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An overview of methods used in aquaculture for the prevention and treatment of bacterial infections, especially those caused by Aeromonas sp.

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Abstract:

Aeromonas species frequently infect farmed fish and are to blame for global economic losses. The best way to avoid infectious diseases is immunization, however, there are still very few vaccinations that are commercially available in the aquaculture industry. Currently, the production of aquaculture depends significantly on antibiotics, which adds to the global problem of the rise of bacteria that are resistant to medicines and resistance genes. To decrease the usage of antibiotics in aquaculture systems, it is crucial to create efficient antibiotic substitutes. Bacteriophage (or phage) therapy is a promising method for controlling pathogenic bacteria in farmed fish. Even though phages are quite common, the aquatic environment has usually been considered their most natural habitat. Water encourages Brownian motion in multiple directions and raises the likelihood that phage particles may come into contact with their bacterial hosts, but it calls for a deep understanding of several variables, including the choice of phages, the number of infections necessary to effectively inactivate bacteria, safety concerns, bacterial resistance, host immune response, mode of administration, phage stability, and influence.

Keywords: Aeromonas species, aquaculture, bacterial infections, fish, phage therapy

Introduction:

For more than a century, research on the Gram-negative, oxidase-positive, rod-shaped members of the genus Aeromonas has been slowed by controversy and ambiguity. The late 19th and early 20th centuries saw the identification of Aeromonas species as belonging to at least 15 additional genera. It is challenging to pinpoint the first incidence of the isolation of any Aeromonas species in a laboratory due to the uncertainty surrounding the categorization of *Aeromonas spp*. and the controversy around whether or not Aeromonas should be considered a separate genus[1]. When a then-novel species was recovered from drinking water sources in the city of Chemnitz, Germany, and identified as Bacillus punctatus by Zimmerman, the earliest plausible mention of an Aeromonas bacterium was in an 1890 publication[2]. Ernst and Sanarelli separately reported and identified further early reports of possible Aeromonas species that were connected to sickness in frogs and later other cold-blooded animals. Kluyver and van Niel suggested the genus Aeromonas in 1936 based on the then-known fermentative characteristics of Gram-negative rods with polar flagella[3]. Based on their growth traits and the outcomes of DNA-DNA hybridization tests, aeromonads could be broadly divided into two primary groups within the first few decades after Aeromonas were identified as a species. The nonmotile psychrophilic strains were often linked to fish illnesses and contained isolates of the

species *Aeromonas salmonicida*. The mesophilic group was linked to several human infections and was more similar to *Aeromonas hydrophila*[4]. Liu isolated *Aeromonas caviae* for the first time in a laboratory setting in 1936. He did so from infected guinea pigs and initially gave the strain the name Pseudomonas caviae[5]. These subsequent isolates were finally granted their own nomenclature after being reclassified as a subspecies of Aeromonas punctata by Popoff and Véron in 1976[6]. A total of 14 species of Aeromonas are described in the most recent edition of Bergey's Manual of Systemic Bacteriology, identified mostly through DNA-DNA hybridization and 16S sequence analysis[7]. Recent advancements in genome sequencing, more multi-locus sequence typing (MLST), the use of several housekeeping genes, and other factors have broadened the scope of this classification. As a result, as of 2017, the list of prokaryotic nametags standing in nomenclature and accessible via the Leibniz Institute DSMZ-German recognizes of 36 species and 12 subspecies at this time. In recent years, several clinical laboratories have adopted the potent technique for the identify and contrast logical isolates, use (MALDI-TOF MS). This method is generally used to find proteins linked to the 16S rRNA gene, hence the method's resolution is also impacted by the using this gene's low resolution to identify closely related *Aeromonas species*[8]. To determine whether two isolates of Aeromonas are related epidemiologically, different molecular techniques have been used, such are the RAPD-PCR, ERIC-PCR, and AFLP and the pulsed-field gel electrophoresis (PFGE)[9]. In silico DNA-DNA hybridization (isDDH) described by Kolthoff et al.[10]. Species demarcation is frequently accomplished using the experimental DDH[11]. However, this method wastes time and results in mistakes. The German Collection of Microorganisms and Cell Cultures GmbH, Leibniz Institute DSMZ, Braunschweig, Germany, developed the genome-to-genome distance calculator (GGDC), which has shown to be an outstanding tool for comparing the genetic makeup of two bacterial genomes[12]. Even though the genus Aeromonas is widely dispersed over several habitats, it is most frequently found in different watery environments[13]. Additionally, these microbes have been isolated from a number of environmental and mseveralles[14]. Because of its ability to regrow in the sewage system, Aeromonas was identified through metagenomic analyses of wastewater as one of the predominant bacteria[15]. Fruits, vegetables, dairy products, meats and sausages, seafood, and shellfish have all been shown to contain Aeromonas[16,17,18,19,20,21]. The amount of Aeromonas in these foods is influenced by the temperature, salinity, or pH[22]. In fact, a number of publications claimed several can live in temperatures as low as 2 to 10 ˚C. Sodium chloride (NaCl), sometimes known as salt, is a typical preservative for raw fish and meat items, although Aeromonas can grow in solutions containing up to 4% NaCl. Finally, another factor that regulates bacterial development in food is pH. Aeromonas has been shown to live at pH 5[23]. Numerous research have looked into how often Aeromonas appears in meat products[24]. The most common species were *A. hydrophila, A. caviae, A. veronii*, and, *A. salmonicida* based on the sequencing of the HKG genes like rpoD and gyrB[25]. Non-biting midges, generally referred to as chironomids (Diptera: Chironomidae), are one of the bug species that are most prevalent in aquatic settings. Aeromonas and Vibrio species can be found naturally in chronomids, which act as a reservoir of chironomids according to many research, contaminated water and fruits and vegetables have been found to have Aeromonas, which is the primary cause of diarrhoea[26].

Aeromonas Infections:

Ever since it was originally discovered in frogs and sick fish suffering from septicemia, the genus Aeromonas has been regarded as an animal pathogen[27]. The significant fish pathogens are *A. salmonicida* and *A. hydrophila*, which are especially harmful to salmonids and cause ulcers, haemorrhages, furunculosis, and septicemias[28]. Infections like these cost the aquaculture sector a lot of money. The significant fish pathogens are *A. salmonicida* and *A. hydrophila*, which are especially harmful to salmonids and cause ulcers, haemorrhages, furunculosis, and septicemias[29]. Worldwide outbreaks of *A. hydrophila* in warm-water fishes are thought to be caused by a particularly virulent strain. Other fish species have been isolated in several studies: *A. veronii* isolated from Catfish [30], Salmonid-isolated *A. piscicola* [27], isolated *A. sobria* from tilapia [31], Snakehead fish-specific *A. schubertii*[32], Isolated from common carp include *A. veronii, A. bestiarum, A. encheleia,* and *A. sobria*, and *A. trota, A. caviae, A. veronii, A. hydrophila, A. jandaei, A. media*, and *A. dhakensis* were isolated from eels. [33,34, 35,36,37]. Due to outbreaks in rabbit farms, Aeromonas was isolated and recovered from dog, cat, and horse faeces[38,39,40.] Numerous papers characterized it as a disease that affects crocodiles, echinoderms, and mollusks and is linked to copepods [41,42,43,44]. Aeromonas are an easing pathogen that can infect both immunocompetent and immunocompromised patients and produce a variety of diseases in people, with gastroenteritis, septicemia, and wound infections being the most common. While Staphylococcus aureus predominates in wound infections, Aeromonas co-occurs with Campylobacter and Salmonella in cases of diarrhoea[4]. Children under the age of three are particularly at risk for gastroenteritis caused by *Aeromonas sp.*, which has an incidence ranging from 2.3% to 13% in places like Taiwan and Nigeria. After the oral-fecal route, wounds are Aeromonas' second-most-common entrance point for humans[45]. Any skin or mucosal surface can become infected by Aeromonas, however the extremities are the most prevalent places[46]. The majority of occurrences affect healthy individuals, and they are frequently linked to traumatic experiences, burns, and scolds related to water and soil[47]. In addition, 7–20% of leech therapy treatments resulted in Aeromonas infections. This is because the leeches can digest the blood since Aeromonas is a symbiont of the leeches[48]. Aeromonas-caused wound infections can rapidly worsen into necrotizing fasciitis (NF), usually in patients with impaired immune systems[49]. NF, often known as the "flesh-eating illness," is a potentially fatal infection that can induce hypotension, fever, necrosis, and gangrene[50]. Numerous cases of bacteremia/septicemia caused by Aeromonas have been reported; the most common underlying conditions discovered in these cases were cancer, hepatobiliary disease, and diabetes. Additionally, according to Janda and Abbott, the symptoms most frequently linked to Aeromonas bacteremia were fever (74–89%), jaundice (57%) abdominal discomfort (16–45%), septic shock (40–45%), and dyspnea (12–24%)[51]. There have also been reports of other, less common illnesses linked to Aeromonas, including bacterial spontaneous peritonitis, respiratory tract infections, and urinary tract infections (SBP)[45]. Many underlying illnesses, including spina bifida, bilateral renal dilatation, and diabetes mellitus, were reported in the majority of the cases. Last but not least, 16% of cirrhotic individuals experience SBP[52]. Numerous virulence factors allow Aeromonas to subvert the immune response of the host and result in infections[53]. One of the most important steps in the initial stage of infections by many

pathogens is the attachment of bacteria to host tissues. To begin colonization, bacteria attach to host tissues and cells and change their defense mechanisms. The flagella, pili, capsule, S layer, and lipopolysaccharides of Aeromonas that are involved in colonization are the structural elements that have received the most research (LPS)[54]. Shiga toxins, proteases, lipases, hemolysins, and enterotoxins, among others, are examples of extracellular components and toxins produced into the extracellular space that serve as indications of the contact between pathogenic bacteria like Aeromonas and host cells.[55,56.] There are now six secretion systems known in Gram-negative bacteria, and each of these systems is involved in virulence component distribution into extracellular medium or directly into the host cell. Despite being Sec independent, the (T1SS), (T3SS), (T4SS), and (T6SS) transport their proteins directly to the cell surface or host cell. Sec or Tat proteins are required for the T2SS and (T5SS), which means that the virulence factors released by these processes contain signal peptides that are recognized by these proteins[57]. A strategy for controlling genomic expression in response to cell population density is called quorum sensing (QS)[54]. The implicated cells secrete and generate chemicals known as auto inductors that act as a chemical signal to activate group genetic expression[58]. N-acyl homoserine-lactone (AHL), the "signal" molecule in Gram-negative bacteria, can control the host immunological response in *Aeromonas sp.*[59]. Numerous studies have demonstrated the significance of AHLs in controlling a variety of biological processes, including biofilm development, the generation of antibiotics, and swarming motility. The influences of the various QS systems also vary[60,61]. The biological process known as "metallostasis" in microorganisms requires metal ions to function properly. They are crucial in the host-pathogen relationship as well. During an infection, the host limits the availability of essential metals by deactivating the bacterial pathogen's metal-dependent activities, which make up for this restriction by producing alternative proteins[62]. Aeromonas' nickel-binding protein HypA may aid in both acid tolerance and protection against macrophages[63]. The TLR4 signaling pathway was studied in a previous study using a fish model after infection with *A. hydrophila*, revealing the importance of this receptor in boosting the innate immune response to bacterial invasion. Considering information from earlier investigations: TLR4 expression may be induced by *A. hydrophila* infection in the blunt snout bream *Megalobrama amblycephala* and the *minnow Gobiocypris rarus*.[64,65]. Genes, some of which are found on plasmids, integrons, or the bacterial genome, mediate a genetic-evolutionary response to antimicrobial medicines. Aeromonas are categorized as ampicillin-resistant bacteria, with a few strains and the species A. trota being the outliers.[66]. Moreover, these bacteria are resistant to first-generation cephalosporins. and penicillins[67]. Gene cassettes that are primarily engaged in the spread of antimicrobial resistance genes and have contributed to multidrug resistance can be combined again by the mobile integron encoding integrase[68]. Class 1 integron, which can be discovered in *Aeromonas sp*. from ornamental fish and other sources, is a frequently detected integron[69]. Class 1 integron is typically found in motile Aeromonas species, which carry a range of antimicrobial resistance gene cassettes, in ornamental fish[70]. Aquaculture is one of the most productive agricultural sectors with the quickest growth rates, with increased global output needed to counteract ongoing global declines in the quantity and quality of natural seafood populations. Aquaculture has recently developed quickly, fueling economic growth and helping to ensure food security around the world. Nearly 38% of all fish captured

or raised globally were traded on international markets in 2018, bringing in a total of USD 164 billion[71]. However, bacteria constitute the biggest problem in the aquaculture industry and the main cause of diseases, resulting in significant financial losses that affect the industry's viability and severely damage fish farming facilities[72]. Several tactics and management options, like as vaccinations and antibiotics, are used in aquaculture species to avoid disease. Since vaccination is one of the primary methods used to prevent and manage infections in aquaculture, it is regarded as essential[73]. There are already more than 26 vaccinations authorized for several fish species. Fish are successfully protected by this combination of vaccines against several serious diseases. The majority of vaccines use adjuvants and inactivated microorganisms that are injected or submerged. Live vaccines are more effective because they can be administered orally or by immersion and produce a significant immune response by simulating the infection caused by a natural virus. There have been several attempts at prophylactic immunization for bacterial infections in farmed fish against *Yersinia ruckeri* and *A. salmonicida*[74,75]. To get over these restrictions, aquacultures have turned to antimicrobial drugs to treat bacterial fish diseases or as a prophylactic measure administered in feeds[76]. These have the effect of releasing these compounds into the environment and selecting for resistance. In the polybacterial matrices of ponds, sediments, or biofilms, even at concentrations below the inhibitory activity, the sustained presence of antibiotics can exert an evolutionary advantage on bacterial communities, leading to the transfer of antibacterial genes between microorganisms[77]. There is a higher danger of widespread drug resistance due to the transfer of antimicrobial residues, bacteria resistant to antimicrobials, and resistance genes from aquatic environments to terrestrial cattle and people[78]. Antibiotic-resistant Aeromonas strains have been seen to arise in aquaculture[79]. Finding other means of infection control is therefore necessary.

Disease control and alternative approach:

Infection control and prevention can be challenging due to the fish pathogenic bacteria' rapid spread and ubiquity[80]. The following factors make disease prevention and management in aquaculture more difficult: (1) Many chemotherapeutic medicines are inefficient against endospores and zoospores, there is substantial fecal contamination in fish farm waters, and few pharmaceuticals are approved for use in fisheries, all of which contribute to treatment failure in the event of infection[81]. (2) Unusual environmental factors that may contribute to illness outbreaks include high temperatures, salinity changes, low oxygen concentrations, and a high organic load. These factors are frequently attenuated by the sensitive fish's intrinsic defense mechanism[82]. (3) Fish farming systems frequently use high fish densities (beyond what is recommended for each species), which lowers infection resistance[83]. (4) The frequency of infections rises due to different periods in the fish life cycle that affect the immune system's development[84]. (5) The concern over antibiotic distribution in the environment and the indiscriminate and preventative use of antibiotics worsens the problem of resistance in common pathogenic bacteria[85]. Despite mounting worries about the emergence of antimicrobial resistance in bacteria, antibiotic use remains the primary method of disease control in aquaculture[86]. In several nations, like China, Vietnam, and India, antibiotics have been and still are inappropriately used to treat and prevent disease as well as to stimulate growth[87,88,89]. As per Lulijwa et al. (2020), 55% of the major aquaculture-producing nations used amoxicillin, erythromycin, enrofloxacin, and sulfadimethoxine, while 73% used florfenicol, sulphadiazine,

and oxytetracycline[85]. Antibiotic use is governed by stringent laws in the United States, the European Union, and Japan, which also places restrictions on the lowest dosages permitted for aquaculture[76]. Only florfenicol, oxytetracycline, and sulfadimethoxine/ormetoprim are permitted by the U.S. Food and Drug Administration (FDA) for use in the aquaculture sector[76,90]. Only amoxicillin, cotrimazine, oxolinic acid, oxytetracycline, and sarafloxacin are permitted in the United Kingdom[90]. The European Union outlawed the use of antibiotics as livestock animal development enhancers in 2006[91]. Due to vaccination and improved husbandry procedures, particularly in Norway, certain countries have drastically reduced their use of antibiotics in recent years[92]. Contrarily, China and Vietnam are the two countries that consume the most antibiotics, which may account for their widespread prophylactic usage[85]. 13 antibiotics (doxycycline, enrofloxacin, florfenicol, flumequine, neomycin, norfloxacin, oxolinic acid, sulfamethazine, sulfamonomethoxine, thiamphenicol, and trimethoprim) are presently permitted by the Chinese government[93]. Thirty antibiotics were permitted in Vietnam up until 2014. However, despite their 2016 bans on both ciprofloxacin and fluoroquinolones, later research has continued to find their use. Other Asian nations, such as India, Thailand, South Korea, and Bangladesh, must use fewer antibiotics to comply with the stringent regulations of their trading partners, namely the United States, the European Union, and Japan, even though Vietnam and China have relatively large domestic markets[94]. Aeromonas resistance strains have emerged as a result of the frequent and widespread use of antibiotic prophylaxis in aquaculture systems, rendering any antibiotic treatment useless in several fish including catfish, koi carp, and tilapia[95]. Aeromonas strains from trout, tilapia, and koi from South African aquaculture systems had significant rates of resistance to tetracycline (78.3%), amoxicillin (89.2%), and augmentin (86.5%), according to Jacobs and Chenia's 2007 research.

Comparing isolates of *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas veronii*, *Aeromonas media*, and *Aeromonas ichtiosoma*, it was shown that the latter two had higher levels of resistance to various antimicrobial drugs[96].

This is probably due to the extensive usage of antibiotics like trimethoprim, quinolones, and oxytetracycline to treat furunculosis in diseased salmonids throughout time[98]. There have been more instances of quinolone resistance in fish-associated aeromonads over the past 20 years[98,99,100,101]. *Aeromonas sp.* diseases can be prevented with vaccination, although there are still relatively few vaccines approved for use in aquaculture. Aquaculture has effectively utilized vaccines to reduce the usage of antibiotics, especially during the production of salmon[87]. Inactivated vaccines are presently very commonly employed in aquacultures because they offer improved biosafety and therefore are simpler to obtain a license for[102]. Vaccines that are live or attenuated have demonstrated significant promise since they can immunize fish with a single dose and have cheap manufacturing costs. Few live bacteria are, meanwhile, approved for commercial usage since using them poses a risk to the natural environment[74,102,103]. For use in different fish species, there are presently over 26 registered and commercially available fish vaccines that have effectively immunized fish against a number of fish illnesses. The majority of approved vaccines include adjuvants that can be injected or immersed to administer attenuated microorganisms[74,102,104-105,]. Sadly, vaccinations are yet to be produced for several types of harmful bacteria and farmed fish[87]. The use of vaccinations against *A. salmonicida* in cutthroat trout (*Oncorhynchus clarki*), had first been documented by Duff in 1942. In these

tests, inactive A. salmonicida was fed to the fish[106]. The very first salmonid vaccines that have been administered by immersion were developed using the same bacterial inactivation method as the Atlantic salmon (Salmo salar)[107]. Furthermore, according to Bricknell et al., the injection-based bacterial vaccines were ineffective against *A. salmonicida* in Atlantic salmon. The extracellular polysaccharide vaccines in this experiment produced an antibody response and remained effective for almost two months after injection[108]. Numerous vaccines against common *A. salmonicida* strains have been created in recent times to offer long-lasting protection in commercial salmonid farming[86,109]. The first aquatic bacterial vaccination for this species, a killed whole-cell vaccine for A. hydrophila (J-1 strain), received the national class I novel veterinary medication certificate from China in 2011[110]. An oil-based, non-mineral vaccine called AQUAVAC® FNM comprises two strains of A. salmonicida, the organism that causes furunculosis in Atlantic salmon[111]. Vietnam licenced the injectable vaccine Alpha Ject Panga 2 in 2017 to provide protection against *Aeromonas hydrophila* and *Edwardsiella ictaluri*[112]. Similar to inactivated vaccines, recombinant protein vaccines, and bacterial lysates, DNA vaccines based on yeast or carbon nanotubes have been shown to promote protection against A. hydrophila[103,113-114]. Due to the biochemical and serological heterogeneity of *A. hydrophila* in fish, commercial vaccine development has proven difficult[115]. Although being an efficient method of preventing Aeromonas infections, immunization has a number of adverse consequences, including scarring, inflammation, pigment accumulation, decreased development, and fibrous adhesions in internal organs[116-117]. Immune systems must be established and functioning in order to respond to vaccinations, which are unlikely to be effective in larval or fry stages. Additionally, they may not always provide complete protection and can be challenging to inject[118,104]. Due to phytotherapy becoming acknowledged as a practical and feasible alternative to chemotherapy since it is affordable, effective, non-resistance forming, renewable, eco-friendly, and farmer-friendly, phytochemicals are another option to antibiotics. Together with the herbs employed as biopesticides and immunostimulants, a study on medicinal plants with antibacterial, antiviral, and antifungal activity is compiled. Since ancient times, medicinal plants have been used extensively as phyto-therapeutic agents to treat infectious diseases in both humans and animals. It is also safe to use these plants therapeutically to cure bacterial disorders in fish. The improvement in fish growth and resistance to MAS serve as indicators of the therapeutic effects of medicinal herbs, which can prevent illnesses and limit the emergence of *A. hydrophila* strains[119]. Additionally, multiple studies have demonstrated the strong antibacterial potential of herbs as an alternate form of biomedicine in aquaculture[120]. This table highlights the effectiveness of phytotherapy in Malaysia against microorganisms that are pathogenic to aquaculture (Table 1) [127-135].

Table 1. Malaysian phytotherapeutic research on harmful microorganisms in aquaculture.

Isolates sources/host	**Plants**	**Isolates Microorganisms**	**Method**	**Plant preparation**	**Findings**	**Reference**
Red tilapia (Oreochromis *sp*.)	The curry tree (*Murraya koenigii)*	*Aeromonas hydrophilla*	*In vitro* (disk diffusion technique)	Aqueous and methanolic extracts	A potent inhibitor of *A. hydrophil*a growth	[121]

African catfish *Clarias gariepinus* (Bloch) fingerlings	Garlic peel (*Allium sativum*)	*Aeromonas hydrophilla*	*In vivo* (Diet and challenge)	Plant powder was incorporated into fish feed	Significantly higher survival rates were recorded in all the fish fed with garlic peel feed	122
Culture stock	Eight seaweeds species (*Dictyota dichotoma, Padina minor, Halimeda macroloba, Caulerpa racemose, Caulerpa macrophysa, Ulva intestinalis, Amphiroa fragilissima, Sargassum duplicatum* and three seagrass species *Thalassia hemprichii, Cymodocea rotundata, Enhalus acoroides*).	*Aeromonas hydrophila* (ATCC35654)	*In vitro* (disk diffusion method, MIC and MBC)	Methanolic extract	Most of the seaweeds and seagrass possesses antibacterial activity against *Aeromonas hydrophila*	123
Diseased red hybrid tilapia (Oreochromis *sp*.)	Pepper elder or Shining bush plant or Man to man	*Aeromonas hydrophila*	*In vivo* (Diet and challenge)	Methanolic extracts coated onto fish pellet	P. pellucida leaf extract may be used to manage	124

	(*Peperomia pellucida*)				motile septicemia.	
Culture stock (Common freshwater pathogen)	(*Vitex trifolia*, *Aloevera*, *Strobilanthes crispus*, *Clinacanthus nutans*, *Pereskia grandifolia* and *Peperomia pellucida*)	*Aeromonas hydrophila*	*In vitro* (disk diffusion technique)	Aqueous and methanolic extracts	Strong antibacterial activity in *V. trifolia*, *A. vera* and *S. crispus* extracts	125
Culture stock	Piper betle	*Aeromonas hydrophila*	*In vitro* (TLC agar overlay bioautography assay)	Methanolic extracts	successful bacterial eradication	126
Culture stock obtained from Universiti Malaysia Terengganu	King's salad (*Cosmos caudatus*)	*Aeromonas hydrophila* (KF146350)	*In vitro* (disk diffusion assay and brine shrimp lethality bioassay)	Methanolic extract	The bacterial growth is inhibited by every concentration that was tested. Less than 100 g mL-1 of *C. caudatus* extract is required for it to be effective and safe.	127
Culture stock obtained from the Laboratory of Fish Health in	Clove and peel (*Allium sativum*)	*Aeromonas hydrophila*	*In vitro* (disk diffusion method, MIC and MBC)	Aqueous and methanolic extracts	According to the research, A. sativum clove extract may one day be	128

the Aquaculture Department, University Putra Malaysia					employed as a phytobiotic to inhibit the growth of marine diseases.	
Bacterial Collection of the Aquatic Animal Health Unit, University Putra Malaysia	(*Polygonum chinense*, *Syzygium polyanthum*, *Premna foetida*, *Pimenta dioica*, *Brucea javanica*, *Vitex negundo*, *Alpinia conchigera* and *Clinacanthus nutans*)	*Aeromonas hydrophila* (EU696781.1)	*In vitro* (disk diffusion method and brine shrimp cytotoxicity assays)	Methanolic and aqueous extracts	Fish culture may benefit from the high bactericidal activity and low toxicity of extracts of *P. chinense* and *P. foetida.*	[129]

MIC=Minimal Inhibitory Concentration and MBC=Minimal Bactericidal Concentration values.

Since more effective methods are required to control Aeromonas diseases, the use of virulent phages to prevent and/or treat infections thus appears to be a potential technique[130,131,132,133,134,135].

The Use of Phages in Therapies:

Pathogenic bacteria are rendered inactive with the use of phages, viruses that only infect prokaryotes (bacteria and archaea). They lack a host-independent metabolism, are unable to make proteins, and cannot replicate themselves as a result[136-137]. Frederick W. Twort in England in 1915 and Felix d'Herelle in Paris in 1917 each independently discovered phages[138]. However, because of the effectiveness of antibiotics, Western European nations eventually abandoned their phage usage plans[139]. It wasn't until the latest generations that interest in it was reignited as a result of developing antibiotic resistance concerns[139,140]. The International scientific community has now been motivated to rethink phage therapy for the treatment of bacterial infections due to the rise of pathogenic bacteria that are resistant to antibiotics. To do this, further research is required on a number of phage ecology-related topics, including abundance, viral decay rates, repair processes, lysogeny, and impact on bacterial

populations[141,142]. Research and development of new phage-based technologies for pathogen control have increased since the regulatory approval of ListShieldTM produced by Intralytix Inc (Baltimore, MD, USA), the first phage-based product (a mixture of six different virulent phages approved to control Listeria in meat and poultry products)[143,144]. Currently, phage therapy's potential applications include:

medicine[145-146], aquaculture[71,87,130,147-148], foodsafety[139,149-150], agriculture[149,151-152], veterinary[153-154], and wastewater treatments[138,155] has begun to be investigated globally. Phages were characterized as antibacterial agents for both people and animals as soon as they were identified by Twort and d'Herelle in the early 1920s[156]. Investigations employing phages to control harmful bacteria in aquaculture were also started more than fifty years after the original studies. Following the 1981 publication of the first report on the use of phages to control *A. hydrophila* in aquaculture[157]. The major bacterial pathogens found in aquacultures, including *Aeromonas spp.*, *Edwardsiella spp*., *Flavobacterium spp.*, *Pseudomonas spp.*, and *Vibrio spp.*, are infected by a number of virulent phages that have been isolated and described for possible therapeutic use[158-159]. The current research examined the isolation and characterization of lytic phages, cocktails, and possible uses of phages as a therapeutic agent to treat illnesses in aquaculture, as well as the dosage and delivery of these agents[135,160-161]. *Edwardsiella tarda*, a bacterium that causes edwardsiellosis in the loach *Misgurnus anguillicaudatus*, has been successfully treated by administering phages as a preventative measure[162]. Yellowtail *Seriola quinqueradiata* suffers from lactococcosis due to *Lactococcus garvieae*[163]. *Plecoglossus altivelis* suffered pseudomonosis due to *Pseudomonas plecoglossicida*[164,165]. *O. mykiss* suffers from flavobacteriosis due to *Flavobacterium psychrophilum* and *Flavobacterium column*[161,166-167]. Sea cucumber *Apostichopus japonicus* infections with *Vibrio parahaemolyticus* and *Vibrio splendidus* are also prevalent[168,169]. Phage therapy in aquaculture has previously been covered by other successful investigations[71,87,170,171].

Few companies have made investments in phage-based strategies to manage and/or prevent bacterial infections in aquaculture over the past few years, as seen by the paucity of products created thus far (Table 2) [171-178]

Table 2. Companies are involved in developing phage-based products to control or prevent bacterial infections in aquaculture:

Company	Country	Target Application	References
Intralytix Inc.	USA (Baltimore, MD)	Application of phages to treat *Vibrio coralliilyticus* and *Vibrio tubiashii* infections in oysters reared in hatcheries.	[172]
BASF New Business GmbH	Germany (Ludwigshafen)	Solutions for the treatment of diseases brought on by Vibrio, Yersinia, Aeromonas, Rickettsia, Moritella, Lactococcus, Piscirickettsia, Flavobacterium, Pseudomonas, or Photobacterium blend phages bonded to particles into the diet.	[173]

Proteon Pharmaceuticals S.A.	Poland (Łódz)	Commercial aquaculture can employ a natural feed additive called BAFADOR® to prevent bacterial illnesses brought on by *Pseudomonas spp.* and *Aeromonas spp.* serotypes.	[174]
Fixed Phage Ltd.	Scotland (Glasgow)	To treat bacterial infections in aquacultures, such as Early Mortality Syndrome in shrimp and Flavobacteria infections in salmonids, phage particles immobilized in pellets that can be introduced to fish and crustacean feed are available.	[173]
ACD Pharma	Norway (Oslo)	Several aquaculture infections are combated by phage-based treatments; CUSTUS® YRS is a product that decreases the infective potential of *Yersinia ruckeri* in aquaculture water.	[175]
Mangalore Biotech Laboratory	India (Karnataka)	Luminous vibriosis caused by *Vibrio harveyi* can be prevented and treated using a product called LUMI-NIL MBL.	[176]
Phage Biotech Ltd.	Israel (Rehovot)	Phage treatment for *Vibrio harveyi* in aquaculture shrimps.	[171]
Biologix	Australia	Phage treatment for *Vibrio sp.* associated with mortalities in aquaculture.	[177]
Aquatic Biologicals	Greece	Treatment with phages for a number of diseases linked to aquaculture fatalities	[178]

Application of Aeromonas sp. Phages in Aquaculture (Especially *Aeromonas hydrophila* Phages):

According to phage application studies, aquatic Aeromonas species are the third most targeted bacterial pathogen[87]. In aquaculture, *A. hydrophila* was the first target for phage therapy in 1981. Eight *A. hydrophila* phages were isolated by these scientists, and AH1 was chosen to explore the biological control of disease in the loach *M. anguillicaudatus*[157]. Since then, numerous researches have examined and demonstrated the positive outcomes that various Aeromonas phages offer as substitute biocontrol agents and their therapeutic (or prophylactic) potential in aquaculture. However, research using phages to prevent or eradicate Aeromonas spp. in aquaculture has only so far focused on two harmful bacterial species, *A. salmonicida* and *A. hydrophila*. Numerous researches have examined phenotypic and genotypic characteristics and assessed the efficiency of phages against *A. hydrophila*, including phages pAh1-C and pAh6-C[179]; Ahp1[180]; pAh-1[181]; Φ2[160]; AP1, AP2, AP3 and AP4[158]; CT45P and TG25P[182]; MJG[131]; AHP-1[183]; Akh-2[134]; PVN-02[133,184]; AhyVDH1[185] and pAh6.2T[132]. Phages can be utilised to biocontrol *A. hydrophila* infections in loach, according to earlier research *M. anguillicaudatus*[186,157,179]; Nile tilapia (*O. niloticus*)[158,187]; striped catfish (*P. hypophthalmus*) [160,133] and rainbow trout (*O. mykiss*)[131,132]. In 1981, phages were used for the first time to

control *A. hydrophila* in loaches[157]. After more than 30 years, Jun et al. (2013) demonstrated that phages pAh1-C or pAh6-C simple suspensions enhanced life expectancies against *A. hydrophila* disease. Phage pAh6-C, meanwhile, managed *A. hydrophila* infection more successfully than pAh1-C[179]. Recent research by Akmal and colleagues demonstrated the protective benefits of phage Akh-2 treatment on *M. anguillicaudatus* with *A. hydrophila* infection and enhanced survival rates[134]. El-Araby et al. (2016) applied a mixture of two phages by immersion to control *A. hydrophila* in Nile tilapia (*O. niloticus*) and decreased the mortality rate from 68% to 18% following a 15-day treatment, further demonstrating the protective impact of Aeromonas phages. Another work by Hassan et al. (2018) shown the phage AP2's potential to treat Nile tilapia with motile Aeromonas septicemia brought on by A. hydrophila[158,187]. Phages have just lately been researched for their ability to prevent and treat bacterial infections in catfish, according to Le et al. (2018). (*P. hypophthalmus*). In particular, the capacity of phages Φ2 and Φ5 to inactivate and regulate *A. hydrophila* in striped catfish through injection and the observed cumulative death of fish decreases with the increase in MOI (cumulative mortality of 0%, 45%, and 68% with a MOI of 100, 1 and 0.01)[160]. Similar findings were made by Dang and colleagues, who noted that fish mortality is influenced by the dose of phage utilised during therapy[133]. The difference, nevertheless, is not as significant as that seen in Le et al (2018)[160]. When fed with feed that had been sprayed with the phage PVN02, Dang et al. (2021) showed that the relative survival rate of catfish with *A. hydrophila* was (75.6-87.8%)[133]. In order to suppress *A. hydrophila* in rainbow trout, Cao et al. (2020) used phage MJG by injection, immersion, and oral administrations, achieving relative percent survival of 100%, 66.7%, and 50%, respectively[131]. Furthermore, Dien et al. (2021) demonstrated that phage pAh6.2TG treatments dramatically increased Nile tilapia survival after exposure to lethal concentrations of *A. hydrophila* (10^7 CFU/mL), with relative percent survival of 73.3% and 50% at a MOI of 1.0 and 0.1, respectively[132]. Numerous studies have demonstrated the ability of various phages, including phage cocktails, to biologically regulate *A. salmonicida* strains in both in vitro and in vivo tests[130,135,188-189]. The use of phages to treat (or prevent) *A. salmonicida* infections in Senegalese sole (Solea senegalensis), rainbow trout (O. mykiss), brook trout (Salvelinus fontinalis), and Atlantic salmon (S. salar), are some few examples[130, 189-190]. Despite the fact that Atlantic salmon treated with a combination of phages O, R, and B had no protective effects against *A. salmonicida*, Verner-Jeffreys et al. (2007) did identify any such effects[189]. Fish death rates decreased from 100% to 10% after 45 days, according to Imbeault et al. (2006), who demonstrated that phage HER110 postponed the development of furunculosis by 7 days[190]. With higher survival rates (0-26%) and mean times to death in rainbow trout infected with *A. salmonicida subsp. salmonicida* (2.5×10^2 CFU/fish) and treated with phage PAS-1 (2.4×10^6 PFU/fish), Kim and colleagues in 2015 demonstrated considerable therapeutic results[191]. Similar findings were confirmed with Senegalese sole (S. senegalensis) treated with phage AS-A, which displayed considerably lower mortality (36% to 0%)[130]. Phages have demonstrated the ability to control *A. hydrophila* and *A. salmonicida* from the alternatives currently available, even with certain restrictions.

Conclusions and Future Perspectives:

As an increasing number of strains become resistant to antibiotics, antibiotic potency is continuously declining. As a result, alternative therapies like phage therapy ought to be

investigated. The majority of the research examined in this paper demonstrated the role that phages play in the management of Aeromonas species on fish, offering a favourable outlook on the potential uses of this technology in the future to cure aquaculture diseases. However, only two species of Aeromonas—*A. salmonicida* and *A. hydrophila*—are the subject of the currently conducted studies. Therefore, further research is required to improve phage application in the field and to comprehend how host fish, bacteria, and phage interact with one another. Phage selection, MOI, environmental parameters that affect lytic phage viability (e.g., temperature, salinity, pH, UV radiation), delivery methods, and bacterial resistance to phages are only a few of the variables that affect phages' capacity to control Aeromonas species in aquaculture systems. Additionally, neither in vivo data for one phage nor in vitro data for another phage can be extrapolated to apply to in vivo experiments. Phages must go through efficacy testing to prove their efficiency and safety before this method is used commercially. It is necessary to standardize and take into account a number of variables, including cost-effectiveness, delivery method, the MOI that effectively inactivates bacteria, and the stability of phage preparations. To facilitate its incorporation as a novel antimicrobial processing technique in aquaculture, it is also required to examine the potential impact on the natural bacterial population and fish health, depending on the type of bacteria and various environmental conditions. Phage therapy is economical, environmentally beneficial, and safe for end users including people and animals as well as aquaculture species. Even while certain bacteria have become resistant to phages, the negative impacts are insignificant in comparison to the rise of antibiotic resistance. Bacteria are ten times more likely to become resistant to phages than antibiotics, and bacteria that have developed resistance to one phage can still be infected by other phages with related target ranges.

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