilled water, 10.52g of Moeller Decarboxylase Broth Base was suspended, followed by 10g of either L-Arginine or L-

Ornithine. pH was adjusted with to 6.0 ± 0.2 before heating the media to dissolve completely in water. 5ml of media was dispensed in pre-autoclaved 10ml test tubes, capped and autoclaved at 110^0C for 15 minutes. After allowing the autoclaved tubes with media to cool down, they were inoculated with single colonies of the cultures, followed by a few drops of parafilm oil to create anaerobic conditions, capped and incubated (NB-205QMC) at 30^0C for 48 hours. Tubes in which media turned yellow and later purple after 48 hours were Arginine dihydrolase and Ornithine decarboxylase positive whereas those that remained yellow after or retained the original media colour pre-inoculation and incubation were negative.

3.2.1.3.6 Salt tolerance

Sodium chloride was added to TSB to make 6% and 1% concentrations of salt. Media was autoclaved at 121^0C for 15 minutes after which an inoculating loop was used to inoculate the tubes with the test cultures respectively. Incubation (NB-205QMC) was done at 30^0C for 24 hours after which were observed for the presence of turbidity as an indicator of growth. Test tubes that were turbid were tolerant cultures whereas those that were clear were not for a given salt concentration.

During media preparation, aseptic conditions were strictly followed by continuously disinfecting contact surfaces and materials and working under a sterile operational laminar airflow hood to avoid contamination.

Cultures that were Gram-negative, catalase-positive, oxidase-positive, fermented glucose with production of a gas bubble in inverted Durham's tubes, and were capable of growing at 1% sodium chloride concentration and not at 6%, Arginine dihydrolase positive, and Ornithine decarboxylase negative, were biochemically considered as *Aeromonas sp.* isolates.

3.2.1.4 Antibiotic susceptibility test

All isolates were tested for antibiotic sensitivity according to (Biemer, 1973; Hudzicki, 2016) by using Mueller Hinton Broth (MHB) and Agar, (MHBA,1%) (Himedia, M391-500G).

3.2.1.4.1 Media

Table 8: Mueller Hinton Broth (MHB) composition (Himedia Laboratories, PVT. Ltd., Mumbai India, M391-500G)

Ingredient	gL^{-1}
HM infusion B from #	300
Acicase™	17.5
Starch	1.5
Final pH (at 25°C)	7.3

**Formula adjusted, standardized to suit performance parameters # Equivalent to Beef, infusion from, $ Equivalent to Casein acid hydrolysate.

Mueller Hinton Broth (21.0g) was suspended in 1000ml of distilled water and heated to boil so as to dissolve the media. Media was thoroughly mixed, dispensed into test tubes, capped and autoclaved at 121^0C for 15 minutes. For MHBA, 1% Agar was added to the broth, heated to dissolve, and autoclaved at 121^0C for 15 minutes. After autoclaving, the agar was poured onto sterile Petri-dishes in a sterile laminar airflow hood., allowed to cool down and solidify.

MHB was used to suspend colonies from different cultures MHB in sterile test tubes and incubated at 30^0C in a shaking incubator (NB-205QMC) and later swabbed on prelabelled MHBA plates, on which antibiotic disks were placed. Plates with disks were incubated (NB-205QMC) at 30^0C for 24 hours. Antibiotics used included: nalidixic acid (NA,30µg), chloramphenicol (C, 30µg), gentamycin (GN, 10µg), streptomycin (S, 10µg), tetracycline (TE, 30µg) and erythromycin (COT, 25µg). Basing on zones of inhibition/clear zones measured, isolates were designated as Resistant, Intermediate or Susceptible according to (Hudzicki, 2016).

Table 9: Zone size reference with respect to antibiotic susceptibilities of microbes (Diagnostics Hardy, 2011; Hudzicki, 2016; Lamy et al., 2012)

Antibiotic	Concentration (ml)	Resistant	Intermediate	Susceptible
		(Zone <…mm)	(Zone <…mm)	(Zone ≥…mm)
Amoxicillin		13	14-17	18
Ampicillin		11	Dec-13	14
Carbenicillin		17	18-22	23
Cefoxatime		14	15-22	23
Cephalothin		14	15-17	18
Chloramphenicol		12	13-17	18
Erythromycin		13	14-22	23
Kanamycin		9	Oct-16	17
Gentamycin		16	16-17	18
Penicillin		28	-	29
Streptomycin		11	12-14	15
Sulfamethoxazole-trimethoprim		10	11-15	16
Tetracycline		17	17-18	19
Nalidixic acid		15	15–19	20

3.2.1.5 Virulence factors

Haemolytic, lipolytic and proteolytic activities of the bacterial isolates were tested as key contributors to virulence of *Aeromonas sp*.

3.2.1.5.1 Haemolytic activity

All bacteria isolates were tested for haemolysin production on blood base agar supplemented with 5% sheep blood. Overnight grown bacteria cultures were streaked on the agar plates and incubated at 30^0C for 24 hours. The plates were observed for β haemolysis which was evidenced by a clearance zone around the bacterial culture growth.

3.2.1.5.2 Proteolytic activity

This was determined by growing the bacterial cultures in on 10% (w/v) skimmed milk agar and incubated at 30^0C for 48 hours. Clearance in the opaque agar by the bacteria cultures after streaking on the plates was indicative of protease production.

3.2.1.5. 3 Lipase activity

Phenol red olive oil agar was used for this test (g/L): 0.01% (w/v) phenol red, 0.1% (w/v) Calcium Chloride, 2% (w/v) agar, pH adjusted to 7.4 with 0.1 N Sodium hydroxide and 1% (v/v) of Olive oil added after autoclaving at 121^0C for 15 minutes, gently mixed prior to pouring onto the plates. Bacteria cultures were streaked on the plates and incubated at 30^0C for 48 hours. A change in colour of phenol red indicator from pink to yellow was an indicator of lipase activity.

3.2.1.6 Growth curve of bacterial isolates

The main aim of this curve was to ascertain the time at which the bacteria isolate undergoes the early log phase, as well as to establish a relationship between OD and cell counts at the early log phase time, to facilitate appropriate phage infection during the one-step phage growth curve. One hundred (100) ml of TSB was suspended into each of the two 500ml conical flasks and autoclaved at 121^0C for 15 minutes. In one flask, 100µl of the bacterial isolate from an overnight grown culture was added whereas in the other that acted as a control, 100µl of sterile water was added, and incubated at 30^0C for 5 hours. Optical density (OD) measurement was taken using an Eppendorf BioSpectrometer at the beginning (T=0). Serial dilutions were made and plated on TSA to enumerate the bacterial cell numbers at the start of the experiment (T=0). The measurements were taken hourly for 5 hours in duplicate.

3.2.2 Phage isolation

Phage isolation followed an enrichment technique with modifications according to Ghugare et al., 2017.

3.2.2.1 Sample collection

The same samples from which bacteria were isolated were used for phage isolation. They were centrifuged at 8000 revolutions per minute (RPM) for 20 minutes at 4^0C (Eppendorf 5810R, Germany). The supernatant was filtered through a Millipore membrane filter (0.22 microns, 47mm diameter) by the membrane filtration technique, stored at 4^0C in a refrigerator, and used for further phage isolation work.

3.2.2.2 Enrichment technique

From each of the isolated bacteria on TSA plates, a single colony was inoculated into labelled sterile TSB test tubes with a sterile inoculating loop and incubated at 30^0C in a shaking N-BIOTEK incubator (NB-205QMC), 150RPM, until the early log phase (~2 hours). One ml of the supernatant from each centrifuged water sample was added to the bacteria cultures except one for each isolate that was left as a control and incubated in a shaking incubator for 4 hours at 30^0C. One (1)ml was pipetted from the tubes into which the supernatant was added prior to incubation into sterile 1.5ml Eppendorf vials and centrifuged at 10,000 RPM at 4^0C (Eppendorf 5810R, Germany) for 5 minutes. The supernatant was transferred into fresh sterile 1.5ml Eppendorf vials, stored at 4^0C in a refrigerator, and was used for phage purification and sensitivity assays.

3.2.2.3 Phage purification

The control tubes (4 hours grown) were used to make lawns of each culture on labelled sterile TSA plates and were allowed to air dry under a sterile lamina airflow. Five (5)µl of the phage lysate was spotted on each plate with the culture lawns to test for phage sensitivity, allowed to air dry and then incubated at 30^0C overnight. Plaques that were formed on bacterial lawns were carefully transferred and suspended into 1.5ml sterile vials containing 1ml of TSB, vortexed for 5 minutes to breakdown the agar, spun down at 12,000 RPM for 10 minutes at room temperature. One ml of the supernatant was transferred into 5ml TSB tubes with respective pre-log grown cultures (~2 hours) from which the plaques originated. After shaking to mix, they were incubated in a shaking incubator at 30^0C for 4 hours. 1ml was pipetted from the tubes, into sterile 1.5ml Eppendorf vials, centrifuged at 10,000 RPM at 4^0C for 5 minutes, the supernatant transferred into fresh sterile 1.5ml Eppendorf vials and stored at 4^0C in a refrigerator. Using the control TSB culture tubes for each bacterial isolate that yielded a plaque, more lawns were made and lysates serially diluted up to a (10^{-5}) dilution. On each lawn, the different concentrations of lysates were spotted, allowed to air dry, and incubated at 30^0C overnight. Plaques formed by the highest dilution of lysate that yielded a clear zone on the lawn was gently picked, and the procedure of suspending it in TSB, vortexing, centrifuging and supernatant storage as phage stocks in sterile Eppendorf vials repeated. A three (3) time purification cycle was conducted while repeating the above steps and the final stocks were later cryopreserved for future use.

3.2.2.4 Cryopreservation

Glycerol was autoclaved at 121^0C for 15 minutes and later at a 50% composition, used to make glycerol phage lysate stocks (50% glycerol and 50% Phage lysate), sealed off with parafilm, and stored at -80^0C from where they were retrieved for further use in case needed. At the same time, a portion of the lysates was stored in the refrigerator (4^0C) for more experiments.

3.2.3 Host range

All the bacteria cultures that were cryopreserved were revived from the -80^0C by pipetting 20µl into sterile TSB tubes and incubated in a static incubator at 30^0C overnight. The cultures were used to make airdried lawns onto which each phage lysate was spotted (5µl) in duplicate. After the lysate spots thoroughly dried, the plates were incubated at 30^0C for 24 hours after which they were examined for presence or absence of clearance at the points where the phage lysates were spotted. Plates with clear zones were indicative of sensitive cultures to specific lysates whereas those without were insensitive.

3.2.4 One step phage growth curve

In order to estimate the phage titres in the phage stocks, serial dilutions were made from the phage stocks and a standard double-layer agar overlay method was used (Clokie & Kropinski, 2009).

3.2.4.1 Overlay technique for enumerating phage

After knowing the time taken for the bacteria isolates to reach the log phase, cultures for the assay were cultured in a shaking incubator (100RPM) for the predetermined period (~ 2 hours). Two hundred (200) µl of the culture was pipetted from each tube into 7ml sterile test tubes and 100µl of the different serially diluted phage lysate was added. To the contents in the tubes, 5ml of sterile soft agar (3g TSB, 0.8g Agar into 100 ml of distilled water) was added and the mixture immediately poured onto labelled and sterile TSA plates in duplicate for each lysate serial dilution. The plates were allowed to stand and solidify for 30 minutes and then incubated at 30^0C for 24 hours. After 24 hours, the plates were removed, plaques identified, marked and counted on the petri dish to ascertain the phage titre (PFU/ml) for each lysate. The same technique was used during phage titre enumeration in the one-step phage growth curve.

3.2.4.2 The growth curve experiment

To the sterile 100ml of TSB in a 500ml flask, 100µl of an overnight grown bacteria culture was inoculated and incubated in a BIOBEE shaking incubator (SLM-INC-OS-250), 100RPM for two hours to reach the early log phase. OD was measured at that time, and the bacterial cell

count (CFU/ml) estimated by using the OD/cell count established relationship in the bacteria growth curve. This was used to estimate the MOI during phage infection, to allow for easy (countable) enumeration of plaques and deciding on the dilutions to make while making phage overlays for phage titre estimation.

At the time of phage infection, the culture was seeded with a phage titre targeting a concentration of approximately 100PFU/ml, so that countable titres on overlays could be made with minimal dilutions. At the time of infection (T=0) and every 20 minutes of incubation for 4 hours in a shaking incubator, 1ml of the mixture was pipetted into a sterile Eppendorf vial, centrifuged at 10000 RPM for 2 minutes at room temperature, overlays were made in duplicate for the undiluted (neat) phage stock, (10^{-1}, 10^{-2} and 10^{-3}) serial dilutions with 5µl of lysate, allowed to airdry in a sterile laminar airflow hood and incubated at 30^0C overnight. In order to ascertain the number of phages that adsorbed onto the bacteria surface, two sterile vials (1.5ml) were filled with 1ml of culture from the flask after 10 minutes of mixing the phage lysate with the bacteria culture in the 500ml flasks. To one of the vials, 1µl of chloroform was added while to the other, nothing was added. The vials were centrifuged at 10000RPM for 2 minutes at room temperature, and 5µl of lysate used in making overlays in duplicate for estimation of phage titres. The difference between the PFU/ml of the chloroform treated samples and untreated ones gave an estimate of adsorbed phages. This was done for all the lysates and a graph of PFU/ml against time for the different lysates was plotted and burst size calculated from (equation 1).

Equation 1: Burst size (PFU/ml) equation

$$\text{Burst size} = \frac{\text{Number of PFU/ml after burst} - \text{Number of Adsorbed phages/ml}}{\text{Number of Adsorbed phages/ml}}$$

3.2.4 CFU reduction assay

The aim of this experiment was to test the effectiveness of the phage lysates in reducing the bacteria colony-forming units at different multiplicities of infection (MOIs) in vitro. The MOIs tested were (10, 1, 0.1 and 0.01) with hourly OD measurements as well as bacterial CFU enumeration. A control treatment was composed of the bacteria culture and instead of adding phage lysates, sterile saline was added of an equal volume as the phage lysates in the treatments. To five conical flasks (Control, MOIs 10, 1, 0.1 and 0.01) each with 100ml of TSB that was autoclaved at 121^0C for 15 minutes, 100 µl of an overnight grown bacteria culture was added and incubated at 30^0C in a shaking incubator for two hours (early log phase). OD

measurements were taken after the 2 hours of growth and before phage inoculation (T=0), to help estimate the bacterial cell load, to allow for MOI estimation as well. Bacterial cell counts were also estimated by spotting different serial dilutions of the culture at (T=0) in duplicate. The bacteria cultures were then infected with phage lysates at the different estimated MOIs, and incubated at 30^0C in a shaking incubator. Immediately after phage infection in flasks of different MOIs, 1ml was centrifuged at 10000RPM for 2 minutes, the supernatant discarded, pellet mixed with 100µl of sterile saline water, gently mixed and used to make different serial dilutions up to (10^{-7}), spotted on TSA plates in triplicate, and incubated in a static incubator at 30^0C overnight. This, with OD measurements, were repeated after every one hour for 6 hours for each phage lysate. The OD measurements and the bacteria cell counts (CFU/ml) which were made the following day, were plotted against time for the different MOIs to observe bacteria CFU reduction by the different phage lysates for the different MOIs.

3.2.5 Viable Phage resistant bacteria development

After the MOI experiment for phage lysate 10 and 2 (6 hours), the flasks with their content were further incubated for 5 hours. 1ml from each flask was centrifuged at 10,000RPM for 2 minutes, the supernatant discarded, and pellet suspended in sterile saline water. Serial dilutions were made, spotted on sterile TSA plates and incubated overnight at 30^0C overnight. Single colonies were picked the following day, dissolved in sterile TSB, and incubated for two hours in shaking incubator at 30^0C. They were both in duplicate used to make lawns onto which all phage lysates (2, 3 and 10) were spotted, incubated at 30^0C in a static incubator overnight, to check whether they would still form clearance zones as before. Lawns with clear zones implied phage sensitivity, while those without, implied phage resistance.

3.2.6 Data analysis

Data were entered using Microsoft excel 2016 and statistical analyses were performed using R Studio, version 1.2.1335 for Mac. The existence of significant differences in the inactivation of Aeromonad isolates by single phage lysates at different multiplicities of infection was assessed by one-way analysis of variance (ANOVA) on log-transformed CFU/ml data. Normality of the data was checked by normal QQ plots whereas homogeneity of variances was checked by Levene's test. A p-value < 0.05 was considered significant. Graphs were drawn by Sigmaplot 13 with means and standard deviations as error bars.

4.0 Results

4.1 Bacteria isolation, purification and growth

Twenty-four (24) isolates of *Aeromonas species* were isolated from samples collected from Aquatic biosystems ornamental aquaculture farm, Mangalore India (12°54'35.64" N, 74°54'54.70" E), and others from home freshwater fish aquaria. They were all Gram-negative, catalase, oxidase, D-Glucose fermenting with the gas formation and arginine dihydrolase positive, but ornithine decarboxylase negative (Figures 6, 7 and 8). They were able to grow at 1% sodium chloride and not at 6% (Figure 9) with varying antibiotic susceptibilities and virulence factors.

4.2 Antibiotic susceptibility

The proportion of isolates that were resistant, intermediate and susceptible for the different antibiotics used as shown in Table 10. Most isolates were resistant to Nalidixic acid (NA 30μg), followed by Tetracycline (TE 30 μg), Chloramphenicol (C 30 μg), and Streptomycin (S 10 μg). No resistance was observed for Erythromycin (E 25 μg) and Gentamycin (GN 10 μg) whereas the susceptibility was greatest with Gentamycin (GN 10 μg), Erythromycin (E 25 μg), Chloramphenicol (C 30 μg), Streptomycin (S 10 μg), Tetracycline (TE 30 μg) and Nalidixic acid (NA 30μg).

Table 10: Proportion (%) of isolates with their antibiotic susceptibilities from the disk diffusion method (n=24)

Antibiotic	Resistant(%)	Intermediate(%)	Susceptible(%)	Total (%)
Nalidixic acid (NA 30 μg)	66.60	4.16	29.20	100
Chloramphenicol (C 30 μg)	4.16	-	95.80	100
Gentamycin (GN 10 μg)	-	-	100.00	100
Streptomycin (S 10 μg)	4.16	4.20	91.67	100
Tetracycline (TE 30 μg)	25.00	8.33	66.67	100
Erythromycin (E 25 μg)	-	4.16	95.83	100

4.3 Virulence factors

For virulence factors, all isolates were positive for lipolytic activity (100%) whereas (87.5%) and (60%) were positive for hemolytic and proteolytic activities respectively (Figures 10, 11 and 12) and (appendix 8.2).

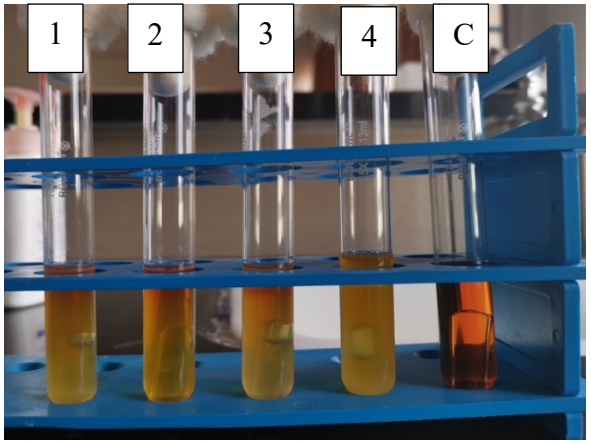

Figure 6: Glucose fermentation by Aeromonad isolates (1-4) and the negative control (C)

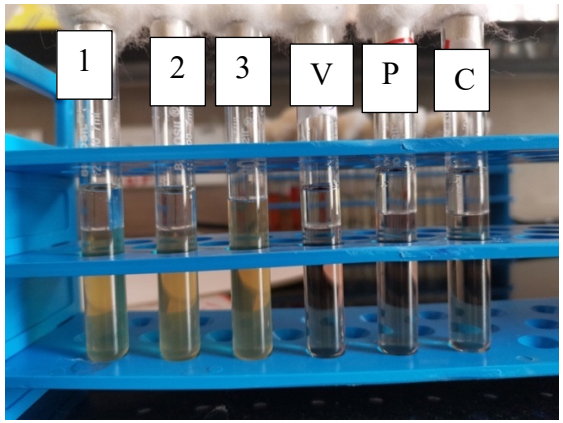

Figure *8*: Ornithine utilization by Aeromonad isolates (1-3), *Vibrio harveyi* BB120, *Pseudomonas aeruginosa* ATCC 27853 (P) and the negative control (C)

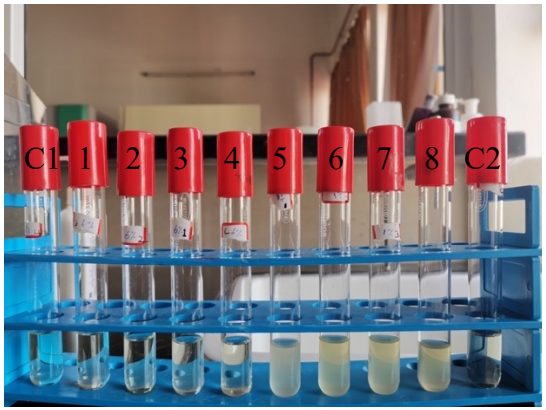

Figure 7: Salt tolerance by Aeromonad isolates, 6% (1-4) & 1% (5-8), C1 and C2 were negative controls

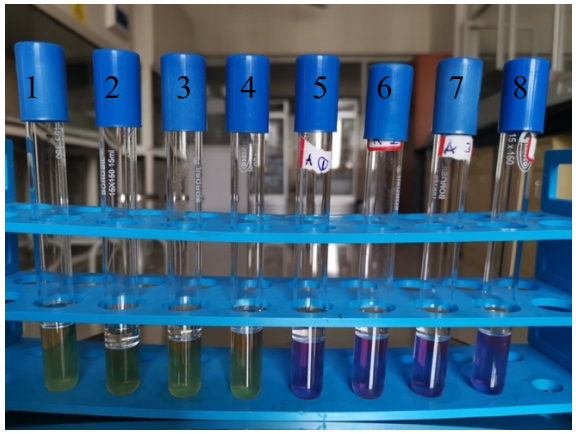

Figure 9: Arginine dihydrolase positive, Aeromonad isolates (4-8) and negative (1-4) bacteria isolates

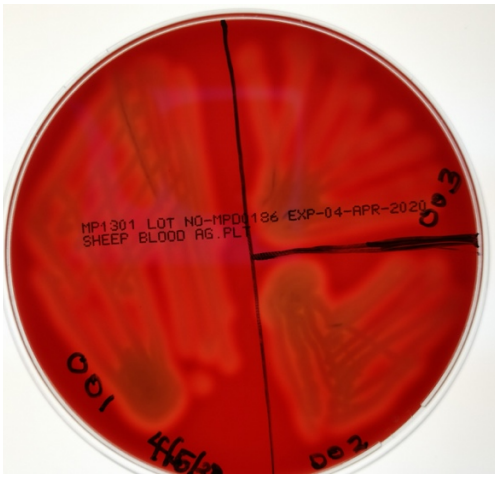

Figure 10: β heamolysis of Aeromonad isolates on Blood base agar supplemented with 5% sheep blood incubated at 30^0C for 24 hours

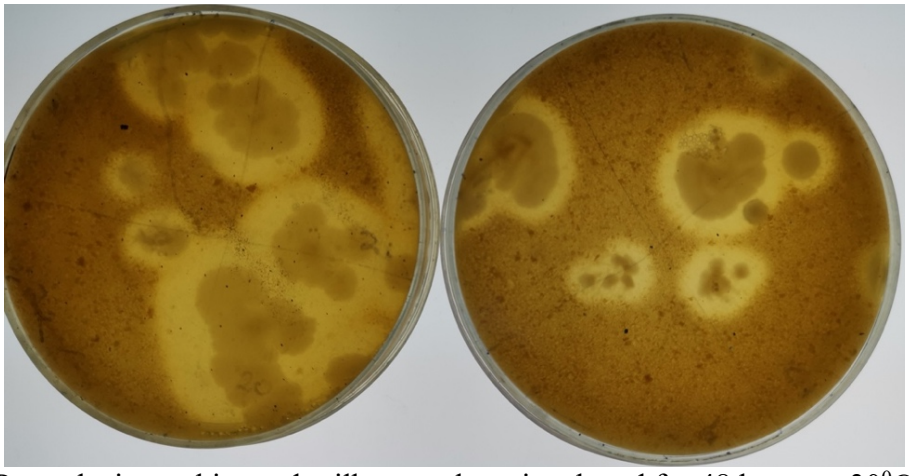

Figure 11: Proteolysis on skimmed milk agar plates incubated for 48 hours at 30^0C for 48 hours

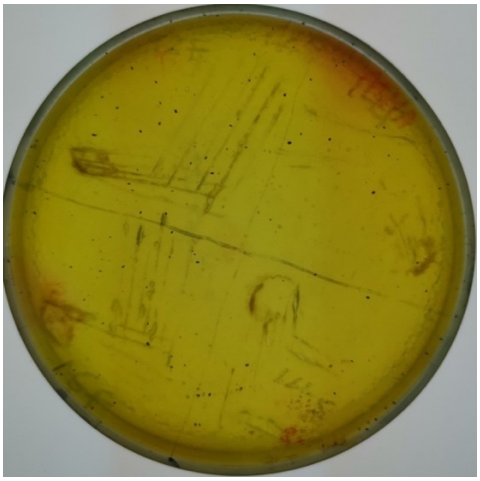

Figure 12: Lipolysis on Phenol red Olive oil agar plates by Aeromonad isolates incubated at 30^0C

4.4 Bacteria isolate growth curve

The optical density (OD) at 600nm (Figure 13) and bacteria cell counts (CFU/ml) (Figure 14) were made hourly for 5 hours. OD at 600nm and CFU/ml were plotted against the culture time to produce the growth curves. The early log phase was estimated to occur after 2 hours of culture, for which an equivalent cell count for one OD during the early log phase was estimated to be $3.56x10^8$ CFU/ml.

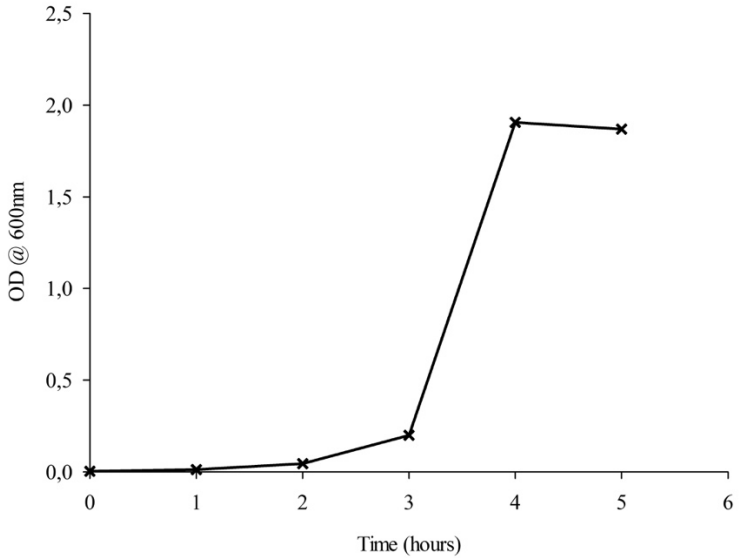

Figure 13: Evolution of Optical density (OD) at 600nm for the Aeromonad isolate used as a host during the one step phage growth curves

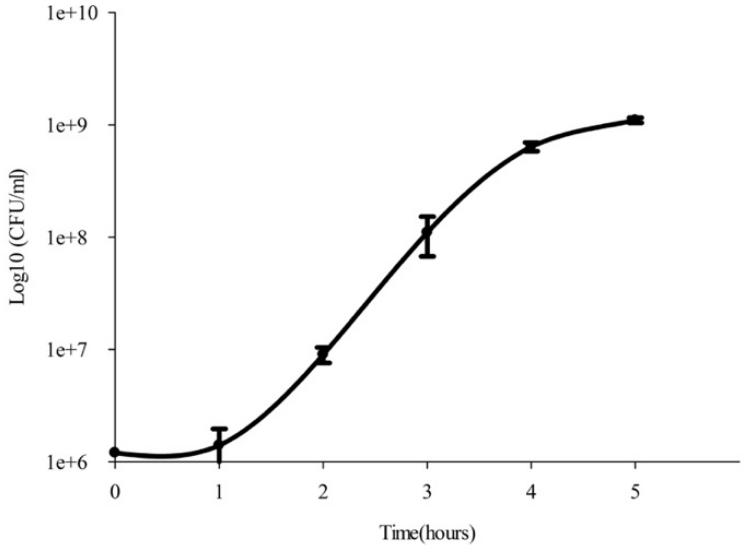

Figure 14: Evolution of Colony Forming Units (CFU/ml) of the Aeromonad isolate used as a host in the one step phage growth curves after 5 hours of incubation at 30^0C

4.5 Phage isolation, enrichment, purification and enumeration

Phage isolation and enumeration were done according to (Adams, n.d.; Ghugare et al., 2017) with modifications. Three phage lysates (2, 3 and 10) were finally isolated and after three cycles of single phage purification together with a spot assay of serial dilutions of the different lysates (Figure 15 and 16), phage stocks were cryopreserved in 50% glycerol and stored at -80^{0}C for future use. Some phage lysate stocks that were stored in a refrigerator at 4^{0}C were used for further phage characterization experiments and to estimate their titers. Phage enumeration was done by both a spot assay and standard double-layer agar overlay method (Clokie & Kropinski, 2009). The phage titers for phage lysate 2, 3 and 10 were 4x10^7, 6x10^7 and 8.5x10^7 PFU/ml respectively.

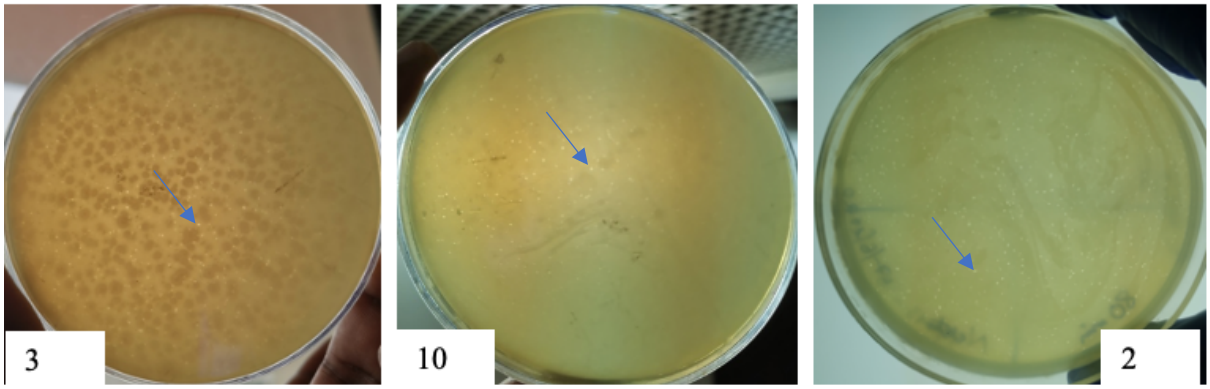

Figure 15: Plaques of phage lysates 3, 10 and 2 after a standard double layer agar overlay

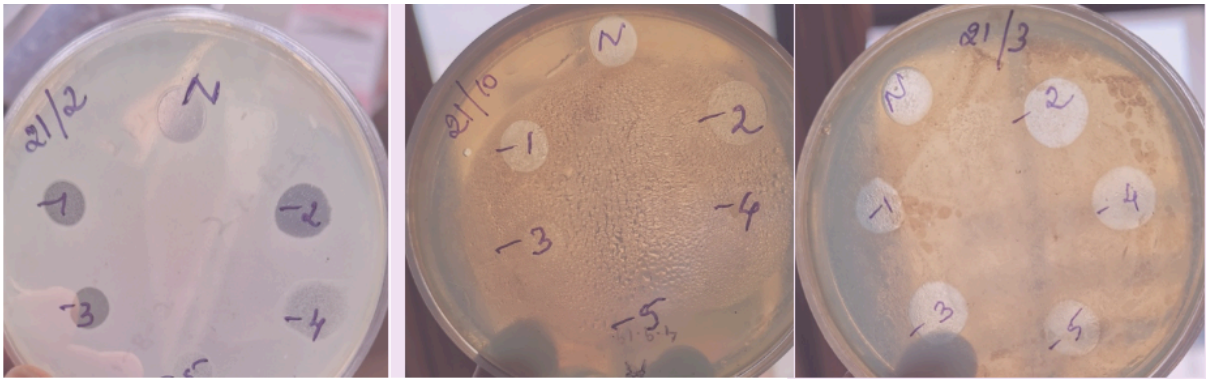

Figure 16: Clearance by different serial dilution of phage lysates 2, 10 and 3 used for phage titre estimation

4.6 Host range

The bacteriolytic spectra of phage lysates 2, 3 and 10 were tested on all the 24 aeromonad isolates. Only four (4) out of the 24 isolates were sensitive to phage lysates 2 and 3 whereas only one (1) isolate was sensitive to phage lysate 10 (Figure 17).

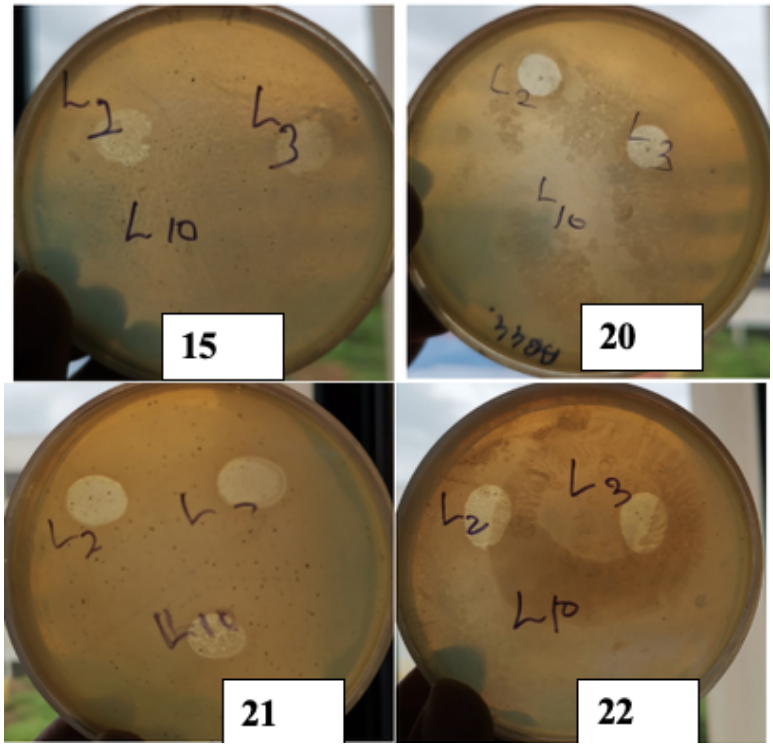

Figure 17: Sensitivity of the different Aeromonad isolates to phage lysates 2, 3 and 10 as seen by presence of a clear zone where phage lysates were spotted on lawns made on TSA plates

4.7 Single step growth curve of phage

The one-step growth curve has three stages: Inoculation; where viruses attach to host cells, Eclipse; when virus genome enters the cell and burst; when sufficient numbers of new viruses are produced and emerge from the host cell. The Multiplicities of infection (MOIs) used during the curve were 0.0001, 0.000045 and 0.00005 for phage lysates 10, 2 and 3. The latent period was shortest (40 minutes) for phage lysate 10 as compared to (60) minutes for lysates 2 and 3, and burst size was highest for phage lysate 10 (271 PFU/cell), 3 (187 PFU/cell) and 2 (56 PFU/cell) (Table 11 and Figure 18). The number of adsorbed phages and their percentages for phage lysate 2, 3 and 10 was 1190 (89%), 1500 (42%) and 1860 (51%) (Table 11).

Table 11: The number of adsorbed phages, burst size and their latent period, incubated at 30^0C, 100RPM for 6 hours

Lysate	Adsorbed phages	Adsorbed phages (%)	Burst size PFU/cell	Latent period (Minutes)
2	1190	89.0	56.0	60
3	1500	42.0	187	60
10	1860	51	271	40

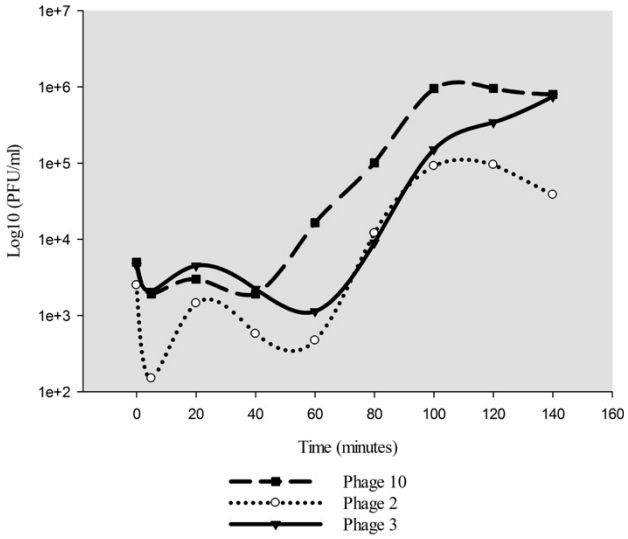

Figure 18: One time one-step phage growth curves for phage lysates 2, 3 and 10, a double layer agar overlay method was used for enumeration of phage titres every twenty minutes

4.8 Single phage lysate inactivation of the Aeromonad isolate

A CFU reduction assay (Figures 20 to 25) showed that for the control treatment, both OD @ 600nm and bacteria CFU/ml for all phage lysate MOI experiments followed the sigmoid growth curve. In contrast, the OD @ 600nm, and CFU/ml reduced for all phage lysates at all MOIs but after variable periods. Table 12 shows the results of statistical analysis, wherein general terms, there was a significant difference in CFU/ml reduction for all phage lysates at the different MOIs as compared to the control (ANOVA, $p < 0.05$). For phage lysate 3, there was a significant difference in the reduction of CFU/ml for all MOIs as compared to the control (ANOVA, $p < 0.05$). MOI (10) showed the least CFU/ml, followed by 1, 0.1 and 0.01 respectively. Furthermore, there was a significant difference in CFU/ml reduction at MOI (10) as compared to MOI (0.1) and (0.01) (ANOVA, $p = 0.033$ and $0.002 < 0.05$), implying that CFU/ml reduction was significantly better at MOI (10) than 0.1 and 0.01 but not 1. For Phage lysate 10, the higher the MOI, the higher was the CFU/ml reduction ($10 > 1 > 0.1 > 0.01$) and there was a significant difference in CFU/ml reduction at all MOIs as compared to the control (ANOVA, $p < 0.05$), as the case was for phage lysate 3. In contrast, for phage lysate 2, MOI (0.1) had the best CFU/ml reduction, followed by 1, 10 and 0.01 as compared to the control, and there was no significant difference in CFU/ml reduction between the different MOIs when compared to each other (ANOVA, $p > 0.05$).

The OD plots (Figures 20, 22 and 24) showed that MOI (10) was the most efficient at retarding bacteria growth, followed by 1, 0.1 and 0.01 after 3 and 5 hours for MOI (10 and 1) and (0.1

and 0.01) respectively for all phage lysates, except for phage lysate 10, where it took 2 and 4 hours for MOIs (10 and 1) and (0.1 and 0.01). In contrast, the CFU/ml plots (Figures 21, 23 and 25), a relatively different trend for each MOI for a given phage lysate was observed. For phage lysate 2 (Figure 20), the minimum CFU/ml was attained from the MOIs (10 and 1) after 3 hours, followed by 0.1 and 0.01 after 4.5 and 6 hours. The multiplicity of infection (10), (0.1), (1) and (0.01) after 5, 4.5, 4 and 6 hours for phage lysate 3, obtained the minimum CFU/ml of the bacteria isolate (Figure 22), whereas for phage lysate 10, after 3, 4.5, 4.5 and 6 hours, MOI 10, 0.1 and 0.01 were able to generate the least CFU/ml (Figure 23). For all phage lysates at MOI 10, 1 and 0.1, there was observed regrowth after reaching the minimum CFU/ml with the highest regrowth observed at MOI 10 for all lysates as compared to the rest except for MOI (0.01) that didn't show any regrowth at all for all lysates for the 6 hours of the experiment.

4.9 Phage resistance evolution in vitro

Finally, the evolution of phage resistance in the Aeromonad isolate was examined by the presence of viable phage-resistant bacteria after phage infection for 5 more hours after the MOI experiments of phage lysates (10 and 2). Survivors were spotted on TSA plates in triplicate, incubated overnight at 30^0C. Lawns were made for the different survivors and by the spot assay, their sensitivity to all the phage lysates was tested. Viable bacteria colonies were obtained from MOIs (10 and 1) for both lysates. Whereas bacteria survivors of phage lysate 10 were resistant to phage lysate 10 but sensitive to phage lysate 2 and 3, the survivors of the phage lysate 2 MOI experiment were still sensitive to all the phage lysates (Figure 19).

Table 12: The (average±standard deviation) of the log10 (CFU/ml) and their mean differences between MOI (10, 1, 0.1 and 0.01) as compared to the control for phage lysates 3, 2 and 10 after a 6 hours CFU reduction assay experiment. P-values with a * indicate no significant difference in comparisons, level of significance 0.05, one way ANOVA, n=60

Phage lysate	MOI	CFU/ml	Mean difference	P value
10	Control	10.058±1.751		
	10	6.667±0.546	-3.391	0.000
	1	7.210±1.557	-2.848	0.000
	0.1	7.783±2.020	-2.275	0.005
	0.01	8.084±1.959	-1.974	0.020
2	Control	8.470±1.233		
	10	7.168±0.797	-1.302	0.019
	1	7.002±0.916	-1.468	0.006
	0.1	6.735±1.182	-1.735	0.001
	0.01	7.263±1.229	-1.207	0.036
3	Control	9.329±91.101		
	10	6.014±0.987	-3.315	0.000
	1	7.144±0.966	-2.185	0.001
	0.1	7.501±1.908	-1.828	0.006
	0.01	8.035±1.240	-1.294	0.119*

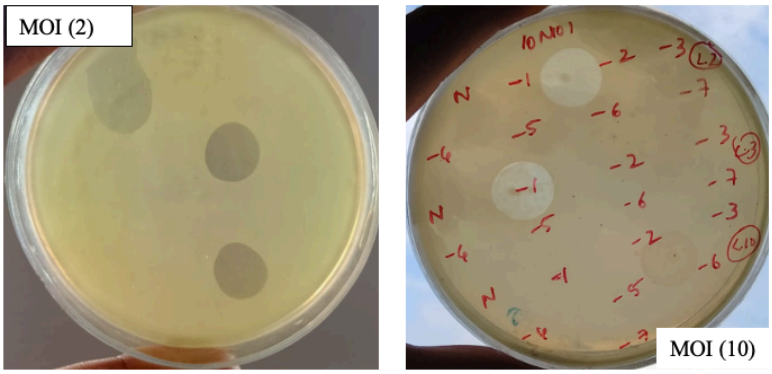

Figure 19: Phage sensitivity test on bacteria survivors after 11 hours of phage lysate (2) and (10) infection. The survivors of phage lysate 10 were resistant to phage lysate 10 but sensitive to 2 and 3 (MOI (10)). Survivors of phage lysate 2 were still sensitive to all phage lysates (MOI (2))

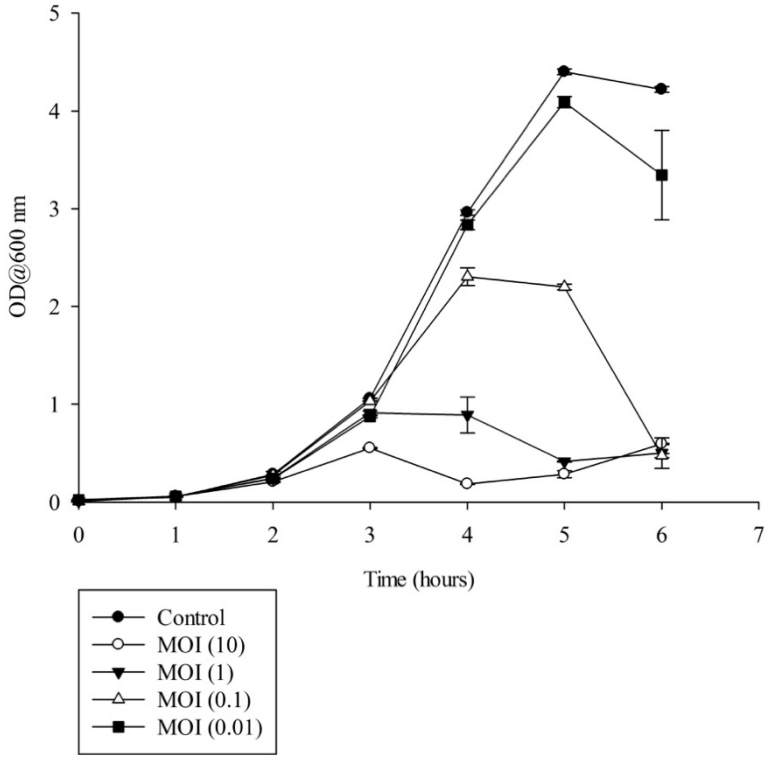

Figure 20: Evolution of OD @ 600nm at different MOIs for phage lysate 2. The experiment was done once and the readings taken in duplicate. Error bars denote the standard deviation of the mean

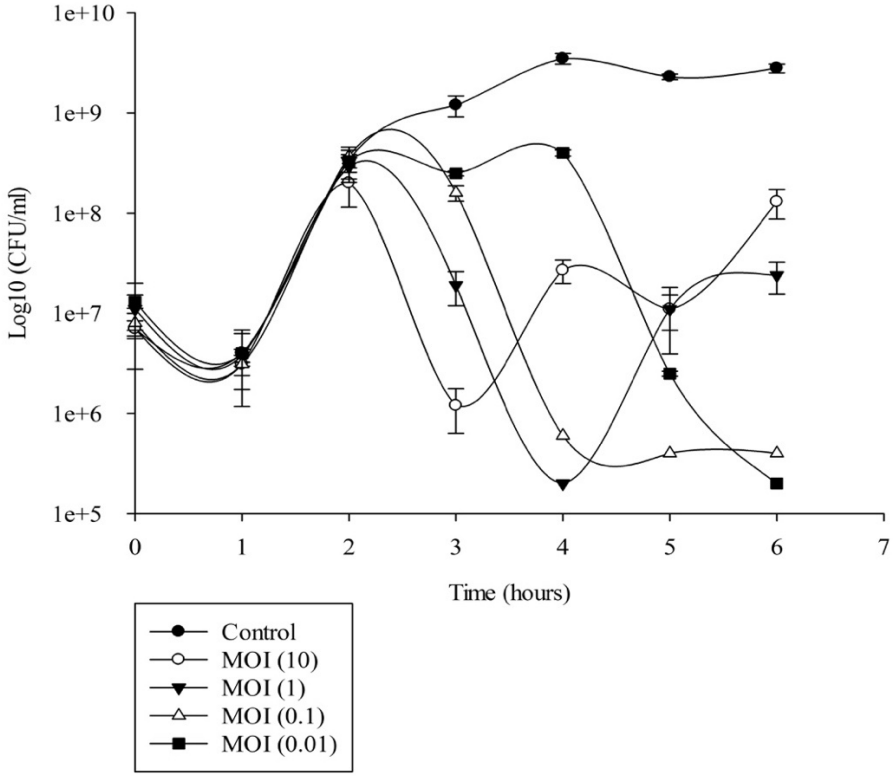

Figure 21: Evolution of bacterial CFU/ml at different MOIs for phage lysate 2. The experiment was conducted once, CFU/ml recorded from the plates with countable colonies < 300. The error bars denote the standard deviation of the mean

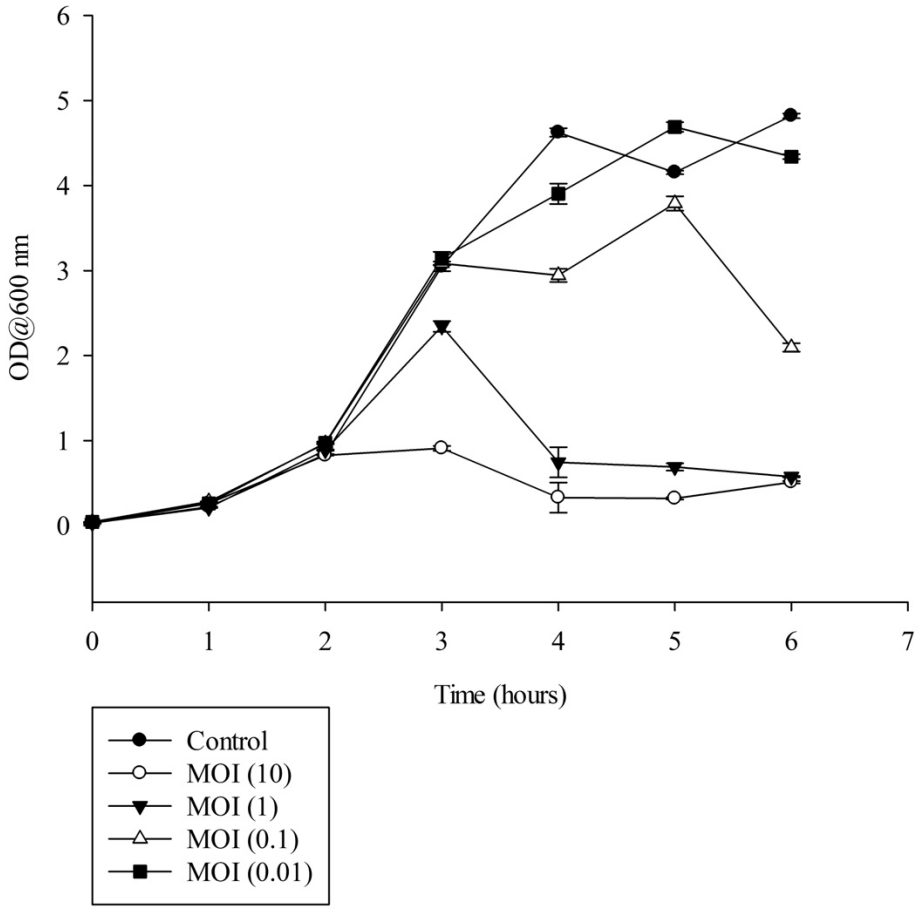

Figure 22: Evolution of OD @600nm at different MOIs for phage lysate 3. The experiment was conducted once and readings taken in duplicate. Error bars denote the standard deviation of the mean

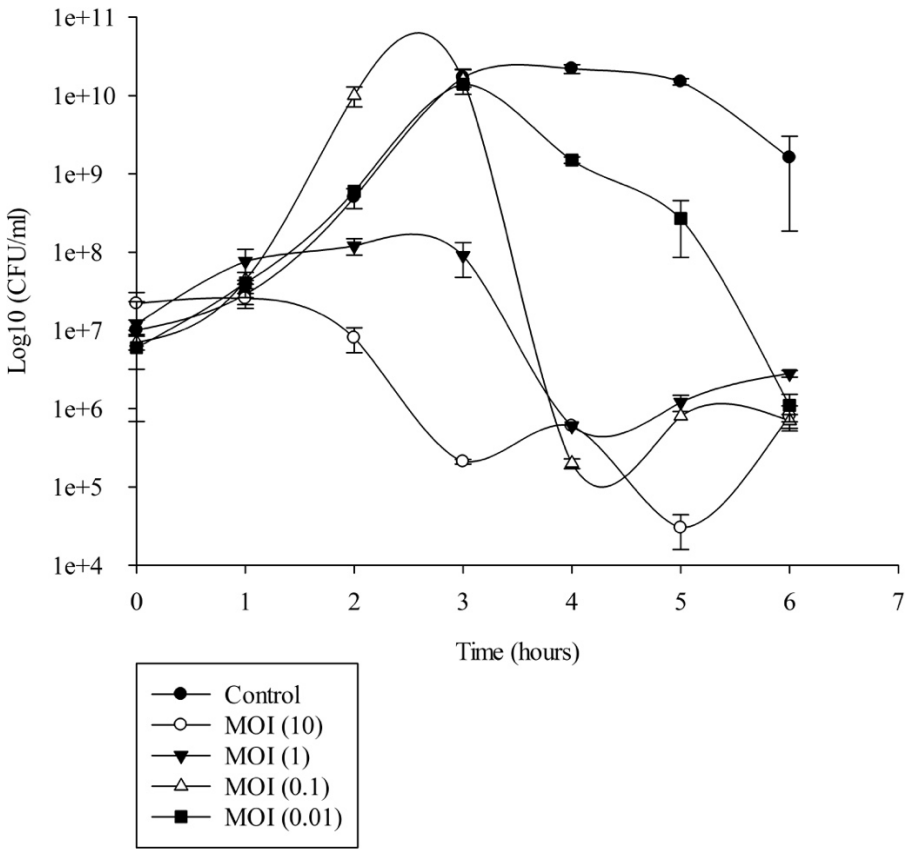

Figure 23: Evolution of bacterial CFU/ml at different MOIs for phage lysate 3. The experiment was done once, CFU/ml recorded from the plates with countable colonies < 300. The error bars denote the standard deviation of the mean

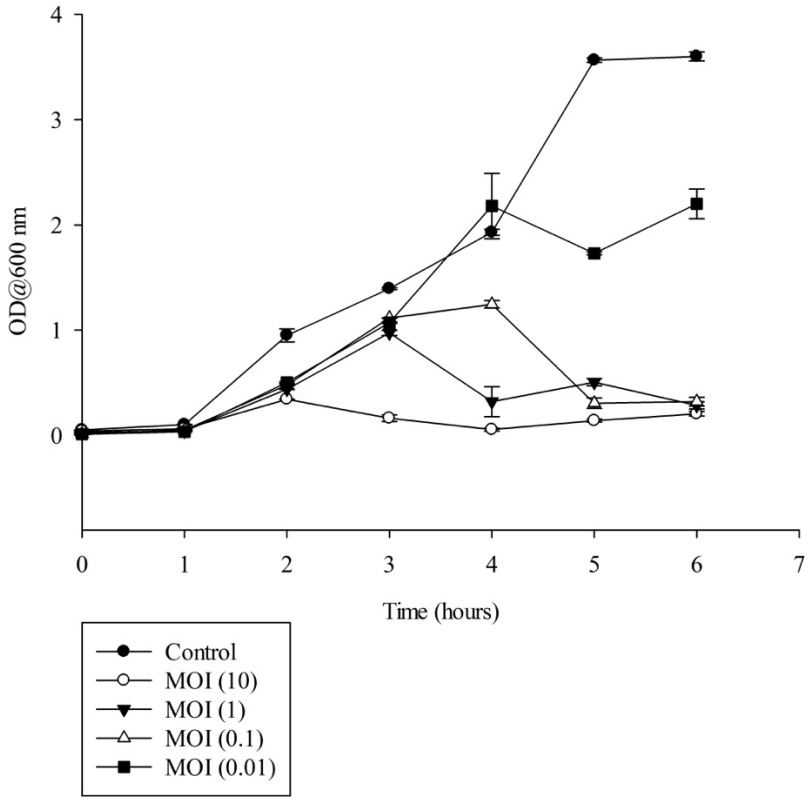

Figure 24: Evolution of OD @ 600nm at different MOIs for phage lysate 10. The experiment was conducted once and readings taken in duplicate. Error bars denote the standard deviation of the mean

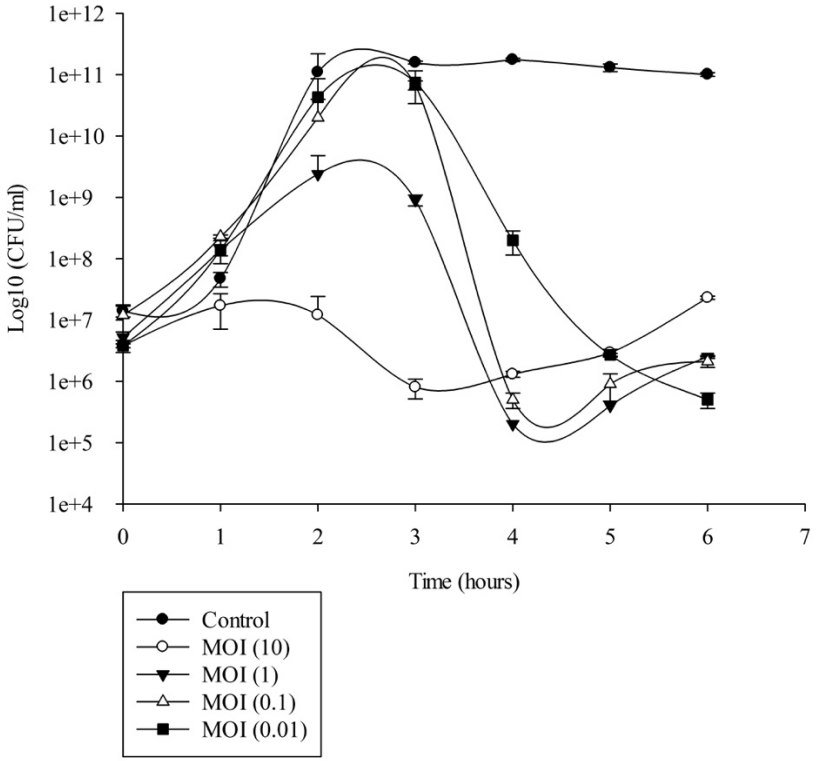

Figure 25: Evolution of CFU/ml at different MOIs for phage lysate 10. The experiment was conducted once, CFU/ml recorded from the plates with countable colonies < 300. The error bars denote the standard deviation of the mean

5.0 Discussion

Motile Aeromonad species, which are known to be opportunistic, and water being their primary habitat, have become key aquaculture pathogens. However, treatment with antibiotics, the most predominant therapeutic method, has become increasingly difficult and expensive due to emergence and widespread of antibiotic resistance (Defoirdt et al., 2011; Paul & Sullivan, 2005; Ran et al., 2018). Their effect, together in synergy with other disease-causing agents like viruses, other bacteria, and fungi are responsible for aggravated severity of diseases of farmed aquatic animals including fish and associated economic losses (Cao et al., 2019, 2020). Notwithstanding, the carriers of antibiotic resistance genes can potentially transfer the resistance to other microbes of the same or different species (Chuah et al., 2016; Huddleston, 2014). As a consequence, development of novel therapeutic and or prophylactic compounds to combat economic losses from infectious diseases in aquaculture is long overdue (Chuah et al., 2016; Hoseinifar et al., 2015). Therefore, this study was aimed at isolating and characterizing novel motile Aeromonad bacteriophages and examining their in vitro therapeutic potential against motile Aeromonad bacterial isolates of aquaculture importance. Bacteriophages are viruses that infect and either kill bacteria; lytic, and or integrate their DNA into the host chromosome; temperate (DA & Didamony, 2016). Lytic phages have been proven to be bactericidal against many bacterial pathogens including antibiotic-resistant bacteria (Easwaran et al., 2017) as seen in isolates 20 and 22 (Figure 17 and appendix 8.1). This could probably be due to differing mechanisms of bacteriolysis as compared to those of antibiotics, with the capability of self-replicating and self-limiting depending on availability of hosts and the nature of the environment (Atterbury et al., 2003; Cao et al., 2019; Easwaran et al., 2017). Phages live close to their hosts according to Duffy & Hay, 1998, and isolation of phage lysate 2, 3 and 10 was centred to aquaculture water and fish samples, showing conformity to the presence of Aeromonad phages in one of the primary habitats of their hosts (Yang et al., 2017). Latent period and burst size are some of the key considerations while choosing a phage for phage therapy (Gon Choudhury et al., 2017). Therefore, from this study, phage lysate 10 had the highest burst size (271) PFU/cell and shortest latent period (40) minutes as compared to the burst sizes of 56 and 187 PFU/cell for phage lysates 2 and 3. Hence, more lytic and more appropriate as a candidate for phage therapy against the isolated motile Aeromonad in this regard (Hoang et al., 2019). However, (Abedon et al., 2001) reported that high burst sizes are

usually followed by longer latency periods, questioning the potency of burst size as a parameter while choosing a candidate phage for phage therapy. As opposed to antibiotics and other chemotherapeutics that indiscriminately kill everything, including the beneficial gut microflora, causing dysbiosis, phage therapy has attracted attention in aquaculture due to the phage host specificity to particularly targeted bacteria (Atterbury et al., 2003; Nikapitiya et al., 2019; Phumkhachorn & Rattanachaikunsopon, 2010). However, phage therapy generally requires phages that are of a relatively broader host range, increasing the possibility of infecting multiple strains of highly diverse hosts like Aeromonads, either in sole or cocktail treatments (Phumkhachorn & Rattanachaikunsopon, 2010). While all phage lysates were able to lyse at least an indicator host, phage lysate 2 and 3 showed an identical and relatively broader host range by lysing 4 out of the 24 Aeromonad isolates (16.6%) (Figure 17), probably due to similarity in recognition receptor structures on the host bacteria they lysed (Kalatzis et al., 2016). In contrast, phage lysate 10 only lysed 1 out of the 24 isolates (4.1%) (Figure 17), thus, more specific than 2 and 3, and more capable of being diagnostic for the Aeromonad isolate host (Yasuike et al., 2014). Variations in tail fibre proteins and their complementarity to the host membrane receptors is one of the reasons for host specificity in phage therapy (Hassan et al., 2018; Lee et al., 2011). According to (Kalatzis et al., 2016), a phage that is capable of lysing almost all target strains of a given bacteria isolate is a more ideal candidate for phage therapy, thus phage lysate 2 and 3, in this case, would be more appropriate. Additionally, the in vitro inactivation of the motile Aeromonad by the phage lysates at multiplicities of infection (MOIs) of 10, 1, 0.1 and 0.01 revealed that all the phage lysates were capable of significantly inactivating the growth of Aeromonad isolates at all MOIs (Table 12). This led to the attainment of peak growth by the Aeromonad isolate at a relatively lower CFU/ml as compared to the corresponding control treatments. However, the Optical density (OD) and cell count (CFU/ml) plots depicted a somewhat different pattern of inactivation for the different MOIs, with OD plots showing that inactivation was optimal at MOI (10) and least with MOI (0.01) (Figures 19, 21 and 23), whereas cell count (CFU/ml) plots had a variable pattern of inactivation with some regrowth patterns observed especially for MOI 10 and 1 for all phage lysates. Optical density (OD) is based on absorbance that depends on turbidity of the culture, dead bacterial cells and debris in suspension and observed regrowth of probably phage resistant mutants especially at higher MOIs and these could have contributed to the OD readings, explaining the difference (Beal et al., 2019; Hoang et al., 2019). Another probable explanation could be the limitation of OD of not being informative about cell viability and being affected

by cell states like protein expression and growth patterns (Beal et al., 2019), unlike direct cell count, that also comes with restrictions such as viable but not culturable cells and cell clumping, leading to a CFU being representative of more than one cell (Rajapaksha et al., 2019). For phage lysates 3 and 10, higher MOIs were better at bacteria reduction than lower ones (10 > 1 > 0.1 and > 0.01). Similar observations have been reported with *Vibrio alginolyticus* phages (Kalatzis et al., 2016), although phage lysate 2 with an MOI (0.1) being the best, contradicted with their findings. The relatively lower inactivation of the Aeromonad isolate by high MOIs of phage lysate 2 could have been due to rapid lysis of bacteria, producing debris onto which phages could have adsorbed to, becoming immobilized and unavailable for further bacteria lysis (Christiansen et al., 2016). Nonetheless, phage lysate 2 would be more useful in phage therapy at low dosages with minimal incidences of phage resistance (Nikapitiya et al., 2019). The proliferation of phage resistant bacteria and the possibility of prophage induction, mutations, and horizontal gene transfer are naturally occurring events (Chan et al., 2013). Using poorly characterized phages for phage therapy could hasten the occurrence of the above-mentioned phenomena, although the use of carefully studied phage cocktails could delay or inhibit their occurrence (Chan et al., 2013; Kalatzis et al., 2016). All MOIs were capable of significantly retarding bacterial growth, with MOIs (0.1 and 0.01) showing no occurrence of phage resistant bacterial colonies for the duration of the culture (11 hours) as compared to MOI (10 and 1). A similar finding was reported by (Kim et al., 2012), and it can be deduced that low MOIs (0.1 and 0.01) of the phage lysates could probably have delayed or inhibited the occurrence of viable phage-resistant bacteria, contrary to high MOIs (10 and 1) for which they were formed (Nikapitiya et al., 2019). Although little is still known about the properties, diversity and temporal dynamics of phage resistant strains in response to phage infection, resistance to phages occurs due to enhanced selection pressure for phage resistance (Christiansen et al., 2016). This succeeds rapid lysis and decay of sensitive phage particles at high but not low MOIs (Christiansen et al., 2016; Laland & Sterelny, 2006; Lenski, 1984). Mutation in bacteria host membrane structures whose role is primarily nutrient uptake from the environment and virulence, but also as receptors for phage adsorption ensues, making resistant strains less competitive as compared to the sensitive ones (Christiansen et al., 2016; Middelboe et al., 2001). Therefore, regrowth and dominance of the culture by resistant strains at the expense of the sensitive strains is likely to happen (Middelboe et al., 2001). Besides, presence of viable bacteria colonies after 11 hours for phage lysates 2 and 10, MOI (10 and 1), justified occurrence of phage resistance at higher MOIs, as reported by (Kim et al., 2012; Nikapitiya et

al., 2019). Nevertheless, phage lysate 10 resistant bacteria were sensitive to phage lysates 2 and 3 whereas the phage lysate 2 survivor bacteria were still sensitive to all phage lysates (Figure 19). This highlights the possibility of using phage cocktail treatments to overcome resistance to one phage by another phage which could probably overcome the resistance mechanisms used by the phage resistant bacteria to another phage (Hoang et al., 2019; Madhusudana Rao & Lalitha, 2015).

6.0 Conclusion

With the current speed at which aquaculture is growing, the use of highly intensive systems with high stocking densities of high-value fish, aquatic plant and invertebrate species is also increasing steadily. As a result, disease outbreaks with resultant associated economic losses in billion dollars have been reported. Several attempts to minimize the drastic effects of diseases in aquaculture were made, largely relying on antibiotic usage. However, the development of antibiotic resistance by most pathogens of aquaculture and public health concern to most used antibiotics reduced their efficacy. Horizontal and vertical transfer on mobile genetic materials, bioaccumulation and magnification in aquatic produce consumers and the environment and other health associated risks, made antibiotic use undergo strict legislation and abandonment. The indiscriminative nature of antibiotics and other chemotherapeutics like disinfectants also led to dysbiosis involving killing commensal and nontarget bacteria, leading to microbial imbalance. To overcome and or minimize the negative attributes and side effects of antibiotics and other chemotherapeutics, biological control measures like phage therapy, whose strength relies upon their self-replicating and self-limiting nature and high specificity had to be used innovated in aquaculture. From this study, 3 lytic phage lysates (2, 3 and 10) were isolated from fish and water samples against motile Aeromonad isolates from the same samples. Phage lysate 2 and 3 had an equal and longer latent period than 10 whereas the adsorption efficiency was highest with phage lysate 2, 10 and 3. Phage lysate 3 was more lytic with the highest burst size and more host-specific as compared to 3 and 2. All phage lysates were capable of significantly retarding bacterial growth at all multiplicities of infection, except for phage lysate 3 for which MOI (0.01) didn't significantly inactivate bacterial growth as compared to the control treatment. For phage lysate 3 and 10, high MOIs 10 and 1 were better than lower ones 0.1 and 0.01 at reducing bacteria CFU/ml while for phage lysate 2, MOI 0.1 was better than the rest at bacteria CFU/ml reduction. Phage lysate 10 and 2 produced viable bacteria colonies after 11 hours of phage infection that were resistant to phage lysate 10 but sensitive to 2 and 3, and sensitive to all phage lysates respectively.

7.0 Recommendations

- Molecular characterization of phage lysates should be done to classify them and check for presence toxin and hazardous gene occurrence in the genome.
- Screening for prophage induction in the bacterial isolates and survivors of phage treatment is also key in deciding whether a bacteriophage would be beneficial in phage therapy.
- Repeating the experiments several times would provide information about reproducibility and consistency of the results.
- Isolation of Aeromonas phages with better host coverage.
- Doing a host range test on other unrelated bacteria isolates would offer a broader picture about the host range of the phage lysates.
- Morphological examination under a transmission electron microscope would be beneficial in classifying the phage lysates.
- Phage lysate stability at different pH values and temperatures would also give an insight into the potential of phage lysates to resist harsh host immune responses.
- An in vivo assessment of the efficacy of the phage lysates should be done to augment the conclusion about the bactericidal ability of the phage lysates against motile Aeromonads.

7.0 References

Abedon, S. T., Herschler, T. D., & Stopar, D. (2001). Bacteriophage Latent-Period Evolution as a Response to Resource Availability. *Applied and Environmental Microbiology*, *67*(9), 4233–4241. https://doi.org/10.1128/AEM.67.9.4233-4241.2001

Adams, M. (n.d.). Bacteriophages. *Sidalc.Net*. Retrieved July 9, 2020, from http://www.sidalc.net/cgi-bin/wxis.exe/?IsisScript=BFHIA.xis&method=post&formato=2&cantidad=1&expresion=mfn=011724

Aich, N., Ahmed, N., & Paul, A. (2018). Issues of Antibiotic Resistance in Aquaculture Industry and Its Way Forward. *International Journal of Current Microbiology and Applied Sciences*, *7*(08), 26–41. https://doi.org/10.20546/ijcmas.2018.708.004

Aly, S. M., & Albutti, A. (2014). Antimicrobials use in aquaculture and their public health impact. *Journal of Aquaculture Research and Development*, *5*(4). https://doi.org/10.4172/2155-9546.1000247

Anid, R. (n.d.). *Guía de búsquedas avanzadas*.

Atterbury, R. J., Connerton, P. L., Dodd, C. E. R., Rees, C. E. D., & Connerton, I. F. (2003). Isolation and characterization of Campylobacter bacteriophages from retail poultry. *Applied and Environmental Microbiology*, *69*(8), 4511–4518. https://doi.org/10.1128/AEM.69.8.4511-4518.2003

Austin, B., & Allen-Austin, D. (1985). A review: Bacterial pathogens of fish. *Journal of Applied Bacteriology*, *58*(5), 483–506. https://doi.org/10.1111/j.1365-2672.1985.tb01490.x

Beal, J., Farny, N. G., Haddock-Angelli, T., Selvarajah, V., Baldwin, G. S., Buckley-Taylor, R., Gershater, M., Kiga, D., Marken, J., Sanchania, V., Sison, A., & Workman, C. T. (2019). *Robust Estimation of Bacterial Cell Count from Optical Density*. 1–17. https://doi.org/10.1101/803239

Bedekar, M. K., Kole, S., & Tripathi, G. (2020). Biotechnological approaches to fish vaccine. *Genomics and Biotechnological Advances in Veterinary, Poultry, and Fisheries*, 407–419. https://doi.org/10.1016/b978-0-12-816352-8.00017-5

Biemer, J. J. (1973). Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method. *Annals of Clinical Laboratory Science*, *3*(2), 135–140.

Cao, Y., Li, S., Han, S., Wang, D., Zhao, J., Xu, L., Liu, H., & Lu, T. (2020). Characterization and application of a novel Aeromonas bacteriophage as treatment for pathogenic Aeromonas hydrophila infection in rainbow trout. *Aquaculture*, *523*(December 2019). https://doi.org/10.1016/j.aquaculture.2020.735193

Cao, Y., Li, S., Wang, D., Zhao, J., Xu, L., Liu, H., Lu, T., & Mou, Z. (2019). Genomic characterization of a novel virulent phage infecting the Aeromonas hydrophila isolatfile:///Users/macbook/Downloads/pharmaceuticals-12-00035-v2.pdfed from rainbow trout (Oncorhynchus mykiss). *Virus Research*, *273*, 197764. https://doi.org/10.1016/j.virusres.2019.197764

Carbone, D., & Faggio, C. (2016). Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of Sparus aurata and Dicentrarchus labrax. *Fish and Shellfish Immunology*, *54*, 172–178. https://doi.org/10.1016/j.fsi.2016.04.011

Chan, B. K., Abedon, S. T., & Loc-carrillo, C. (2013). *fmb.13.47 - Kopie*. 769–783. https://doi.org/10.2217/fmb.13.47

Christiansen, R. H., Madsen, L., Dalsgaard, I., Castillo, D., Kalatzis, P. G., & Middelboe, M. (2016). Effect of Bacteriophages on the Growth of Flavobacterium psychrophilum and

Development of Phage-Resistant Strains. *Microbial Ecology*, *71*(4), 845–859. https://doi.org/10.1007/s00248-016-0737-5

Chuah, L. O., Effarizah, M. E., Goni, A. M., & Rusul, G. (2016). Antibiotic Application and Emergence of Multiple Antibiotic Resistance (MAR) in Global Catfish Aquaculture. In *Current environmental health reports* (Vol. 3, Issue 2, pp. 118–127). https://doi.org/10.1007/s40572-016-0091-2

Clokie, M. R. J., & Kropinski, A. M. (2009). Bacteriophages : methods and protocols. *Methods in Molecular Biology*, *501*, xxii, 307 p. https://doi.org/10.1007/978-1-60327-164-6

Cohen, Y., Joseph Pollock, F., Rosenberg, E., & Bourne, D. G. (2013). Phage therapy treatment of the coral pathogen Vibrio coralliilyticus. *MicrobiologyOpen*, *2*(1), 64–74. https://doi.org/10.1002/mbo3.52

Czajkowski, R., Ozymko, Z., & Lojkowska, E. (2016). Application of zinc chloride precipitation method for rapid isolation and concentration of infectious Pectobacterium spp. and Dickeya spp. lytic bacteriophages from surface water and plant and soil extracts. *Folia Microbiologica*, *61*(1), 29–33. https://doi.org/10.1007/s12223-015-0411-1

DA, E. A., & Didamony, G. El. (2016). New Approach to Use Phage Therapy against Aeromonas hydrophila Induced Motile Aeromonas Septicemia in Nile Tilapia. *Journal of Marine Science: Research & Development*, *6*(3), 6–11. https://doi.org/10.4172/2155-9910.1000194

Dafale, N., Lakhe, S., Yadav, K., Purohit, H., & Chakrabarti, T. (2008). Concentration and Recovery of Coliphages from Water with Bituminous Coal. *Water Environment Research*, *80*(3), 282–288. https://doi.org/10.2175/106143007x220923

Dalmo, R. A. (2018). DNA vaccines for fish: Review and perspectives on correlates of protection. In *Journal of Fish Diseases* (Vol. 41, Issue 1, pp. 1–9). Blackwell Publishing Ltd. https://doi.org/10.1111/jfd.12727

Das, S., Aswani, R., Jasim, B., Sebastian, K. S., Radhakrishnan, E. K., & Mathew, J. (2020). Distribution of multi-virulence factors among Aeromonas spp. isolated from diseased Xiphophorus hellerii. *Aquaculture International*, *28*(1), 235–248. https://doi.org/10.1007/s10499-019-00456-5

Dash, P., Sahoo, P. K., Gupta, P. K., Garg, L. C., & Dixit, A. (2014). Immune responses and protective efficacy of recombinant outer membrane protein R (rOmpR)-based vaccine of Aeromonas hydrophila with a modified adjuvant formulation in rohu (Labeo rohita). *Fish and Shellfish Immunology*, *39*(2), 512–523. https://doi.org/10.1016/j.fsi.2014.06.007

de Jonge, P. A., Nobrega, F. L., Brouns, S. J. J., & Dutilh, B. E. (2019). Molecular and Evolutionary Determinants of Bacteriophage Host Range. *Trends in Microbiology*, *27*(1), 51–63. https://doi.org/10.1016/j.tim.2018.08.006

Defoirdt, T., Sorgeloos, P., & Bossier, P. (2011). Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology*, *14*(3), 251–258. https://doi.org/10.1016/j.mib.2011.03.004

Diagnostics Hardy. (2011). Hardydisk ™ Antimicrobial Sensitivity Test (Ast). *Hardy Diagnostics*, *553*, 1–16.

Duffy, J. E., & Hay, M. E. (1998). *The Ecology and Evolution of Interactions*.

Easwaran, M., Dananjaya, S. H. S., Park, S. C., Lee, J., Shin, H. J., & De Zoysa, M. (2017). Characterization of bacteriophage pAh-1 and its protective effects on experimental infection of Aeromonas hydrophila in Zebrafish (Danio rerio). *Journal of Fish Diseases*, *40*(6), 841–846. https://doi.org/10.1111/jfd.12536

Ertör-Akyazi, P. (2020). Contesting growth in marine capture fisheries: the case of small-scale fishing cooperatives in Istanbul. *Sustainability Science*, *15*(1), 45–62. https://doi.org/10.1007/s11625-019-00748-y

FAO. (2019). The State of World fisheries and aquaculture 2018 - Meeting the sustainable development goals. In *Nature and Resources*.

Fe, A., Ko, J., Mudro, D., Popelka, P., & Toropilová, J. (2017). *The use of probiotic bacteria against Aeromonas infections in salmonid aquaculture*. *469*, 1–8. https://doi.org/10.1016/j.aquaculture.2016.11.042

Fortier, L. C., & Sekulovic, O. (2013). Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence*, *4*(5), 354–365. https://doi.org/10.4161/viru.24498

Garlock, T., Asche, F., Anderson, J., Bjørndal, T., Kumar, G., Lorenzen, K., Ropicki, A., Smith, M. D., & Tveterås, R. (2020). A Global Blue Revolution: Aquaculture Growth Across Regions, Species, and Countries. *Reviews in Fisheries Science & Aquaculture*, *28*(1), 107–116. https://doi.org/10.1080/23308249.2019.1678111

Ghugare, G. S., Nair, A., Nimkande, V., Sarode, P., Rangari, P., & Khairnar, K. (2017). Membrane filtration immobilization technique—a simple and novel method for primary isolation and enrichment of bacteriophages. *Journal of Applied Microbiology*, *122*(2), 531–539. https://doi.org/10.1111/jam.13344

Gill, J., & Hyman, P. (2010). Phage Choice, Isolation, and Preparation for Phage Therapy. *Current Pharmaceutical Biotechnology*, *11*(1), 2–14. https://doi.org/10.2174/138920110790725311

Gon Choudhury, T., Tharabenahalli Nagaraju, V., Gita, S., Paria, A., & Parhi, J. (2017). Advances in Bacteriophage Research for Bacterial Disease Control in Aquaculture. *Reviews in Fisheries Science and Aquaculture*, *25*(2), 113–125. https://doi.org/10.1080/23308249.2016.1241977

Gonçalves Pessoa, R. B., de Oliveira, W. F., Marques, D. S. C., dos Santos Correia, M. T., de Carvalho, E. V. M. M., & Coelho, L. C. B. B. (2019). The genus Aeromonas: A general approach. *Microbial Pathogenesis*, *130*(February), 81–94. https://doi.org/10.1016/j.micpath.2019.02.036

Gudding, R, Lillehaug, A., & Evensen, O. (2014). *Fish vaccination*. https://books.google.com/books?hl=en&lr=&id=lzUKAwAAQBAJ&oi=fnd&pg=PR15&dq=importance+of+vaccines+in+aquaculture+&ots=alNsQDb2GY&sig=SVUhP9kFFJqv8IGr05rOo9XVKjI

Gudding, Roar, & Van Muiswinkel, W. B. (2013). A history of fish vaccination: Science-based disease prevention in aquaculture. *Fish and Shellfish Immunology*, *35*(6), 1683–1688. https://doi.org/10.1016/j.fsi.2013.09.031

Gui, L., & Zhang, Q. Y. (2018). Disease Prevention and Control. *Aquaculture in China: Success Stories and Modern Trends*, 577–598. https://doi.org/10.1002/9781119120759.ch7_3

Hancock, R. E. W. (1998). Resistance Mechanisms in Pseudomonas aeruginosa and Other Nonfermentative Gram-Negative Bacteria . *Clinical Infectious Diseases*, *27*(s1), S93–S99. https://doi.org/10.1086/514909

Hassan, S. W. M., Ali, S. M., & AlMisherfi, M. M. (2018). Isolation and molecular characterization of some marine Aeromonas phages: Protective effects for Nile tilapia infected with Aeromonas hydrophila. *Journal of Pure and Applied Microbiology*, *12*(3), 1175–1185. https://doi.org/10.22207/JPAM.12.3.17

Hatfull, G. F. (2012). The Secret Lives of Mycobacteriophages. In *Advances in Virus Research* (1st ed., Vol. 82). Elsevier Inc. https://doi.org/10.1016/B978-0-12-394621-8.00015-7

Henein, A. (2013). What are the limitations on the wider therapeutic use of phage? *Bacteriophage*, *3*(2), e24872. https://doi.org/10.4161/bact.24872

Higuera, G., Bastías, R., Tsertsvadze, G., Romero, J., & Espejo, R. T. (2013). Recently discovered Vibrio anguillarum phages can protect against experimentally induced vibriosis in Atlantic salmon, Salmo salar. *Aquaculture*, *392–395*, 128–133. https://doi.org/10.1016/j.aquaculture.2013.02.013

Hoang, H. A., Xuan, T. T. T., Nga, L. P., & Oanh, D. T. H. (2019). Selection of phages to control aeromonas hydrophila-an infectious agent in striped catfish. *Biocontrol Science*, *24*(1), 23–28. https://doi.org/10.4265/bio.24.23

Hoseinifar, S. H., Esteban, M. Á., Cuesta, A., & Sun, Y. Z. (2015). Prebiotics and Fish Immune Response: A Review of Current Knowledge and Future Perspectives. In *Reviews in Fisheries Science and Aquaculture* (Vol. 23, Issue 4, pp. 315–328). Taylor and Francis Inc. https://doi.org/10.1080/23308249.2015.1052365

Huddleston, J. R. (2014). Horizontal gene transfer in the human gastrointestinal tract: Potential spread of antibiotic resistance genes. *Infection and Drug Resistance*, *7*, 167–176. https://doi.org/10.2147/IDR.S48820

Hudzicki, J. (2016). *Kirby-Bauer Disk Diffusion Susceptibility Test Protocol*. *December 2009*, 1–23.

Hyman, P. (2019). Phages for phage therapy: Isolation, characterization, and host range breadth. *Pharmaceuticals*, *12*(1). https://doi.org/10.3390/ph12010035

Jäckel, C., Hammerl, J., & Hertwig, S. (2019). Campylobacter Phage Isolation and Characterization: What We Have Learned So Far. *Methods and Protocols*, *2*(1), 18. https://doi.org/10.3390/mps2010018

Janda, J. M. (1985). Biochemical and exoenzymatic properties of Aeromonas species. *Diagnostic Microbiology and Infectious Disease*, *3*(3), 223–232. https://doi.org/10.1016/0732-8893(85)90034-3

Jones, B. L., & Wilcox, M. H. (1995). Aeromonas infections and their treatment. *Journal of Antimicrobial Chemotherapy*, *35*(4), 453–461. https://doi.org/10.1093/jac/35.4.453

Kalatzis, P. G., Bastías, R., Kokkari, C., & Katharios, P. (2016). Isolation and characterization of two lytic bacteriophages, φst2 and φgrn1; Phage therapy application for biological control of vibrio alginolyticus in aquaculture live feeds. *PLoS ONE*, *11*(3), 1–18. https://doi.org/10.1371/journal.pone.0151101

Kalatzis, P. G., Castillo, D., Katharios, P., & Middelboe, M. (2018). Bacteriophage interactions with marine pathogenic vibrios: Implications for phage therapy. *Antibiotics*, *7*(1), 1–23. https://doi.org/10.3390/antibiotics7010015

Karunasagar, I., Shivu, M. M., Girisha, S. K., Krohne, G., & Karunasagar, I. (2007). Biocontrol of pathogens in shrimp hatcheries using bacteriophages. *Aquaculture*, *268*(1-4 SPEC. ISS.), 288–292. https://doi.org/10.1016/j.aquaculture.2007.04.049

Khushiramani, R., Girisha, S. K., Karunasagar, I., & Karunasagar, I. (2007). Cloning and expression of an outer membrane protein ompTS of Aeromonas hydrophila and study of immunogenicity in fish. *Protein Expression and Purification*, *51*(2), 303–307. https://doi.org/10.1016/j.pep.2006.07.021

Kim, J. H., Son, J. S., Choi, Y. J., Choresca, C. H., Shin, S. P., Han, J. E., Jun, J. W., Kang, D. H., Oh, C., Heo, S. J., & Park, S. C. (2012). Isolation and characterization of a lytic myoviridae bacteriophage PAS-1 with broad infectivity in Aeromonas salmonicida. *Current Microbiology*, *64*(5), 418–426. https://doi.org/10.1007/s00284-012-0091-x

Kobayashi, M., Msangi, S., Batka, M., Vannuccini, S., Dey, M. M., & Anderson, J. L. (2015). Fish to 2030: The Role and Opportunity for Aquaculture. *Taylor & Francis*, *19*(3), 282–300. https://doi.org/10.1080/13657305.2015.994240

Kutter, E., & Guttman, B. (2004). Bacteriophages : Biology and Applications BACTERIOPHAGES : BIOLOGY AND APPLICATIONS Elizabeth Kutter and Alexander Sulakvelidze. *CRC Press*, *January*, 29–66.

Labh, S. N., & Shakya, S. R. (2014). Application of immunostimulants as an alternative to vaccines for health management in aquaculture. *International Journal of Fisheries and Aquatic Studies*, *2*(1), 153–156.

Lafferty, K. D., Harvell, C. D., Conrad, J. M., Friedman, C. S., Kent, M. L., Kuris, A. M., Powell, E. N., Rondeau, D., & Saksida, S. M. (2015). Infectious Diseases Affect Marine Fisheries and Aquaculture Economics. *Annual Review of Marine Science*, *7*(1), 471–496. https://doi.org/10.1146/annurev-marine-010814-015646

Laland, K. N., & Sterelny, K. (2006). International Journal of Organic Evolution. *Evolution. International Journal of Organic Evolution*, *60*(9), 1751–1762. https://doi.org/10.1554/05-570.1

Lambert, P. A. (2002). Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. *Journal of the Royal Society of Medicine, Supplement*, *95*(41), 22–26.

Lamy, B., Marchandin, H., Lamy, B., Laurent, F., Lamy, B., Laurent, F., Kodjo, A., Lamy, B., Roger, F., Jumas-Bilak, E., Marchandin, H., Laurent, F., Laurent, F., Kodjo, A., & Jumas-Bilak, E. (2012). Which antibiotics and breakpoints should be used for Aeromonas susceptibility testing? Considerations from a comparison of agar dilution and disk diffusion methods using Enterobacteriaceae breakpoints. *European Journal of Clinical Microbiology and Infectious Diseases*, *31*(9), 2369–2377. https://doi.org/10.1007/s10096-012-1578-x

Le, T. S., Nguyen, T. H., Vo, H. P., Doan, V. C., Nguyen, H. L., Tran, M. T., Tran, T. T., Southgate, P. C., & İpek Kurtböke, D. (2018). Protective effects of bacteriophages against Aeromonas hydrophila species causing motile aeromonas Septicemia (MAS) in striped catfish. *Antibiotics*, *7*(1), 1–11. https://doi.org/10.3390/antibiotics7010016

Lee, W. J., Billington, C., Hudson, J. A., & Heinemann, J. A. (2011). Isolation and characterization of phages infecting Bacillus cereus. *Letters in Applied Microbiology*, *52*(5), 456–464. https://doi.org/10.1111/j.1472-765X.2011.03023.x

Lenski, R. E. (1984). Coevolution of bacteria and phage: Are there endless cycles of bacterial defenses and phage counterdefenses? *Journal of Theoretical Biology*, *108*(3), 319–325. https://doi.org/10.1016/S0022-5193(84)80035-1

Li, Z., Li, X., Zhang, J., Wang, X., Wang, L., Cao, Z., & Xu, Y. (2016). Use of phages to control Vibrio splendidus infection in the juvenile sea cucumber Apostichopus japonicus. *Fish and Shellfish Immunology*, *54*, 302–311. https://doi.org/10.1016/j.fsi.2016.04.026

Madhusudana Rao, B., & Lalitha, K. V. (2015). Bacteriophages for aquaculture: Are they beneficial or inimical. *Aquaculture*, *437*(2015), 146–154. https://doi.org/10.1016/j.aquaculture.2014.11.039

Maiti, B., Shetty, M., Shekar, M., Karunasagar, I., & Karunasagar, I. (2011). Recombinant outer membrane protein A (OmpA) of Edwardsiella tarda , a potential vaccine candidate for fish , common carp. *Microbiological Research*, *167*(1), 1–7. https://doi.org/10.1016/j.micres.2011.02.002

Mao, I. L. (2016). *AQUACULTURE* *. http://147.162.137.15/cassandro/Corso_PROD_ANIMALI/AquacLecture 2P.pdf

Marvin, D. A., Symmons, M. F., & Straus, S. K. (2014). Structure and assembly of filamentous bacteriophages. *Progress in Biophysics and Molecular Biology*, *114*(2), 80–122. https://doi.org/10.1016/j.pbiomolbio.2014.02.003

Middelboe, M., Hagström, A., Blackburn, N., Sinn, B., Fischer, U., Borch, N. H., Pinhassi, J.,

Simu, K., & Lorenz, M. G. (2001). Effects of bacteriophages on the population dynamics of four strains of pelagic marine bacteria. *Microbial Ecology*, *42*(3), 395–406. https://doi.org/10.1007/s00248-001-0012-1

Monk, A. B., Rees, C. D., Barrow, P., Hagens, S., & Harper, D. R. (2010). Bacteriophage applications: Where are we now? *Letters in Applied Microbiology*, *51*(4), 363–369. https://doi.org/10.1111/j.1472-765X.2010.02916.x

Moreirinha, C., Osório, N., Pereira, C., Simões, S., Delgadillo, I., & Almeida, A. (2018). Protein expression modifications in phage-resistant mutants of Aeromonas salmonicida after AS-A phage treatment. *Antibiotics*, *7*(1). https://doi.org/10.3390/antibiotics7010021

Muktar, Y., & Tesfaye, S. (2016). Present Status and Future Prospects of Fish Vaccination: A Review. *Journal of Veterinary Science & Technology*, *07*(02). https://doi.org/10.4172/2157-7579.1000299

Munro, J., Oakey, J., Bromage, E., & Owens, L. (2003). Experimental bacteriophage-mediated virulence in strains of Vibrio harveyi. *Diseases of Aquatic Organisms*, *54*(3), 187–194. https://doi.org/10.3354/dao054187

Mzula, A., Wambura, P. N., Mdegela, R. H., & Shirima, G. M. (2019). Current State of Modern Biotechnological-Based Aeromonas hydrophila Vaccines for Aquaculture: A Systematic Review. *BioMed Research International*, *2019*. https://doi.org/10.1155/2019/3768948

Naas, T., Oueslati, S., Bonnin, R. A., Dabos, M. L., Zavala, A., Dortet, L., Retailleau, P., & Iorga, B. I. (2017). Beta-lactamase database (BLDB)–structure and function. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *32*(1), 917–919. https://doi.org/10.1080/14756366.2017.1344235

Nakai, T. (2016). *for Control of Infectious Diseases in Aquaculture*. *July*, 257–272.

Nakai, T., & Park, S. C. (2002). *Bacteriophage therapy of infectious diseases in aquaculture*. *153*, 13–18.

Nale, J. Y., Spencer, J., Hargreaves, K. R., Trzepiński, P., Douce, G. R., & Clokie, M. R. J. (2016). Bacteriophage combinations significantly reduce Clostridium difficile growth in vitro and proliferation in vivo. *Antimicrobial Agents and Chemotherapy*, *60*(2), 968–981. https://doi.org/10.1128/AAC.01774-15

Nascimento, I. P., & Leite, L. C. C. (2012). Recombinant vaccines and the development of new vaccine strategies. *Brazilian Journal of Medical and Biological Research*, *45*(12), 1102–1111. https://doi.org/10.1590/S0100-879X2012007500142

Nayak, S. K. (2020). Current status of Aeromonas hydrophila vaccine development in fish: An Indian perspective. *Fish and Shellfish Immunology*. https://doi.org/10.1016/j.fsi.2020.01.064

Newaj-Fyzul, A., Al-Harbi, A. H., & Austin, B. (2014). Review: Developments in the use of probiotics for disease control in aquaculture. *Aquaculture*, *431*, 1–11. https://doi.org/10.1016/j.aquaculture.2013.08.026

Nikapitiya, C., Dananjaya, S. H. S., Chandrarathna, H. P. S. U., Senevirathne, A., De Zoysa, M., & Lee, J. (2019). Isolation and Characterization of Multidrug Resistance Aeromonas salmonicida subsp. salmonicida and Its Infecting Novel Phage ASP-1 from Goldfish (Carassius auratus). *Indian Journal of Microbiology*, *59*(2), 161–170. https://doi.org/10.1007/s12088-019-00782-5

Nirmal Kumar, G. P., Sundarrajan, S., Paul, V. D., Nandini, S., Saravanan, R. S., Hariharan, S., Sriram, B., & Padmanabhan, S. (2012). Use of prophage free host for achieving homogenous population of bacteriophages: New findings. *Virus Research*, *169*(1), 182–187. https://doi.org/10.1016/j.virusres.2012.07.026

Novriadi, R. (2016). Vibriosis in aquaculture. *Omni-Akuatika*, *12*(1), 1–12. https://doi.org/10.20884/1.oa.2016.12.1.24

Odeyemi, O. A., & Ahmad, A. (2017). Antibiotic resistance profiling and phenotyping of Aeromonas species isolated from aquatic sources. *Saudi Journal of Biological Sciences*, *24*(1), 65–70. https://doi.org/10.1016/j.sjbs.2015.09.016

Okocha, R. C., Olatoye, I. O., & Adedeji, O. B. (2018). Food safety impacts of antimicrobial use and their residues in aquaculture. In *Public Health Reviews* (Vol. 39, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40985-018-0099-2

Oliveira, J., Castilho, F., Cunha, A., & Pereira, M. J. (2012). Bacteriophage therapy as a bacterial control strategy in aquaculture. *Aquaculture International*, *20*(5), 879–910. https://doi.org/10.1007/s10499-012-9515-7

Paul, J. H., & Sullivan, M. B. (2005). Marine phage genomics: What have we learned? *Current Opinion in Biotechnology*, *16*(3 SPEC. ISS.), 299–307. https://doi.org/10.1016/j.copbio.2005.03.007

Pękala-Safińska, A. (2018). Contemporary threats of bacterial infections in freshwater fish. *Journal of Veterinary Research (Poland)*, *62*(3), 261–267. https://doi.org/10.2478/jvetres-2018-0037

Pérez-Sánchez, T., Mora-Sánchez, B., & Balcázar, J. L. (2018). Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. *Trends in Microbiology*, *26*(11), 896–903. https://doi.org/10.1016/j.tim.2018.05.002

Phumkhachorn, P., & Rattanachaikunsopon, P. (2010). Isolation and partial characterization of a bacteriophage infecting the shrimp pathogen Vibrio harveyi. *African Journal of Microbiology Research*, *4*(16), 1794–1800.

Plumb, J. A. (1999). Overview of Warmwater Fish Diseases. *Journal of Applied Aquaculture*, *9*(2), 1–10. https://doi.org/10.1300/J028v09n02_01

Prado, S., Barja, J. L., Luzardo, A., Dubert, J., & Blanco, J. (2020). Encapsulation of live marine bacteria for use in aquaculture facilities and process evaluation using response surface methodology. *Applied Microbiology and Biotechnology*, *104*(5), 1993–2006. https://doi.org/10.1007/s00253-019-10332-0

Pruden, A., Joakim Larsson, D. G., Amézquita, A., Collignon, P., Brandt, K. K., Graham, D. W., Lazorchak, J. M., Suzuki, S., Silley, P., Snape, J. R., Topp, E., Zhang, T., & Zhu, Y. G. (2013). Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environmental Health Perspectives*, *121*(8), 878–885. https://doi.org/10.1289/ehp.1206446

Rajapaksha, P., Elbourne, A., Gangadoo, S., Brown, R., Cozzolino, D., & Chapman, J. (2019). A review of methods for the detection of pathogenic microorganisms. *Analyst*, *144*(2), 396–411. https://doi.org/10.1039/c8an01488d

Ran, C., Qin, C., Xie, M., Zhang, J., Li, J., Xie, Y., Wang, Y., Li, S., Liu, L., Fu, X., Lin, Q., Li, N., Liles, M. R., & Zhou, Z. (2018). Aeromonas veronii and aerolysin are important for the pathogenesis of motile aeromonad septicemia in cyprinid fish. *Environmental Microbiology*, *20*(9), 3442–3456. https://doi.org/10.1111/1462-2920.14390

Richards, G. P. (2014). Bacteriophage remediation of bacterial pathogens in aquaculture: a review of the technology. *Bacteriophage*, *4*(4), e975540. https://doi.org/10.4161/21597081.2014.975540

Rong, R., Lin, H., Wang, J., Khan, M. N., & Li, M. (2014). Reductions of Vibrio parahaemolyticus in oysters after bacteriophage application during depuration. *Aquaculture*, *418–419*, 171–176. https://doi.org/10.1016/j.aquaculture.2013.09.028

Scarano, C., Piras, F., Virdis, S., Ziino, G., Nuvoloni, R., Dalmasso, A., De Santis, E. P. L., & Spanu, C. (2018). Antibiotic resistance of Aeromonas ssp. strains isolated from

Sparus aurata reared in Italian mariculture farms. *International Journal of Food Microbiology*, *284*(January), 91–97. https://doi.org/10.1016/j.ijfoodmicro.2018.07.033
Secombes, C. J., & Belmonte, R. (2016). Chapter 2. Fish Vaccines. In *Fish Vaccines, Birkhäuser Advances in Infectious Diseases*. https://doi.org/10.1007/978-3-0348-0980-1
Silva, Y. J., Costa, L., Pereira, C., Mateus, C., Cunha, Â., Calado, R., Gomes, N. C. M., Pardo, M. A., Hernandez, I., & Almeida, A. (2014). Phage therapy as an approach to prevent Vibrio anguillarum infections in fish larvae production. *PLoS ONE*, *9*(12), 1–23. https://doi.org/10.1371/journal.pone.0114197
Silva, Y. J., Moreirinha, C., Pereira, C., Costa, L., Rocha, R. J. M., Cunha, Â., Gomes, N. C. M., Calado, R., & Almeida, A. (2016). Biological control of Aeromonas salmonicida infection in juvenile Senegalese sole (Solea senegalensis) with Phage AS-A. *Aquaculture*, *450*, 225–233. https://doi.org/10.1016/j.aquaculture.2015.07.025
Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines*, *4*(1), 89–101. https://doi.org/10.1586/14760584.4.1.89
SS, M. (2017). Present Status of Fish Disease Management in Freshwater Aquaculture in India: State-of-the-Art-Review. *Aquaculture & Fisheries*. https://doi.org/10.24966/aaf-5523/100003
Stalin, N., & Srinivasan, P. (2017). Efficacy of potential phage cocktails against Vibrio harveyi and closely related Vibrio species isolated from shrimp aquaculture environment in the south east coast of India. *Veterinary Microbiology*, *207*, 83–96. https://doi.org/10.1016/j.vetmic.2017.06.006
Stenholm, A. R., Dalsgaard, I., & Middelboe, M. (2008). Isolation and characterization of bacteriophages infecting the fish pathogen Flavobacterium psychrophilum. *Applied and Environmental Microbiology*, *74*(13), 4070–4078. https://doi.org/10.1128/AEM.00428-08
Stickney, R. (2001). Aquaculture. In *Kirk-Othmer Encyclopedia of Chemical Technology*. John Wiley & Sons, Inc. https://doi.org/10.1002/0471238961.0117210119200903.a01.pub2
Suttle, C. A., Chan, A. M., & Cottrell, M. T. (1991). Use of ultrafiltration to isolate viruses from seawater which are pathogens of marine phytoplankton. *Applied and Environmental Microbiology*, *57*(3), 721–726. https://doi.org/10.1128/aem.57.3.721-726.1991
Thurlow, C. M., Hossain, M. J., Sun, D., Barger, P., Foshee, L., Beck, B. H., Newton, J. C., Terhune, J. S., Saper, M. A., & Liles, M. R. (2019). The gfc operon is involved in the formation of the O antigen capsule in Aeromonas hydrophila and contributes to virulence in channel catfish. *Aquaculture*, *512*(June), 734334. https://doi.org/10.1016/j.aquaculture.2019.734334
Vadstein, O., Øie, G., Olsen, Y., Salvesen, I., Skjermo, J., & Skjåk-Bræk, G. (1993). A strategy to obtain microbial control during larval development of marine fish. *Fish Farming Technology*, *January*, 69–75.
Vignesh, R., Karthikeyan, B., Periyasamy, N., & Devanathan, K. (2011). Antibiotics in Aquaculture: An Overview. *South Asian Journal of Experimental Biology*, *1*(3), 114-120–120.
Vincent, A. T., Paquet, V. E., Moineau, S., & Charette, S. J. (2019). Would Bacteriophages Be a New Old Complement to Antibiotics in Aquaculture? *Microbial Communities in Aquaculture Ecosystems*, 51–68. https://doi.org/10.1007/978-3-030-16190-3_3
Wang, W., Sun, J., Liu, C., & Xue, Z. (2017). Application of immunostimulants in aquaculture: current knowledge and future perspectives. In *Aquaculture Research* (Vol. 48, Issue 1, pp. 1–23). Blackwell Publishing Ltd. https://doi.org/10.1111/are.13161

Wang, Y., Barton, M., Elliott, L., Li, X., Abraham, S., O'Dea, M., & Munro, J. (2017). Bacteriophage therapy for the control of Vibrio harveyi in greenlip abalone (Haliotis laevigata). *Aquaculture*, *473*, 251–258. https://doi.org/10.1016/j.aquaculture.2017.01.003

Williamson, K. E., Corzo, K. A., Drissi, C. L., Buckingham, J. M., Thompson, C. P., & Helton, R. R. (2013). Estimates of viral abundance in soils are strongly influenced by extraction and enumeration methods. *Biology and Fertility of Soils*, *49*(7), 857–869. https://doi.org/10.1007/s00374-013-0780-z

Yang, Q., Zhao, M., Wang, K. Y., Wang, J., He, Y., Wang, E. L., Liu, T., Chen, D. F., & Lai, W. (2017). Multidrug-Resistant Aeromonas veronii Recovered from Channel Catfish (Ictalurus punctatus) in China: Prevalence and Mechanisms of Fluoroquinolone Resistance. *Microbial Drug Resistance*, *23*(4), 473–479. https://doi.org/10.1089/mdr.2015.0296

Yasuike, M., Kai, W., Nakamura, Y., Fujiwara, A., Kawato, Y., Hassan, E. S., Mahmoud, M. M., Nagai, S., Kobayashi, T., Ototake, M., & Nakai, T. (2014). Complete genome sequence of the Edwardsiella ictaluri-specific bacteriophage PEi21, isolated from river water in Japan. *Genome Announcements*, *2*(2), 137–138. https://doi.org/10.1128/genomeA.00228-14

8.0 Appendices

8.1 Antibiotic susceptibility by Aeromonad isolates

Table 13: Antibiotic susceptibilities of the different Aeromonad isolates for the different antibiotics, using the disk diffusion method, data is expressed as mean±standard deviation

Isolate ID	Nalidixic acid (NA 30 µg)	Chloramphenicol (C 30 µg)	Gentamycin (GN 10 µg)	Streptomycin (S 10 µg)	Tetracycline (TE 30 µg)	Erythromycin (E 25 µg)
1	8.5±0.7	31.7±0.6	21.7±0.6	14.7±0.6	19.0±1.0	28.7±1.5
2	10±0.0	29.7±0.6	21±1.0	18±1	17.7±0.6	28±1
3	17.7±0.6	32.7±1.5	24±2	22.7±0.6	20±1	31.7±1.5
4	7.3±0.6	32.7±0.6	23±1	17.5±0.7	29.7±0.6	30.3±0.6
5	25.3±0.6	26±1	27.3±3.1	26.3±0.6	27.7±0.6	27±2.6
6	12.7±0.6	24.3±2.1	27.3±3.2	21±1	24.7±0.6	24.7±0.6
7	24.7±0.6	24±1	34.3±1.2	28.7±0.6	26±1	27.3±1.5
8	36±1.7	28.7±1.5	25.7±0.6	20.7±0.6	26±1	28.7±0.6
9	10.5±0.6	36.5±0.6	26.5±0.6	20.5±1	20.5±0.6	31±1
10	9.5±0.7	31±1	26±2.6	21±1	22±1	31.7±1.5
11	12±1	36.7±1.5	26.7±1.5	21.7±1.5	22±1	35.3±0.6
12	9.5±0.7	32±1	24.7±1.5	11.7±0.6	11.3±0.6	28.7±0.6
13	8±1	7±1	29.3±1.2	19±1	7.7±0.6	14.3±0.6
14	9.3±0.6	32.7±2.5	21.3±1.5	19±1	14.7±0.6	30.3±1.5
15	36±0	43±0	30±0	21.3±0.6	38±0	31.7±1.5
16	9±0	29.7±0.6	24.7±0.6	20.5±0.7	10.5±0.7	29±1
17	9±1	35±0	24.7±0.6	20.3±0.6	18±1	32.3±1.2

18	13.3±0.6	30.3±5.7	26.7±0.6	20.3±0.6	20.3±0.6	31±1
19	29±1.4	36.5±2.1	26.5±0.7	21.5±0.7	15.3±0.6	35.5±0.7
20	9.3±0.6	33±1.4	18±0	18.7±0.6	18.3±0.6	31.5±0.7
21	38±1.4	31.5±0.7	29±1	24.3±1.2	30±1	29.3±2.3
22	10.5±0.7	33.3±1.5	25.3±1.5	20.7±0.6	20.3±0.6	30.3±0.6
23	12±0	33±0	25.7±0.6	19.3±0.6	21.7±0.6	34±1.4
24	33±1	36±1.4	27±1	20.7±1.2	33.7±1.5	31±1

8.2 Virulence factors of aeromonad isolates

	Isolates																						
Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Haemolysis	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+
Proteolysis	+	+	+	+	+	-	-	+	-	-	-	-	+	+	-	+	-	+	+	+	+	+	+	-
Lipolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+