BACTERIOPHAGE AND OZONE-NANOBUBBLE BASED APPROACHES FOR COMBATING MULTIDRUG-RESISTANT *AEROMONAS HYDROPHILA* IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*) CULTURE SYSTEM

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การใช้แบคเทอริโอเฟจและฟองนาโนโอโซนสำหรับต่อต้านเชื้อแอโรโมแนสไฮโดรฟิลลา
ที่ดื้อยาปฏิชีวินะหลายชนิดในการเลี้ยงปลานิล

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ทานฮ์ ดีน เล : การใช้แบคเทอริโอเฟจและฟองนาโนโอโซนสำหรับต่อต้านเชื้อแอโรโมแนสไฮโดรฟิลลาที่ดื้อยาปฏิชีวินะหลายชนิดในการเลี้ยงปลานิล. (BACTERIOPHAGE AND OZONE-NANOBUBBLE BASED APPROACHES FOR COMBATING MULTIDRUG-RESISTANT *AEROMONAS HYDROPHILA* IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*) CULTURE SYSTEM) อ.ที่ปรึกษาหลัก : ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาร่วม : ฮา ทานห์ ดง

การติดเชื้อแอโรโมแนส ไฮโดรฟิลลา (*Aeromonas hydrophila*) ถือได้ว่าเป็นการติดเชื้อที่สำคัญที่ส่งผลกระทบทางเศรษฐกิจต่ออุตสาหกรรมการเลี้ยงปลาน้ำจืดทั่วโลกหลายพันล้านดอลล่า การดื้อยาปฏิชีวนะหลายชนิด (MDR) ของเชื้อแอโรโมแนส ไฮโดรฟิลลา กำลังเป็นเรื่องที่กังวลกันทั่วโลก ทางเลือกอื่นที่ไม่ใช่การใช้ยาปฏิชีวนะสามารถลดความต้องการในการใช้ยาปฏิชีวนะในการควบคุมและรักษาโรคติดเชื้อและเชื้อดื้อยาลงได้ ในการศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบความสามารถในการใช้แบคเทอริโอเฟจประเภทไลติค ร่วมกับการใช้ฟองนาโนโอโซน ในระบบ Modified Recirculation System (MRS-NB-O_3) เพื่อทำลายเชื้อแอโรโมแนส ไฮโดรฟลล่า ที่ดื้อยาปฏิชีวนะหลายชนิดในการเลี้ยงปลานิล ผลการทดลองพบแบคเทอริโอเฟจชนิดใหม่ ได้แก่ *Myoviridae* phage pAh6.2TG แบคเทอริโอเฟจนี้ถูกแยก พิสูจน์ และตรวจสอบคุณสมบัติต่างๆ จากการทดลองใช้แบคเทอริโอเฟจนี้ในการต่อต้านการติดเชื้อ MDR แอโรโมแนส ไฮโดรฟิลลาแก่ปลานิลทดลองที่ถูกเลี้ยงกับเชื้อนี้พบว่าแบคเทอริโอเฟจสามารถลดปริมาณของเชื้อแบคทีเรียในน้ำได้ และปลามีอัตราการรอดชีวิต (RPS) เพิ่มขึ้นเมื่อเทียบกับกลุ่มควบคุมที่ไม่ได้เลี้ยงโดยให้แบคเทอริโอเฟจ โดยพบว่ามีค่า RPS เท่ากับ 50-73.3 % นอกจากนี้เมื่อนำเทคโนโลยีฟองนาโนโอโซนในระบบ Modified Recirculation System (MRS-NB-O_3) มาทดสอบพบว่าไม่เพียงแต่มีความปลอดภัยสูงต่อปลานิลวัยอ่อน (juvenile Nile tilapia) แต่ยังสามารถกระตุ้นภูมิคุ้มกันแบบ innate ของปลานิล และทำให้อัตราการรอดชีวิต (RPS) ของปลานิลทดลองที่ถูกเลี้ยงกับเชื้อ MDR แอโรโมแนส ไฮโดรฟิลลาสูงขึ้นเมื่อเทียบกับกลุ่มควบคุมที่ไม่ได้เลี้ยงโดยใช้ MRS-NB-O_3 โดยพบว่ามีค่า RPS เท่ากับ 64.7- 66.7 % พบว่าปลาที่รอดชีวิตจากการทดลองนี้สามารถสร้างภูมิคุ้มกันแบบจำเพาะชนิด IgM ต่อเชื้อ MDR แอโรโมแนส ไฮโดรฟิลลาได้อีกด้วย การค้นพบนี้บอกได้ว่าแบคเทอริโอเฟจ pAh6.2TG และฟองนาโนโอโซน เป็นวิธีการที่มีประสิทธิภาพในการควบคุมป้องกันโรคที่เกิดจากการติดเชื้อ MDR แอโรโมแนส ไฮโดรฟิลลา โดยไม่ใช้ยาปฏิชีวินะที่ดีสำหรับอุตสาหกรรมการเลี้ยงสัตว์น้ำจืด นอกจากนี้จากการศึกษาผลของเทคโนโลยีฟองนาโนต่อแบคเทอริโอฟาจพบว่าฟองนาโนโอโซนสามารถฆ่าแบคเทอริโอเฟจในน้ำได้ 99.99 และ 100% ภายหลังจากการให้ฟองนาโนโอโซนในน้ำ 5 และ 10 นาที ตามลำดับ ในขณะที่ฟองนาโนออกซิเจนธรรมดา (oxygen nanobubbles ; NB-O_2) ไม่ให้ผลนี้ นอกจากนี้ยังพบว่าการให้ฟองนาโนออกซิเจนธรรมดาสามารถเพิ่มการเกาะติดของแบคเทอริโอเฟจ pAh6.2TG กับพื้นที่ผิวของปลาได้และหลังจากนั้นแบคเทอริโอเฟจจะมีการกระจายไปที่ตับของปลา จากผลการทดลองนี้แสดงให้เห็นว่าการใช้แบคเทอริโอเฟจร่วมกับฟองนาโนออกซิเจน (NB-O_2) เป็นวิธีการที่ดีในการควบคุมการติดเชื้อแบคทีเรียในการเลี้ยงสัตว์น้ำ ในขณะที่การใช้ฟองนาโนโอโซน (NB-O_3) น่าจะเป็นวิธีการที่ดีในการการฆ่าเชื้อไวรัสในน้ำที่ใช้เลี้ยงสัตว์น้ำ จากผลการวิจัยทั้งหมดสรุปได้ว่าการใช้แบคเทอริโอเฟจและเทคโนโลยีฟองนาโน ถือเป็นวิธีการที่มีประสิทธิภาพในการควบคุมป้องกันการติดเชื้อ MDR แอโรโมแนส ไฮโดรฟิลลาในปลานิลและอาจรวมถึงปลาน้ำจืดชนิดอื่นๆ ได้

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6175602031 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY
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Thanh Dien Le : BACTERIOPHAGE AND OZONE-NANOBUBBLE BASED APPROACHES FOR COMBATING MULTIDRUG-RESISTANT *AEROMONAS HYDROPHILA* IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*) CULTURE SYSTEM. Advisor: Assoc. Prof. CHANNARONG RODKHUM Co-advisor: Asst. Prof. Ha Thanh Dong

Aeromonas hydrophila infection is considered one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry. Multidrug-resistant (MDR) *A. hydrophila* is becoming a global issue of concern. Non-antibiotic approaches can minimize the requirement for antimicrobials to combat infectious diseases and antimicrobial resistance. This study aims to investigate the potential application of lytic bacteriophage, ozone nanobubbles (NB-O_3), a potential combination of bacteriophage and nanobubble-based technology in combatting MDR *A. hydrophila* in Nile tilapia. A newly *Myoviridae* phage pAh6.2TG was isolated, identified, and characterized in this study. The application of this phage as a prophylactic agent significantly suppressed bacterial concentration in water and improved survivability of Nile tilapia challenged with the pathogenic MDR *A. hydrophila* with RPS of 50 73.3%. In addition, treatments of NB-O_3 in a modified recirculation system were not only safe for juvenile Nile tilapia, but also modulated fish innate immunity and significantly improved survivability of Nile tilapia challenged with MDR *A. hydrophila* with RPS of 64.7 - 66.7%. The surviving fish from these treatments developed specific antibody IgM against the challenged bacteria. Taken together, this study reveals that phage pAh6.2TG and NB-O_3 are promising non-antibiotic approaches to control diseases caused by MDR *A. hydrophila* in the freshwater fish aquaculture industry. Subsequently, an investigation of the impact of nanobubble technology on bacteriophage revealed that NB-O_3 killed 99.99 and 100% bacteriophage in water after 5- and 10-min treatment, respectively, while oxygen nanobubbles (NB-O_2) did not. The NB-O_2 treatment improved the adherence of phage pAh6.2TG on fish body surface and the uptake of phages into the fish liver. Thus, a combination of NB-O_2 treatment and phage therapy is feasible to control bacterial infections in aquaculture, while NB-O_3 might be a promising viral disinfection method for aquaculture water. In summary, this study demonstrates that both bacteriophage and nanobubble technology are promising strategies for combatting MDR *A. hydrophila* in tilapia and probably other freshwater fish species.

Field of Study:	Veterinary Science and technology	Student's Signature ....
Academic Year:	2020	Advisor's Signature ....
		Co-advisor's Signature ....

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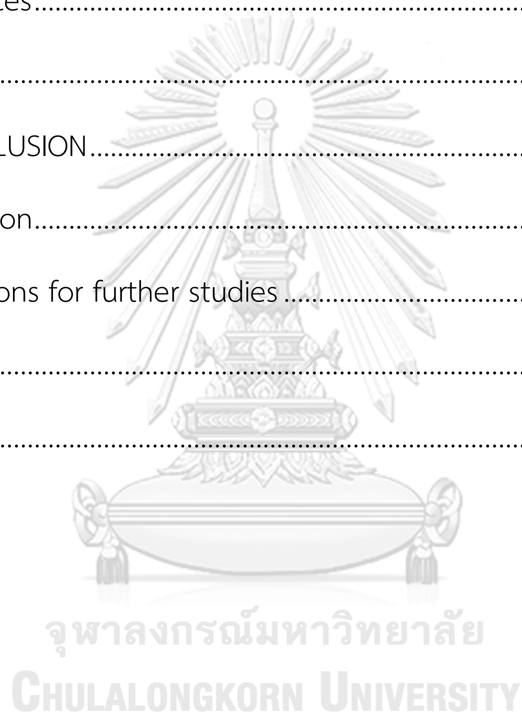

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CHAPTER 1

INTRODUCTION

1.1 Importance and rationale

In 2018, the global population was 7.6 billion people, with projections of 8.5 billion by 2030, 9.7 billion by 2050, and more than 11 billion by 2100 (DESA, 2019). Whereby, the intake of protein is predicted to increase rapidly in the next decade to supply for the growing population (Henchion et al., 2017). Aquaculture is extremely important for global food security and nutrition, especially in low- and middle-income countries (LMICs) (Hicks et al., 2019; FAO, 2020; Webb et al., 2020; Naylor et al., 2021). Intensive culture is becoming increasingly popular, especially in the freshwater fish sector, in the setting of worldwide demographic increase and growing demand for fish products. Nonetheless, intensive fish framing has harmed the physiological status of fish in the cultural system and raised their stress (Martos-Sitcha et al., 2020). Consequently, the cultured fish are vulnerable to infectious diseases due to immunodeficiency (Alexander et al., 2010). Hence, infectious diseases is one of the challenges for sustainable aquaculture (Stentiford et al., 2017; Stentiford et al., 2020). Among bacterial infectious diseases, *Aeromonas hydrophila* infection is considered one of the most important responsible for the loss of millions of dollars in the global freshwater aquaculture industry (da Silva et al., 2012; Pridgeon and Klesius, 2012; Hossain et al., 2014; Peterman and Posadas, 2019). Antibiotics are still used intensively to combat this disease, especially in LMICs. Consequently, multidrug-resistant (MDR) *A. hydrophila* is becoming a global issue of concern (Guz and Kozinska, 2004; Patil et al., 2016; Stratev and Odeyemi, 2016).

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Alternatives to antibiotics are any substance that can be substituted for therapeutic drugs that are increasingly becoming ineffective against pathogenic bacteria due to antimicrobial resistance. Non-antibiotic approaches can minimize the requirement for antimicrobials to combat infectious diseases in both animal and human health

(Hoelzer et al., 2018). Bacteriophage is one of the environmentally friendly approaches which replace or complement chemotherapy to reduce the hazard of bacterial disease and antimicrobial resistance in aquatic animals (Cao et al., 2021). Previous studies have demonstrated that phages can be applied in aquaculture to combat *A. hydrophila* infection (Jun et al., 2013; Anand et al., 2016; Le et al., 2018; Cao et al., 2020; Dang et al., 2021). Hence, strategy using phages for biocontrol of *A. hydrophila* has become increasingly attractive. However, potential application of bacteriophage as a solution to prevent the motile *Aeromonas* septicemia (MAS) disease caused by multidrug-resistant *A. hydrophila* is still unclear.

Nanobubbles (NBs) are bubbles less than 100 nm in diameter filled with chosen gases, neutral buoyancy, and a lengthy residence time in the liquid solutions (Agarwal et al., 2011; Tsuge, 2014). In aquaculture, oxygen nanobubbles (NB-O_2) commonly used to enhance dissolved oxygen (DO) and stimulate the growth of some aquatic animals (Mahasri et al., 2018; Mauladani et al., 2020; Rahmawati et al., 2020). Several studies have recently indicated that ozone nanobubbles (NB-O3) have the potential to reduce pathogenic bacteria, improve DO in water, and modulate immune systems against bacterial infections (Imaizumi et al., 2018; Jhunkeaw et al., 2021; Linh et al., 2021; Nghia et al., 2021). The limitations of direct application of NB-O_3 with high level of ozone (3.5 mg/L, 970 mV ORP (oxidation reduction potential) is the tissue damage and mortality that this gas can cause to animals (Imaizumi et al., 2018). Jhunkeaw et al. (2021) did not observe fish mortality but the fish gill morphology was damaged when fish were exposed directly to multiple NB-O_3 treatments with an ORP range between 860 ± 42 and 885 ± 15 mV.

This study aims to investigate potential application of lytic bacteriophage, ozone nanobubbles (NB-O_3) application in a modified recirculation system, and potential

combination of bacteriophage and nanobubble technology in combatting multidrug-resistant *A. hydrophila* in Nile tilapia**.**

1.2 Objectives of study

- To isolate and characterize lytic bacteriophage infecting multidrug resistant *A. hydrophila.*
- To evaluate efficacy of bacteriophage in reducing concentration of multidrug-resistant *A. hydrophila* and in improving survivability of Nile tilapia upon experimental infection.
- To evaluate efficacy of MRS-NB-O_3 in reducing concentration of multidrug-resistant *A. hydrophila* and in improving survivability of Nile tilapia upon experimental infection.
- To investigate potential combination of bacteriophage and nanobubble technology.

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CHAPTER 2

Non-antibiotic approaches to combat motile *Aeromonas* infections in aquaculture: current state of knowledge and future perspectives

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2.1 Abstract

Aquaculture is one of the fastest-growing food sectors playing a vital role in global food security and nutrition, particularly in low- and middle-income countries (LMICs). Inland aquaculture contributed by three major fish groups, including carps, tilapias, and catfishes is likely irreplaceable in the global 'fish for food' supply chain. However, the sustainable development of this sector is hampered by disease epidemics, especially those caused by bacteria such as *Aeromonas* species. At least eight pathogenic motile *Aeromonas* species (*A. hydrophila*, *A. veronii*, *A. jandaei*, *A. caviae*, *A. sobria*, *A. bestiarum*, *A. dhakensis* and *A. schubertii*) have been reported in aquaculture; some causing up to 100% mortalities during disease outbreaks. Simultaneously, emerging multidrug-resistant *Aeromonas* due to a long-inappropriate use of antibiotics is alarming and highlights a global public health concern and negative socioeconomic impacts. Here, we provide a comprehensive overview of motile *Aeromonas* infections, antibiotic use and antimicrobial resistance of *Aeromonas* species. This contribution also highlights the non-antibiotic approaches to control motile *Aeromonas* infections including, vaccines, probiotics, phytochemicals, bacteriophages and omics-applications. Along with the current state of knowledge and limitations of each prophylaxis/therapy, perspectives for further studies are critically discussed. Some emerging applicable nanotechnology themes such as nanovaccines, nanobioactive compounds, and nanobubbles are also included in this review.

Key words: *Aeromonas*, alternatives to antibiotics, antimicrobial resistance, aquaculture, bacteriophages, nanobubbles

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2.2 Introduction

The global population reached 7.6 billion people in 2018 and is projected to reach 8.5 billion by 2030, 9.7 billion by 2050 and exceed 11 billion in 2100 (DESA 2019). The intake of protein is predicted to increase rapidly in the next decade to supply the increasing population (Henchion *et al.* 2017). Aquaculture is predicted to provide most aquatic protein sources by 2050 (Stentiford *et al.* 2020). Fish consumption accounted for 17% of total animal-based protein intake in 2017 (FAO 2020). Sustainable production of healthy, safe, and nutritious diets in the required quantity is an irrefutable urgency for realizing global food system transformation (Webb *et al.* 2020). Aquatic foods are a rich source of minerals, vitamins, and essential fatty acids, which have enormous potential as a lever for food system transformation to tackle micronutrient deficiencies (Hicks *et al.* 2019). In 2018, about 88% with over 156 million tonnes of total aquatic production (178.5 million tonnes) was used for human food consumption that has increased significantly from 67% since the 1960s. Global aquaculture production attained 114.5 million tonnes in live weight in 2018, in which aquatic animal production account for 71.7% with 82.1 million tonnes, including 51.3 million tonnes of inland and 30.8 million tonnes of marine and coastal aquaculture and is projected to reach 109 million tonnes in 2030 (FAO 2020). Finfish farming dominates the total production of inland aquaculture with 91.5% (47 million tonnes), followed by crustaceans with 7.1%, molluscs with 0.4%. The farming of carps, tilapias and catfishes feature the most important sectors by 35.84% of world aquaculture production with 83 billion dollars (Table 1). Simultaneously, aquaculture has contributed to global socioeconomic development by providing a stable livelihood for approximately 20.5 million people worldwide (FAO 2020).

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Table 1. Top finfish groups by quantity and value in 2018

Species group	Number of countries farming the species group	Production quantity of the species group (live weight, tonnes)	Share of world aquaculture production quantity of all species (%)	Economic value (billion USD)	Top species	Top producers	Average unit value (USD/Kg)		
							Fresh	Frozen	Fillet
Carps, barbs and other cyprinids	93	29,225,694	25.52	62.41	Grass carp Silver carp Common carp	China India Myanmar	1.47	1.21	5.4
Tilapias and other cichlids	124	6,031,432	5.27	11.23	Nile tilapia	China Egypt Indonesia	2.01	2.01	6.38
Catfishes	89	5,761,483	5.05	9.44	Striped catfish Torpedo-sharped-catfish	Vietnam India Bangladesh	1.16	2.23	6.12

Data source: 2020 FAO Yearbook. Fishery and Aquaculture Statistics 2018. Available at http://www.fao.org/fishery/static/Yearbook/YB2018_USBcard/index.htm

In the context of global demographic growth and pressing demand for fish products, intensive culture is becoming increasingly popular, especially in the freshwater fish sector. Nevertheless, intensive fish farming by stocking more fish and adding more food has affected physiological status and increased the stress of fish in the culture system (Martos-Sitcha *et al.* 2020; Boyd *et al.* 2020). Consequently, the cultured fish are vulnerable to infectious diseases due to immunodeficiency (Alexander *et al.* 2010). The epidemics in aquaculture are caused by various agents such as viruses, parasites, fungi and bacteria (Pridgeon & Klesius 2012). In nature, parasites and fishes coexist in a dynamic equilibrium and adverse changes in the environment can alter the host-parasite equilibrium causing outbreaks of disease (Buchmann & Lindenstrøm 2002). Parasites can cause physiological damage to fish and increase the risk of opportunistic bacterial diseases such as *Flavobacterium*, *Vibrio*, especially motile *Aeromonas* (Kotob *et al.* 2017). Besides, synergistic interactions of *Aeromonas* and heterologous bacteria, *Aeromonas* and fungi, *Aeromonas* and viruses potentiate pathogenic effects and lead to serious negative consequences and massive mortalities on the exposed fish (Abdel‐Latif & Khafaga 2020; Kotob *et al.* 2017). Among bacterial infectious diseases, Motile Aeromonas Septicemia (MAS) is one of the most popular and gives rise to economic losses in millions of dollars in the global aquaculture industry (Pridgeon & Klesius 2012; da Silva *et al.* 2012; Hossain *et al.* 2014; Peterman & Posadas 2019).

The control of these bacterial infections still heavily rely on disinfectants and antibiotics. Nonetheless, a global issue of concern is that multidrug-resistant bacteria are becoming increasingly ubiquitous due to massive misuse of antibiotics (Cabello 2006; Cantas & Suer 2014; Malik & Bhattacharyya 2019). Besides, antimicrobial residues in the environment and aquaculture products negatively impacted food safety, human

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health and international trade, especially in developing and aquaculture producing countries (Okocha *et al.* 2018; Ben *et al.* 2019; Heuer *et al.* 2009).

Alternatives to antibiotics (ATAs) are any substance that can be substituted for therapeutic drugs that are increasingly becoming ineffective against pathogenic bacteria due to antimicrobial resistance. Non-antibiotic approaches can minimize the need for antibiotics to combat infectious diseases in animal and human health (Hoelzer *et al.* 2018). In aquaculture, along with promoting biosecurity, water quality management and improving brood stock and fingerling quality, ATAs are urgently needed in both prophylactics and therapeutics. The scientific approaches on fish and causative agents provide comprehensive strategies to combat motile *Aeromonas* infections toward sustainable aquaculture (Figure 1). Vaccines are an ideal approach for preventing infectious diseases. Nonetheless, commercially available vaccines are still very limited in the aquaculture field due to the gaps in efficacy and cost-benefit between research and practical application (Ma *et al.* 2019a). Several environmentally friendly approaches have been investigated and used in aquaculture based on the advancements in science and technology. Among them, nanotechnology and modern biotechnology contribute significantly to research, improvement, and ATAs (Lieke *et al.* 2020; Shah & Mraz 2020). Here, we present an overview of antibiotic usage and antimicrobial resistance of Aeromonads associated with MAS in aquaculture. We then provide a systematic review of the current state of knowledge on various alternatives to antibiotics in MAS control, including vaccines, probiotics, phytochemicals, bacteriophages, omics-approaches, and nanobubble technology. Finally, we highlight important considerations and future directions for effective non-antibiotic strategies to control MAS towards sustainable aquaculture and food security.

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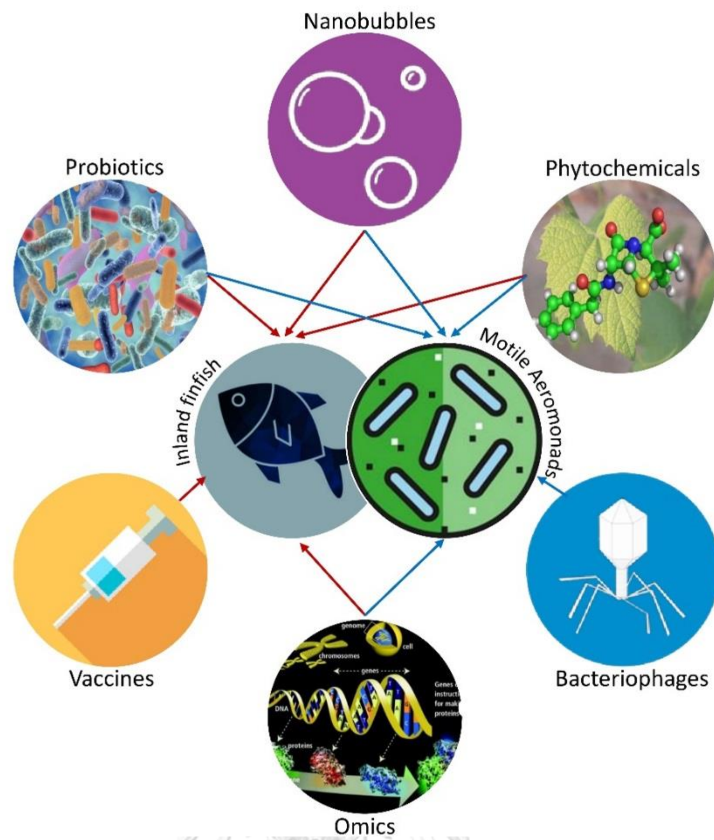

Figure 1. The concept of non-antibiotic approaches to combat MAS in aquaculture. The application of the alternatives to antibiotics can be divided into two directions. The first direction (red arrows) is applied on fish to reduce stress (phytochemicals, oxygen nanobubbles), enhance the immunity system (vaccines, probiotics, phytochemicals, ozone nanobubbles, metabolites), or improve the intestinal microbiome (probiotics). The second direction (blue arrows) is applied directly to inhibit or eradicate bacteria such as lytic phages, ozone nanobubbles, probiotics, phytochemicals or metabolites

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2.3 Overview of motile *Aeromonas* infections in fish aquaculture

2.3.1 Common infectious Aeromonads

Motile Aeromonas Septicemia is one of the most common infectious diseases in aquatic animals, especially in inland aquaculture. Motile Aeromonads cause MAS, the pathogen group frequently observed in a warm water environment, such as *A. hydrophila*, *A. veronii*, *A. caviae, A. jandaei* and *A. schubertii*. Another group is non-

motile *Aeromonas* with *A. salmonicida* as a representative species, seen mostly in cold water (Hanson *et al.* 2014).

Motile Aeromonads can cause the disease in fishes, aquatic invertebrates, amphibians, reptiles and mammals, including humans (Dias *et al.* 2016; Khajanchi *et al.* 2010). Among motile *Aeromonas* species, *A. hydrophila* has been reported as the most common pathogen in at least fifteen freshwater fishes, especially in carps (Harikrishnan *et al.* 2003; Jiang *et al.* 2016b; Song *et al.* 2014; Zheng *et al.* 2012b), tilapias (Abdel-Latif & Khafaga 2020; AlYahya *et al.* 2018; Leung *et al.* 1995) and catfishes (Almaw *et al.* 2014; Angka *et al.* 1995; Crumlish *et al.* 2010; De Figueiredo & Plumb 1977; Laith & Najiah 2013). Also, blunt-snout bream, *Megalobrama amblycephala* (Xia et al. 2017), snakehead fish, *Channa striata* (Duc *et al.* 2013; Samayanpaulraj *et al.* 2019) and a variety of freshwater ornamental fishes (Musa *et al.* 2008) were infected by *A. hydrophila*. The MAS outbreak caused high mortality in farmed grass carp in China, resulting in production losses estimated at 2,200 tonnes with economic losses exceeding five billion Chinese Yuan per year, approximately 74 million USD (Chua *et al.* 2015; Rasmussen-Ivey *et al.* 2016b). A hypervirulent *A. hydrophila* (v*Ah*) strain ST251 was first reported in 1989 in Jiangsu province, China (Chen & Lu 1991). In 2004, the first case of v*Ah* was isolated from channel catfish, *Ictalurus punctatus* in Washington Country, Mississippi, United State (Hossain *et al.* 2014). They were found to cause 35% of mortalities with an estimated 10,500 tonnes in channel catfish in Alabama Fish Farming Center in 2009, 50% - 60% in West Mississippi and Arkansas (Pridgeon & Klesius 2011b; Baumgartner *et al.* 2018). The Aquatic Diagnostic Laboratory at Mississippi State University, USA reported a pond mortality rate of nearly 100% (Abdelhamed *et al.* 2019).

Aeromonas veronii is the second most prevalent causative agent of MAS in food finfish species, such as channel catfish (Hoai *et al.* 2019; Liu *et al.* 2016a; Nawaz *et al.*

2010), Chinese longsnout catfish, *Leiocassius longirostris* (Cai *et al.* 2012), pond loach, Misgurnus anguillicaudatus (Zhu et al. 2016), Nile tilapia, *Oreochromis niloticus* (Dong *et al.* 2017; Hassan *et al.* 2017; Raj *et al.* 2019; Dong *et al.* 2015), red hydrid tilapia, *Oreochromis* spp. (Amal *et al.* 2018; Sewaka *et al.* 2019), crucian carp (Chen *et al.* 2019b; Zhu *et al.* 2016), gibel carp, *Carassius gibelio* (Sun *et al.* 2016) and Northern snakehead fish, *Ophiocephalus argus* Cantor (Chen *et al.* 2019a; Zheng *et al.* 2012a). Other Aeromonads, such as *A. dhakensis* (Carriero *et al.* 2016; Soto-Rodriguez *et al.* 2018), *A. sobria* (Dar *et al.* 2016; Majtán *et al.* 2012), *A. jandaei* or *A. punctata* (Dong *et al.* 2017; Purushothaman *et al.* 2015), *A. bestiarum* (Soriano-Vargas *et al.* 2010), *A. schubertii* (Liu *et al.* 2018; Liu & Li 2012) were also able to cause diseases and mortalitty in many fish species.

2.3.2 Aeromonad transmission and virulence

The uptake of motile Aeromonads may be by oral or dermal routes, which are promoted by disruption of mucosal defense associated with body injury, especially in primary barriers such as skin and gill (Hanson *et al.* 2014). One of the most critical mechanisms of MAS infection is the immunosuppressive event caused by environmental stress. High-density culture, temperature fluctuation, low dissolved oxygen level, increased ammonia, and carbon dioxide concentrations may promote stress in fish (Esch & Hazen 1980; Walters & Plumb 1980). Almost cases of MAS correlated to co-infection with other bacteria, fungi, parasites or viruses. For transmission route, Aeromonads transmit primarily by horizontal mean. They distribute widely in water and sediments of culture ponds and can be transmitted by discharge from the intestinal tract and external lesions on the skin or the direct contact with biological carriers such as duck or bird (Cunningham *et al.* 2018; Jubirt *et al.* 2015).

Motile Aeromonas Septicemia fish expresses various pathological conditions ranging from acute, chronic, or subclinical infection. The clinical manifestations of

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infected fish depend on the species of *Aeromonas*, virulence, physiological conditions of the fish and stressful environmental conditions. A chronic infection lasts throughout the culture period, causes mortality of about 10% of the cultured population, while acute infection can induce mortality up to 100% within two to three days (Camus *et al.* 1998; Hanson *et al.* 2014; Pridgeon & Klesius 2011c; Rasmussen-Ivey *et al.* 2016b). Virulence in motile Aeromonads is related to several bacterial mechanisms involved in producing major virulence factors, including surface-polysaccharides, exotoxins, and other extracellular enzymes, iron-binding systems, secretion systems, adhesions, motility and flagella and biofilm formation. It depends on the species, infection route and host (Rasmussen-Ivey *et al.* 2016a; Tomás 2012).

2.3.3 Pathological signs and diagnostic

The pathological signs are not specific and relatively similar between chronic and acute infection. The pathological lesions in MAS-infected fish may be seen in the skin, internal organs, or other body sites. Surface ulcerative and acute systemic types of bacterial diseases are popular forms of MAS, but their impact depends on the fish host and *Aeromonas* species (Kozinska & Pekala 2012). Kozinska& Pekala (2012) reported that *A. hydrophila* was the most versatile and dangerous among motile Aeromonads. They caused skin ulcers and septicemia, while the other species caused only skin ulcers or some specific internal lesions with or without septicemia. Behaviorally, the diseased fish tend to hang at the water surface and show lethargy and loss of appetite. External signs with typical septicemia include hemorrhages on the skin, fin, eyes, and inflammation of the anus. In internal organs, hemorrhages may occur in the intestine, pale liver, swollen spleen and kidney. Multifocal necrosis is found in histopathological samples of gills, liver and kidney (Abdelhamed *et al.* 2017; Miyazaki & Jo 1985).

The presumptive diagnostic of MAS can be conducted based on clinical symptoms, bacterial isolation using Tryptic Soy Agar or Rimler-Shott Agar supplemented with

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Novobiocin (selective medium for Aeromonads) and identification using biochemical assays such as API-20E, API-32GN, VITEK GN Card, Microscan W/A NFC47, Phoenix ID69, or Omnilog GN2 Microplate (Hanson *et al.* 2014; Lamy *et al.* 2010). Nonetheless, motile Aeromonads have similar morphological, physiological and biochemical characteristics that can lead to species-level misidentification (Lamy *et al.* 2010; Soler *et al.* 2004). Due to the limitations of biochemical-based assays, identification and classification of *Aeromonas* based on proteomic and genotypic characterization have been applied and regularly updated. The proteomics-based technique, namely Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), is recommended for rapid identification of *Aeromonas* at the species level with high accuracy compared with biochemical tests (Benagli *et al.* 2012; Elbehiry *et al.* 2019; Pérez-Sancho *et al.* 2018; Shin *et al.* 2015). In addition, the confirmation diagnostic using sequence analysis of *gyrB* gene or *rpoD* gene has been encouraged in research and diagnostic laboratories (Soler *et al.* 2004; Yanez *et al.* 2003; Persson *et al.* 2015; Saavedra *et al.* 2006). For classification of *Aeromonas* species, Multilocus Phylogenetic Analysis (MLPA) using the sequence of housekeeping genes (*gyrA, gyrB, rpoB, rpoD, recA, dnaJ, dnaK, dnaX, atpD, gltA, radA, zipA, cnp60, mdh, metG, ppsA, tsf*) is arguably one of the most accurate methods with classification accuracy up to 100% (Martinez-Murcia *et al.* 2011; Navarro & Martínez-Murcia 2018; Roger *et al.* 2012; Du *et al.* 2021). In recent years, core genome multilocus sequence typing (cgMLST) or whole-genome multilocus sequence typing (wgMLST) offered an excellent platform for bacterial strain-level identification and characterization (de Sales *et al.* 2020; Kovács *et al.* 2020; Miro *et al.* 2020; Uelze *et al.* 2020; de Melo *et al.* 2019). The novel platform based on whole-genome sequencing should be investigated in Aeromonas-related studies in the future.

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2.3.4 Antibiotic usage and antimicrobial resistance of Aeromonads associated with MAS

Antibiotics or antimicrobials are natural or synthetic compounds that can kill or inhibit bacterial growth (Sapkota *et al.* 2008; Romero 2012). The use of antibiotics as prophylactic and therapeutic agents or growth promoters has become popular in aquaculture (Cabello 2006; Ibrahim *et al.* 2020). In the last decade, at least forty antibiotics belonging to ten groups were used in aquaculture (Table 2). The global consumption of antibiotics used for all purposes is increasing rapidly and is estimated to rise by 67% in 2030 compared to 2010. Based on total antimicrobial consumption, China dominated with 23%, followed by the United States (13%), Brazil (9%), while India and Germany shared the position among the top five biggest consumers, each with 3% (Van Boeckel *et al.* 2015). In aquaculture, the global antimicrobial consumption in 2017 was estimated at 10,259 tons and projected to rise 33% to 13,600 tons by 2030. The Asian-Pacific region accounts for the overwhelming majority of 93.8%. China (57.9%), India (11.3%), Indonesia (8.6%), and Vietnam (5%) are the four countries with the largest share of antimicrobial consumption worldwide (Schar *et al.* 2020). The report of the World Organization for Animal Health on antimicrobial agents intended for use in animals in 2016 showed that 86 out of 146 countries surveyed had not authorized any antimicrobials as growth promoters, 37 countries authorized them, while 23 countries lacked any legislation for antimicrobial growth promoters (OIE 2017). In this report, the top ten antimicrobials commonly used for growth promotion included Bacitracin, Flavophosfolipol, Avilamycin, Tylosin, Virginiamycin, Colistin, Enramycin, Lincomycin, Oxytetracycline and Chlotetracycline (OIE 2017).

Numerous drugs are found relevant to treat motile *Aeromonas* infections in the food fish industry. Oral administration of Oxytetracycline and Sulfadimethoxine/Ormetoprim was recommended for MAS treatment (Cipriano *et al.*

1984; Swann & White 1991). In recent studies, Oxytetracycline and Ciprofloxacin were applied orally to treat *A. hydrophila*, *A. caviae* in Nile tilapia and common carp, *C. carpio* (Julinta *et al.* 2017a; Julinta *et al.* 2017b; Neowajh *et al.* 2015; Roy *et al.* 2019). Ciprofloxacin and Streptomycin are also used to treat *A. hydrophila* and *A. sobria* in the farming of black tiger shrimp, *Penaeus monodon*. The list of authorized and banned antibiotics in the top ten aquaculture producing countries in 2018 is summarized in Table 2. The data show that Oxytetracycline is the most widely approved antibiotic in nine countries except China (88.9%), followed by Oxolinic acid and Sulfamonomethoxine in seven countries (77.8%) and Amoxicillin, Florfenicol, Sulfadimethoxine along with Sulfamothoxazone in six countries (66.7%). Besides, Chloramphenicol and Nitrofurans are banned in all countries (data not available in Egypt). Fluoroquinolones are prohibited in China (exception of Enrofloxacin and Flumequine) and Vietnam.

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Table 2. The list of prohibited and allowed antimicrobials of ten major aquaculture fish producing countries in 2018

Aquaculture fish producers		1 CHN	2 INA	3 IND	4 VIE	5 BLD	6 EGY	7 NOR	8 CHL	9 MYA	10 THA
Annual fish production (thousand tonnes)		47,559.1	7,066	5,426.9	4,134	2,405.4	1,561.5	1,354.9	1,266.1	1,130.4	890.9
Percentage of world total		57.93%	8.61%	6.61%	5.04%	2.93%	1.90%	1.65%	1.54%	1.38%	1.09%
Category	Antibiotic name										
β-lactams	Amoxicillin	NA				A			A	A	A
	Penicillin G	NA									
	Flucloxacillin					A					
Tetracyclines	Tetracycline			NA		A			A	A	
	Chlotetracycline	NA				A			A		
	Oxytetracycline*	NA			A	A		A	A	A	A
	Doxycycline	A			A						
Aminoglycosides	Gentamycin S	NA									
	Neomycin	A	NA								
	Streptomycin	NA									
Macrolides	Erythromycin	NA							A	A	
Chloramphenicols	Chloramphenicol	NA	NA	NA	NA	NA		NA	NA	NA	NA
	Florfenicol*	A			A			A	A		
	Thiamphenicol	A									
Quinolones	Ciprofloxacin	NA	NA		NA	A					
	Enrofloxacin	A			NA					A	A
	Norfloxacin	NA	NA	NA	NA						
	Sarafloxcacin	NA			NA						A

	Lomefloxacin	NA			NA						
	Fleroxacin	NA			NA						
	Difloxacin	NA			NA						
	Ofloxacin	NA	NA		NA						
	Flumequine	A			NA			A	A		
	Nalidixic Acid		NA			A					
	Oxolinic Acid					A		A	A	A	A
Sulfonamides	Sulfadiazine	A			A			A		A	A
	Sulfamerazine				A					A	
	Sulfadimidine	A			A					A	A
	Sulfanilamide				A	A				A	
	Sulfamethoxazole	A	NA		A	A		A		A	
	Sulfathiazole				A	A				A	
	Sulfamonomethoxine	A			A	A				A	A
	Sulfadimethoxine+				A	A				A	A
Glycopeptides	All antibiotics		NA		NA						
Nitrofurans	Furazolidone	NA	NA	NA	NA	NA		NA	NA	NA	NA
	Nitrofuran metabolites	NA	NA	NA	NA	NA		NA	NA	NA	NA
Others	Trimethoprim	A			A	A		A			A
	Ormetoprim*				A						A

A: authorized NA: not authorized Blank: Data not available

+ Approved by United State Food and Drugs Administration

CHN: China (Liu *et al.* 2017c), INA: India (Srivastava *et al.* 2011), IND: Indonesia (ASEAN 2013), VIE: Vietnam (ASEAN 2013; MARD 2014), BLD: Bangladesh (DOF 2019), EGY: Egypt (Data not available), NOR: Norway (EC 2009; Lulijwa *et al.* 2020), CHL: Chile (Lozano *et al.* 2018), MYA: Myanmar (ASEAN 2013), THA: Thailand (ASEAN 2013).

Targets for the antimicrobial reduction in aquaculture and regulations for the use and application of antibiotics have been promulgated in many countries. Nevertheless, the heavily illegal use of banned antibiotics has been reported in major aquaculture producers - Chloramphenicol, Enrofloxacin in Vietnam, Chloramphenicol, Ciprofloxacin, Erythromycin in China; and Penicillin in Thailand. The technological improvements in aquaculture and stricter regulations have reduced the prevalence of antibiotics in Norway and Japan (Lulijwa *et al.* 2020). Therefore, national authorities, especially top aquatic-food producers and exporters, need to formulate policies that strictly limit antibiotic use in aquaculture. Multi-national policies should be considered to reduce antimicrobials for sustainable aquaculture.

2.3.5 The emergence of multidrug-resistant Aeromonads

Chemotherapy in aquaculture should be applied based on antimicrobial susceptibility testing data and an authorized antibiotic list. In addition to benefits, the use of antibiotics in aquaculture has adverse effects on public health, the environment and the national exporting economy of aquaculture producing countries (Rasul & Majumdar 2017). The overuse and misuse of antibiotics cause antibiotic residues in aquaculture products and the environment. The targeted aquatic animals typically absorb only 20%-30% of antibiotics, while the remaining amount diffuses into the environment (Hernandez 2005). The residual antibiotics are accumulated in sediments and water then penetrate the food chain. The accumulation of antibiotic residues can enhance adverse drug reactions and induce antimicrobial-resistant mechanisms of critically important pathogens in humans (Liu *et al.* 2017c). On the other hand, the non-selective effect of antibiotics will eradicate intestinal microorganisms of aquatic animals and destroy the microbial ecosystem's balance (Grenni *et al.* 2018; He *et al.* 2012; Park & Kwak 2018; Song *et al.* 2016). Further, using prohibited antibiotics and the detection of antimicrobial residues in seafood that did not satisfy the critical standards

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of importing countries had led to the loss of export turnover in China, Bangladesh, Indonesia, and Vietnam (Cato & Dos Santos 1998; Hassan *et al.* 2013; Hernandez 2005).

Inappropriate use of antibiotics for disease treatment or growth promoters in aquaculture lead to increased antimicrobial resistance (AMR) and induces multidrug-resistant bacteria. Moreover, fraudulent antibiotics, including counterfeit and substandard antibiotics in aquaculture could exacerbate the risk of inducing antimicrobial-resistant bacteria (Leung *et al.* 2020). Indeed, motile Aeromonads resist numerous antimicrobials approved for food fish such as Amoxicillin, Tetracycline, Oxytetracycline, Ciprofloxacin, Nalidixic acid, and Sulfamethoxazole /Trimethoprim (Table 3). *Aeromonas hydrophila*, *A. sobria* and *A. caviae* were reported to resist prohibited antibiotics, including Chloramphenicol and Nitrofurans. *Aeromonas* species can survive to **β**-lactams combined with **β**-lactamase inhibitors such as Ampicillin and Sulbactam, Piperacillin and Tazobactam as well as carbapenems including Imipenem and Meropenem (Ruzauskas *et al.* 2018; Yano *et al.* 2015).

Aeromonas species isolated from ornamental fish resist to various **β**-lactams including Ceftiofur, Imipenem (Preena *et al.* 2019a; Verner-Jeffreys *et al.* 2009); Tetracycline, Oxytetracycline, Nalidixic acid, Trimethoprim, Chloramphenicol and even Colistin, the ultimate solution for Carbapenem-resistant and extended-spectrum β-lactamase (ESBL)-producing bacteria (Sreedharan *et al.* 2011). Stratev& Odeyemi (2016) reported the high prevalence of antimicrobial resistance of *A. hydrophila* in seafood, meat, meat products, fresh milk, dairy products and vegetables. Besides, the occurrence of multidrug-resistant *Aeromonas* species in river water and wastewater with high-risk assessment has been documented in several publications (Aravena-Román *et al.* 2012; Deng *et al.* 2016a; Goñi-Urriza *et al.* 2000; Skwor *et al.* 2020), indicating the ubiquity of multidrug-resistant Aeromonads in the environment.

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The multidrug-resistant *Aeromonas* can distribute the resistance genes in the aquaculture systems and to the human pathogens through the mobile genetic elements such as plasmids, transposons and integrons via horizontal gene transfer mechanisms, namely transformation, transduction or conjugation (Romero 2012; Piotrowska & Popowska 2014; Stratev & Odeyemi 2016). The vast majority of motile *Aeromonas* specie's plasmids, such as pRA1, pR148 (IncA/C group), RA3, pFBAOT1-17, pFBAOT16, pAS37, p42, pP2G1 (IncU group), pBRST7.6, pAHH04 (IncQ group) carry several resistance determinants and mobile elements. Moreover, they have become a global concern for public health because most of the reported plasmids are of broad-host-range and capable of conjugative transfer (Piotrowska & Popowska 2015).

Different resistant genes encode the plasmid-mediated antibiotic resistance to various antimicrobial groups of *Aeromonas* species. For example, *tet*E gene frequently mediates Tetracyclines-resistance (Agersø *et al.* 2007; Cui *et al.* 2016; Kim *et al.* 2019a) while bla_{TEM}, bla_{SHV}, bla_{CphA}, bla_{OXAB} are ESBL-resistant genes (Tacão *et al.* 2014; Vega-Sánchez *et al.* 2014). Other genes, such as *qnr* (Chenia 2016; Cattoir *et al.* 2008), *qnrs*2 (Dobiasova *et al.* 2014; Wen *et al.* 2016), *qnrs* and *qnrs*5 (Han *et al.* 2012), missense mutation in QRDR regions (Hooper & Jacoby 2015) have also known to involve in Quinolones-resistance of motile Aeromonads. Besides, the evidence related to tetracycline resistance transposon Tn1721 has disseminated between *Aeromonas* species and *Escherichia coli*, showing the potential risk of transferring plasmid-encoded resistance genes from *Aeromonas* to serious human pathogens (Rhodes *et al.* 2000).

Integrons, another class of genetic structures, described as containing one or more gene cassettes located at a specific site on chromosome called chromosomal integrons, or transposons, and plasmids called mobile integrons (Gillings 2014; Stalder *et al.* 2012). Integrons found in motile *Aeromonas* species mainly belong to class 1 and carry many antibiotic resistance gene cassettes (Piotrowska & Popowska 2015).

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Herein, class 1 integrons with gene cassettes *ant*(3'')Ia, *aad*A, *add*A2, *add*2 were reported to resist Streptomycin (Lukkana *et al.* 2011; Ndi & Barton 2011; Schmidt *et al.* 2001; Sarria-Guzmán *et al.* 2014) while *dhfr*I, *dfr*A12 response for Trimethoprim-resistance (Deng *et al.* 2016b; Lukkana *et al.* 2011; Schmidt *et al.* 2001). Other gene cassettes such as *aac*(6')Ia, *sul*1, *lin*F are responsible for Aminoglycosides, Sulfonamides and Lincosamides-resistance, respectively (Jacobs & Chenia 2007; Ndi & Barton 2011; Sarria-Guzmán *et al.* 2014). Although prominent in Aeromonads, gene cassettes *dfr*A1 (Trimethoprim-resistance), *sat*2 (Streptothricin-resistance), *orf*X (Spectinomycin-resistance) and *aad*A1 (Streptomycin-resistance) of class 2 integrons were also reported (Hansson *et al.* 2002; Jacobs & Chenia 2007; Otero-Olarra *et al.* 2020).

The prevalence of *Aeromonas* resistant to important antimicrobials for human medicine in the environment alerted a global public health hazard. The reduction of antibiotics in aquaculture can restrict the flow of AMR genes along the food chain and ultimately to the consumers. Taking cues from the best regulatory model in Norway, national authorities should require pharmacies to sell antibiotics only by prescription from aquatic veterinarians and antimicrobial susceptibility testing. Management policies and strict sanctions should be enforced. Concurrently, the One Health platform that incorporates human, animal, environmental, ecosystem, and wildlife perspectives should be promoted in each country. The enhancement of international cooperation and multinational scientific transfer related to the One Health approach will help combat multidrug-resistant pathogens.

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Table 3. Antibiogram of motile Aeromonads in food aquatic and ornamental sources

Antimicrobials		WHO Classification for human medicine	Food aquatic animals				Food and Ornamental animals		Ornamental animals								
Category	Name		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
β-lactams⁺	β-lactams	Critically important	R^{amp}	R^{m}	R^{m}	R^{m}	R^{m}		R^{amp}	R^{m}	R^{m}	R^{m}	R^{m}		R^{amo}	R^{m}	R^{m}
Tetracyclines	Tetracycline	Highly important	R				R		R	R	R	R	R		R	R	
	Oxytetracycline		R	R							R	R		R			R
	Doxycycline											R					
Aminoglycosides	Amikacin	Critically important (High priority)								R	R	S					
	Gentamycin			S	S	S	S			R	R	S	S	R		S	S
	Kanamycin		R									S					S
	Neomycin								R		R	S			R		
	Streptomycin			R						R	R	S	R			R	
	Tobramycin					S					R	S				S	
	Vancomycin											R					
Polypeptides	Bacitracin	Important										R				R	R
Polymyxins	Colistin	Critically important (Highest priority)										R				S	
	Polymycin B											R					
Macrolides	Erythromycin				R		R		R	R		R			R	R	S

	Azithromycin	Critically important (Highest priority)					R					S				R	S
	Oleandomycin											R					
Lincosamides	Lincomycin	Highly important										R					
	Clindamycin				S							R					
Chloramphenicols	Chloramphenicol	Highly important	S	R	S		R			R	R	S	S	R	R	S	R
	Florfenicol			R							R			R			
Quinilones	Ciprofloxacin	Critically important (Highest priority)		S		S	R	R		S	R	S	S	S			S
	Enrofloxacin										R	S		S	R	R	S
	Norfloxacin				R							S					
	Sarafloxcacin								R								
	Ofloxacin						R	R			R	S					
	Gatifloxacin					S					R						
	Flumequine										R			S			
	Perfloxacin											S				R	
	Nalidixic Acid				R		R	R	R			R	R	S		R	
	Oxolinic Acid			S					R		R			S			
Sulfonamides	Sulfadiazine	Highly important											S				R
	Sulfapiridine																
	Sulfadimidine																
	Sulfamethoxazole			R	R	R	R		R		S			R	R	S	
Nitrofurans	Nitrofurantoin	Important	R	R		S			R		S	S			R	S	S

	Furazolidone										R	S				R	R
Other	Trimethoprim	Highly important		R	R	R	R		R	R	S	S	R	R	R	R	R

S: sensitive; R: resistance; R^{amp}: ampicillin-resistance; R^{amo}: amoxicillin-resistance; R^{m}: multi-β-lactam-resistance

Blank: data not available

+: exception of Penicillins and Cephalosporins (1st and 2nd generation)

Source of samples and references: 1. channel catfish, *Ictalurus punctatus* (McPhearson *et al.* 1991); 2. rainbow trout, *Oncorhynchus mykiss* (Akinbowale *et al.* 2007); 3. white leg shrimp, *Litopenaeus vannamei* and black tiger, *Penaeus monodon* (Yano *et al.* 2015); 4. common carp, *Ciprynus carpio*, rainbow trout, *O. mykiss* and bighead carp, *Hypophthalmichthys nobilis* (Ruzauskas *et al.* 2018); 5. Mozambique tilapia, *Oreochromis mossambicus*, rainbow trout, *O. mykiss* (Jacobs & Chenia 2007); 6. Mozambique tilapia, *O. mossambicus*, rainbow trout, *O. mykiss* and Koi, *Cyprinus rubrofuscus* (Chenia 2016); 7. goldfish, *Carassius auratus* and Koi, *C. rubrofuscus* (Dixon & Issvoran 1993); 8. common carp, *C. carpio*, anchovy, *Engraulis encrasicolus* and sardine, *Sardina pilchardus* (Yucel *et al.* 2005); 9. variety of ornamental fish (Verner-Jeffreys *et al.* 2009); 10. Oscar, *Astronotus ocellatus* (Sreedharan *et al.* 2011); 11. variety of ornamental fish (John & Hatha 2012); 12. variety of ornamental fish (Dobiasova *et al.* 2014); 13. variety of ornamental fish (Jagoda *et al.* 2014); 14. goldfish, *C. auratus* and Koi, *C. rubrofuscus* (Preena *et al.* 2019a); 15. guppy, *Poecilia reticulata* (Preena *et al.* 2019b).

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2.4 Non-antibiotic approaches to combat motile *Aeromonas* infections

2.4.1 Vaccines

Disease prevention by vaccination is one of the most important non-antibiotic preventive measures to control infectious diseases in aquaculture, contributing to a sustainable aquaculture industry (Gudding 2014). Up to now, over 26 licensed vaccines for use in aquaculture are commercially available, mainly for the salmon and trout industry (Ma *et al.* 2019b). Although commercial inactivated vaccines against MAS for some freshwater fish species are available, their protective efficacy is entirely dependent on the specific strain of problematic bacteria (Ma *et al.* 2019a; Stratev & Odeyemi 2017; Wang *et al.* 2020). There is a great effort to develop vaccines against MAS in many countries worldwide, evidenced by thousands of relevant publications and systematic data reported in the previous reviews (Mzula *et al.* 2019; Nayak 2020).

Fish parenteral vaccines, including inactivated bacteria (with or without adjuvant) are accounted for the most available aquaculture vaccines because of their safety and effectiveness (Ma *et al.* 2019a). An inactivated formalized vaccine against *A. hydrophila* J-1 became the first aquatic bacterial vaccine commercialized in China after obtaining the national class I new veterinary drug certificate in 2001. This vaccine has been used to effectively prevent the MAS caused by *A. hydrophila* in silver carp, crucian carp, bighead carp, bream, Chinese soft shell turtles and bullfrogs, with an average relative percentage survival (RPS) of 70% (Wang *et al.* 2020). However, some inactivated vaccines showed a limited ability to enter the fish via immersion or oral routes, resulting in low and non-long-lasting immunity. Therefore, a booster vaccination is required to enhance the immune responses and improve the efficiency of inactivated vaccines (Ma *et al.* 2019a).

Live attenuated vaccines are prepared from an avirulent form of the bacteria, created by physical or chemical processes, serial passage in cell culture, culture under

unfavored conditions, or gene editing (Ma *et al.* 2019a). This whole organism vaccine type showed some advantages in providing a faithful simulation of natural infection and lifelong immunity (Mzula *et al.* 2019). *A. hydrophila* mutants on *aroA* gene, exoenzyme (Liu & Bi 2007), plasmid (Majumdar *et al.* 2007) or attenuated by the serial culture on media containing novobiocin and rifampicin (Jiang *et al.* 2016a; Mu *et al.* 2011; Pridgeon & Klesius 2011a) have been tested as potential live vaccines against *A. hydrophila* infections in different fish species with the protection up to 100%. However, safety concerns and the use of genetically modified organisms (GMO) are major challenges of this vaccine type (Ma *et al.* 2019a; Mzula *et al.* 2019). Hence, controlling the ability to revert to virulence or display residual virulence, and optimizing the current time-consuming attenuation process, are possible solutions that need further investigations to apply attenuated vaccines in aquaculture systems. Although live attenuated vaccines for catfish have been licensed in the USA, in the absence of regulations for their safe use in aquaculture, this type of vaccines' commercial application is yet not feasible in other countries.

The majority of subunit vaccines for MAS targeted the outer membrane proteins (OmpTS, OmpR, Omp38, Omp48, OmpA1, OmpW, S-layer protein, Tdr, 46kDa maltoporin) due to their location at the host–bacterial interface and the cross-protection attributed to their conserved nature of antigenic determinants (Abdelhamed *et al.* 2016; Dash *et al.* 2014; Khushiramani *et al.* 2012; Poobalane *et al.* 2010; Wang *et al.* 2013). Other antigenic proteins potentially used as recombinant protein vaccines were ATPase protein (Abdelhamed *et al.* 2019), iron-related protein A0KIY3 (Guo *et al.* 2018), fimbrial proteins Fim and FimMrfG (Abdelhamed *et al.* 2016), hemolysin co-regulated protein - Hcp (Wang *et al.* 2015), G-protein coupled receptor 18 - GPR18 (Pridgeon & Klesius 2013b), and proaerolysin (Zhang *et al.* 2013). Vaccine efficacy of these recombinant proteins against MAS varied depending on protein types,

host species, immunization regimens and challenge strains. Feng et al. (2017) reported RPS values from 62.5% to 100% in 46 kDa maltoporin-vaccinated eel group after the challenge with five different *A. hydrophila* strains at four weeks post the vaccination. Further research on the synergistic immune response exhibited by combining these outer membrane proteins (OMPs) in subunit vaccine formulation is required (Mzula *et al.* 2019). On the other hand, the ability to stimulate a potent immune response of subunit vaccines is limited due to the lack of components capable of stimulating an immune system as a whole-cell vaccine (Holten-Andersen *et al.* 2004). Besides, the process of production and purification makes subunit vaccines expensive and unaffordable to scale up in developing countries. Therefore, multiple booster vaccination is required to ensure long-term protective immunity. Recombinant proteins could be expressed in a cost-effective manner using newer and safer host systems, such as beneficial bacteria, yeast, edible parts of plants and DNA vectors.

DNA vaccination is a form of genetic immunization that uses a gene or genes encoding protective antigens and can produce protection against intracellular pathogens by mimicking the natural route of infection (Biering & Salonius 2014). Pridgeon and Klesius developed recombinant apolipoprotein A1 plasmid DNA as a useful DNA vaccine against *A. hydrophila* with RPS of 100% in channel catfish, *I. punctatus* through intraperitoneal injection (Pridgeon & Klesius 2013a). In another study, Liu *et al.* (2016b) applied a novel functionalized single-walled carbon nanotubes (SWCNTs) as a delivery vehicle for the intramuscular injection of an *aerA* (a virulence factor that has haemolytic and cytolytic properties) DNA vaccine in juvenile grass carp, *Ctenopharyngodon idella*. Besides, the association between the intramuscular administration of DNA vaccine and the development of myositis is an additional safety concern on utilising this vaccine type that needs to be clarified in further studies (Tonheim *et al.* 2008).

The recombinant live vector vaccines against MAS have been developed and experimentally tested in fish by several research groups (Anuradha *et al.* 2010; Han *et al.* 2019; Ju *et al.* 2020). In these studies, nonpathogenic recombinant organisms *Lactococcus lactis* (Anuradha *et al.* 2010), *Saccharomyces cerevisiae* (Han *et al.* 2019) and *Lactococcus casei* (Ju *et al.* 2020; Kong *et al.* 2019; Tian *et al.* 2020; Zhang *et al.* 2018; Zhang *et al.* 2019b) were used to express antigenic proteins against *A. hydrophila* and *A. veronii* such as aerolysin, OmpG, Omp48, OmPAI, OmpW, flaA, flaB, and Malt. This framework is expected to mimic natural infections and offered intrinsic adjuvant properties than other types of non-replicating vaccines (i.e. inactivated or subunit). Anuradha *et al.* (2010) obtained the RPS values of 70-100% against *A. hydrophila* when feeding tilapia with live recombinant *Lactococcus lactis* vaccine expressing aerolysin genes. However, it is not easy to get approval for their application at the field level since recombinant live vectors are classified as genetically modified organisms in many countries. Appropriate research strategies considering the GMO regulations in each country need to be devised.

Fish vaccination is done *via* different routes such as intramuscular/intraperitoneal injection, dip/bath immersion and oral immunization. Even though the injection route is labour-intensive, costly, unsuitable for small fish, and results in stress to fish, most aquatic vaccines are delivered by this method. Administration by injection appears to impart greater protection, compared to other delivery systems. Other administration routes of vaccines are employed due to the time and cost involved in mass injection immunization of fish, especially the fry (< 2 g) held in hatchery systems. The delivery methods *via* immersion, oral, and cohabitation routes are more feasible for the farmers and minimize handling stress (Wang *et al.* 2020; Mzula *et al.* 2019). Using the immersion route, Dehghani *et al.* (2012) reported that bivalent formalin-killed vaccine against *A. hydrophila* and *A. veronii* biovar *sobria* protected rainbow trout, *Oncorhynchus mykiss*

against *A. hydrophila* with RPS of 67%. The disadvantages of the immersion delivery system are the requirement of large antigen amount and the low protection due to the inadequate vaccine antigen uptake through skin and gills (Mzula *et al.* 2019; Wang *et al.* 2020).

Numerous experiments on oral vaccines in aquaculture have been reported (Nayak *et al.* 2004; Siriyappagouder *et al.* 2014; Pereira *et al.* 2015). The oral delivery route has been suggested as a booster after primary immersion vaccination (Lillehaug 2014). Pereira *et al.* (2015) demonstrated that the oral booster vaccination enhanced the immune response against haemorrhagic septicemia due to *A. hydrophila* infection in the hybrid surubim, *Pseudoplatystoma corruscans* x *Pseudoplatystoma reticulatum*. The limitations of oral administration are insufficient protection, the inconsistency in antigen uptake by fish, and the degradation of naked antigens in the fish's foregut (Mzula *et al.* 2019). Therefore, to improve antigen uptake and allow the slow release of antigens *in vivo*, microencapsulation of oral vaccines in polymers, such as chitosan, micromatrix, alginates, liposome, and poly D,L-lactic-co-glycolic acid (PLGA), could be examined for the extensive application in aquaculture (Embregts & Forlenza 2016). Moreover, various scientists have recorded the oral tolerance in fish, evidenced by the decreased antibody production, due to repeated feeding of small amounts of antigen, vaccination of fish with immature immunity, low temperatures, and unfeasible antigen types (Wang *et al.* 2020).

Several adjuvants have been used to improve vaccine efficacy in aquaculture worldwide. For fish vaccines, oil adjuvants and water-soluble adjuvants are the commonly used emulsion formulations (Tafalla & Evensen 2014). Montanide adjuvants, Freund's adjuvants, and other conventional chemical adjuvants are the most widely used carrier systems in an experimental vaccine against MAS pathogens (*A. hydrophila*, *A. bestiarum*) in fish (Bastardo *et al.* 2012; Kozinska & Guz 2004; LaPatra *et al.* 2010).

Despite the reported efficiency, the adverse effects produced by conventional chemical adjuvants, such as chronic peritonitis, adhesions, and granulomas in extreme conditions have been documented (Mzula *et al.* 2019). Several biological-based adjuvants were investigated in vaccines against *A. hydrophila* to avoid their undesirable side effects. These include the extract of *Asparagus racemosus* (Thangaviji et al., 2012), modified garlic, *Allium sativum* adjuvant (Dash *et al.* 2014), nanomaterials including single-walled carbon nanotubes - SWCNTs (Gong *et al.* 2015; Liu *et al.* 2016b; Zhang *et al.* 2020) and poly lactic-co-glycolic acid nanoparticle (Dubey *et al.* 2016; Yun *et al.* 2017). Although nanoparticles have tremendous potential for application as delivery vehicles of novel vaccines, in-depth studies on antigen release, cellular uptake and intracellular fate of nanovaccines are required (Giri *et al.* 2021).

Most of the vaccination studies considered the production of antibodies and the RPS as the gold standard for determining the vaccine efficiency, as they found the a correlation between RPS and antibody titers (Wang *et al.* 2020). However, besides innate and antibody-mediated immunity, adaptive cellular immunity is also elicited by a vaccine, and its role in the vaccine efficacy should not be undervalued (LaPatra *et al.* 2010). Abdelhamed et al. (2017) suggested the predominance of cellular immunity over the antibody responses when they did not observe the correlation between antibody titers and the protection level in fish immunized with subunit vaccines. Technical difficulties in assessing cell-mediated immunity could be why the measurement of cellular immunity lacked in most studies (Mzula *et al.* 2019).

One of the problems that limit the development of commercial vaccines against MAS is the inter-species and intra-species diversity of the pathogens causing MAS. Some studies developed multivalent vaccines combining heterogeneously serological *A. hydrophila* strains (Chandran *et al.* 2002), a bivalent vaccine of *A. hydrophia* and *A. veronii* bv. *sobria* (Dehghani *et al.* 2012), or a monovalent vaccine containing a

representative strain selected based on the analysis of genotyping, protein and antibiotic resistance testing profiles of many *A. hydrophila* isolates from fish farms (Ciftci *et al.* 2016). With the advances in molecular biology, biotechnology, and vaccine immunology, potential immune antigens can be screened quickly. New hi-tech vaccines can be developed for broad-spectrum and consistent protection in various fish species against multiple pathogens in a single vaccine shot (Mzula *et al.* 2019). Up to now, many vaccines against MAS have been developed and experimentally tested. However, it is hard to compare the results of these studies due to the differences in fish species, vaccine preparations (e.g., immunization dose, type of vaccine, adjuvant), immunization and challenge methods and the duration of vaccination trials. Therefore, standard experimental design and guidelines for specific fish species and other general regulations issued by authorities within each country should be established to help researchers have a common understanding of the protective efficiency of their newly developed fish vaccines (Mzula *et al.* 2019).

2.4.2 Probiotic-based approaches

Probiotics, known as beneficial microbes, are a less complex approach for controlling aquatic diseases and reducing therapeutic chemicals and antibiotics in aquaculture (Pintado et al., 2010). Historically, the application of probiotics has centered on human and terrestrial to improve the host intestinal balance (Verschuere *et al.* 2000). Compared to terrestrial animals, aquatic species have a closer relationship to the external environment, as the water flow passes through their digestive tract (Martínez Cruz *et al.* 2012). The effects of probiotics include activating the immune defense, improving intestinal microbial balance, growth performance and survival rate of the host; competition for space, chemicals or available energy with harmful microorganisms; production of inhibitory substances or interference of quorum sensing of pathogenic bacteria (Di *et al.* 2019; Zorriehzahra *et al.* 2016).

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Beside vaccine developments against *Aeromonas* infections, many probiotic-based products have been developed and used in aquaculture for MAS control in fish (Table 4). *Bacillus* and *Lactobacillus* have been commonly used as probiotics due to their pH and heat-tolerance and long shelf-life (Kavitha *et al.* 2018). Other probiotic microoragnisms, such as *Paenibacillus ehimensis*, *Paenibacillus polymyxa, Rumelibacillus stabekisii, Pediococcus pentocaceus, Pseudomonas chlororaphis, Pseudomonas aeruginosa*, *Saccharomyces cerevisiae, Debaryomyces hansenii, Aspergillus oryzae, Enterococcus faecium* are also employed as putative probiotics against MAS. Moreover, numerous studies combined probiotics and prebiotics such as β-glucan, hemicellulose, chitosan, Jerusalem artichoke (JA), galactooligosaccharides (GOS), fructooligosaccharides (FOS), mannan oligosaccharides (MOS) to improve fish growth performance and enhance immunity against motile *Aeromonas* infections (Table 4).

Although various benefits of potential probiotics have been demonstrated in the management of MAS outbreaks in aquaculture, further studies should aim to determine appropriate methods and optimal doses to improve probiotic's efficacy in different animals (species and sizes) and environmental conditions (Di *et al.* 2019). For example, Farias et al. (2016) suggested not feeding too many probiotics as partial suppression of the immunological responses could happen. The use of several probiotics strains in a combination did not seem to significantly improve the immunostimulant effect, compared with a single probiotic (Aly *et al.* 2015; Park *et al.* 2017). Melo-Bolívar *et al.* (2021) also indicated that it is not possible to conclude whether multistrain probiotics provide enhanced benefits over monostrain probiotics counterparts. However, there is an increasing trend of using multistrain probiotic-based products in aquaculture. As the antagonistic effect of probiotics against MAS pathogens may be host and strain-specific (Habil et al., 2011), the origin, source and type of

probiotics in connection with fish need to be screened *in vitro* and *in vivo* for ensuring their benefits and technical feasibility before being administrated as dietary supplements in aquaculture practice (Di *et al.* 2019; Patel *et al.* 2016). There are limited studies on evaluating of probiotic effects in fish farms, where *Aeromonas* infections could be different between fish and the environmental microflora and the water quality could affect the efficiency of the product. These clinical trial designs in fish farm conditions could also improve the economic models of probiotic utilization (Hayatgheib *et al.* 2020).

Although many studies reported the benefits of probiotics in aquaculture, there are still gaps in elucidating the underlying mechanism of the beneficial effects and potentially harmful effects of the probiotics (Amenyogbe *et al.* 2020; Hayatgheib *et al.* 2020; Wang *et al.* 2019a). The risks of transferring antibiotic resistance genes from probiotics to pathogenic bacteria and producing antibiotics by probiotics should not be underestimated (Martínez Cruz *et al.* 2012; Wang *et al.* 2019a). Notably, due to no specific regulations for probiotics used in aquaculture, an industry-standard is required to normalize and manage the production and usage of commercial probiotic products. A list of selection criteria for aquatic probiotics have been proposed, regarding their safety for both aquatic animals and human; adaptability in both aquatic environment and the host intestinal tract; function on growth, digestion, immunity, disease resistance, water quality or general welfare; and convenience for storage and administration (Wang *et al.* 2019a).

In the authors' view, probiotics play a vital role in sustainable aquaculture, but the viability of probiotics in aquaculture is closely related to downstream processing operations such as centrifugation, drying and storage conditions. The encapsulation technology should be applied to avoid a large fluctuation in the quality of probiotics. Although considerable evidence to show significant improvement of

immunostimulation between single and multiple probiotics is lacking, the combination of variant strains with optimization of their proportions might be more beneficial to enhance the growth performance and health status of aquatic animals. In addition, probiotic-based products including synbiotics (probiotics-prebiotics) and tribiotics (probiotics-prebiotics-postbiotics), need to be investigated on a laboratory scale and industrial scale to optimize feed formulation for aquatic animals. Future research should clarify the interaction between probiotics and host using modern biotechnological approaches such as metagenomics, transcriptomics, proteomics and metabolomics to understand the probiotic activity. The national authorities should promulgate strict policies to enforce probiotic manufactures and suppliers responsible for their products.

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Table 4. Summary of *in vivo* research of probiotics against MAS in major food fish species

Fish species	Probiotics	Prebiotics	Challenged *Aeromonas*	RPS*	References
Nile tilapia (*Oreochromis* niloticus)	*S. cerevisiae*	Non	*A. hydrophila*	40%-75%	Abdel-Tawwab *et al.* (2008) Abdel-Tawwab (2012a) Abass *et al.* (2018)
	L. lactis D1813	Non	*A. hydrophila*	72%-94%	Suprayudi *et al.* (2017)
	L. rhamnosus GG	Non	*A. hydrophila*	66.7%	Ngamkala *et al.* (2010)
	L. plantarum	Non	*A. hydrophila*	40-57%	Liu *et al.* (2016d)
	L. plantarum	Non	*A. hydrophila*	74.4%	Hamdan *et al.* (2016)
	L. plantarum	Non/β-glucan	*A. hydrophila*	64% / 72%	Dawood *et al.* (2020)
	M. luteus	Non	*A. hydrophila*	68.8%	El-Rhman *et al.* (2009)
	B. amyloliquefaciens	Non	*A. hydrophila*	38.3%-46%	Saputra *et al.* (2016)
	B. subtilis	Non	*A. hydrophila*	48.3%	Aly *et al.* (2008)
	L. acidophilus	Non	*A. hydrophila*	43.5%	
	Mixture: *B. subtilis,* *L. acidophilus.*	Non	*A. hydrophila*	52%	
	B. subtilis	Non	*A. hydrophila*	35.2%-62%	Addo *et al.* (2017)
	B. subtilis	Hemicellulose	*A. hydrophila*	59.2%-64.8%	
	P. ehimensis	Non	*A. hydrophila*	26.8%-41.5%	Chen *et al.* (2019c)
	R. stabekisii	Non	*A. hydrophila*	60%	Tan *et al.* (2019)
	Mixture: *B. subtilis,* *S. cerevisiae,* *A. oryzae.*	Non	*A. hydrophila*	19%	Iwashita *et al.* (2015)

	Mixture: *L. acidophilus,* *E. faecium,* *Bifidobacterium* sp.	Non/ MOS/ Chitosan	*A. sobria*	20%/ 40%/ 6.67%	Cavalcante *et al.* (2020)
	S. cerevisiae	Non	*A. sobria*	25.4%-62.7%	Reda *et al.* (2018)
	L. plantarum	Non	*A. sobria*	73.3%-80%	Abou-El-Atta *et al.* (2019)
Red hybrid tilapia (*Oreochromis* spp.)	*L. brevis*	Non/FOS	*A. hydrophila*	19.5% / 28/7%	Liu *et al.* (2017b)
	L. plantarum	Non/FOS	*A. hydrophila*	36.8% / 48.3%	
	L. rhamnosus GG	JA	*A. veronii*	52%	Sewaka *et al.* (2019)
Mozambique tilapia (*Oreochromis mossambicus)*	*B. licheniformis*	Non	*A. hydrophila*	55.7%	Gobi *et al.* (2018)
Grass carp (*Ctenopharyngodon idella*)	*B. subtilis*	Non	*A. hydrophila*	NA	Tang *et al.* (2019)
	Mixture: *B. velezensis,* *B. cereus,* *L. casei.*	Non	*A. hydrophila*	58.8%-87.5%	Chen *et al.* (2020)
	P. pentocaceus	Non	*A. hydrophila*	42.6%	Gong *et al.* (2019)
Common carp (*Cyprinus caprio*)	*B. cereus*	Non	*A. hydrophila*	62.6%-100%	Djauhari *et al.* (2016)
	B. coagulans	Non	*A. hydrophila*	20.8%	Gupta *et al.* (2014)
	B. licheniformis	Non	*A. hydrophila*	25%	
	P. polymyxa	Non	*A. hydrophila*	37.5%	
	P. polymyxa	Non	*A. hydrophila*	29.9%-56.7%	Gupta *et al.* (2016)
	L. plantarum	Non	*A. hydrophila*	8.3%-33.3%	Soltani *et al.* (2017)
	L. plantarum	Non	*A. hydrophila*	23.5%	Kazuń *et al.* (2018)
	L. fermentum	Non Non	*A. hydrophila* *A. hydrophila*	28.6% 45.5%-77.3%	Ahmadifar *et al.* (2019) Krishnaveni *et al.* (2021)
	L. lactis	Non	*A. hydrophila*	31.3%-43.8%	Feng *et al.* (2019)

	E. faecium	Non	*A. hydrophila*	77.8%	Gopalakannan& Arul (2011)
Crucian carp (*Carssius carassius*)	*L. lactis*	Non	*A. hydrophila*	NA	(Dong *et al.* 2018)
	B. velezensis	Non	*A. veronii*	53.3%-75%	Zhang *et al.* (2019a)
Javanese carp (*Puntius gonionotus*)	*E. faecalis*	Non	*A. hydrophila*	53%	Allameh *et al.* (2017)
Rohu (*Labeo rohita*)	*B. licheniformis*	Non	*A. hydrophila*	73.6%	Ramesh *et al.* (2015)
	B. pumilus	Non	*A. hydrophila*	62.1%	Ramesh *et al.* (2017)
	B. aerophilus	Non	*A. hydrophila*	56.2%-66.1%	Ramesh& Souissi (2018)
	B. subtilis	Non	*A. hydrophila*	38.4%-75.8%	Giri *et al.* (2014)
	B. subtilis	Non/GOS	*A. hydrophila*	87.5% / 93.8%	Devi *et al.* (2019)
	B. amiloliquefaciens	Non	*A. hydrophila*	36.4%-72.7%	Nandi *et al.* (2018)
	P. aeruginosa	Non	*A. hydrophila*	15%-50.1%	Giri *et al.* (2012)
	P. aeruginosa	Non	*A. hydrophila*	52%	Hoque *et al.* (2019)
	L. plantarum	Non	*A. hydrophila*	26.1%-73.8%	Giri *et al.* (2013)
	Mixture: *B. subtilis,* *L. plantarum.*	Non	*A. hydrophila*	69.2%	Giri *et al.* (2014)
	Mixture: *B. subtilis* *P. aeruginosa.*	Non	*A. hydrophila*	61.5%	
	Mixture: *B. subtilis,* *L. plantarum,* *P. aeruginosa.*	Non	*A. hydrophila*	84.5%	
	Mixture: *L. lactis,* *S. cerevisiae.*	Non	*A. hydrophila*	60%	Mohapatra *et al.* (2014)

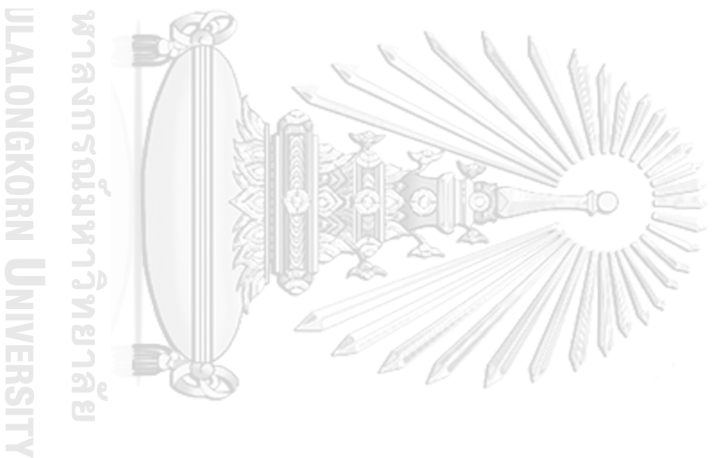
	Mixture: *B. subtilis,* *S. cerevisiae.*	Non	*A. hydrophila*	55%	
	Mixture: *B. subtilis,* *L. lactis.*	Non	*A. hydrophila*	65%	
	Mixture: *B. subtilis,* *L. lactis,* *S. cerevisiae.*	Non	*A. hydrophila*	80%	
	B. methylotrophicus	Non	*A. hydrophila*	38.8%	Mukherjee *et al.* (2019)
	B. amiloliquefaciens	Non	*A. hydrophila*	46.2%	
	B. licheniformis	Non	*A. hydrophila*	57%	
	Mixture: *B. methylotrophicus,* *B. amiloliquefaciens.*	Non	*A. hydrophila*	62.3%	
	Mixture: *B. methylotrophicus,* *B. licheniformis.*	Non	*A. hydrophila*	83%	
	Mixture: *B. amiloliquefaciens,* *B. licheniformis.*	Non	*A. hydrophila*	70%	
	Mixture: *L. acidophilus,* *Bifidobacterium,* *S. thermophiles,*	Non	*A. hydrophila*	46%-67%	Kanwal& Tayyeb (2019)

	L. bulragicus.				
Catla (*Labeo catla*)	*B. circulans*	Non	*A. hydrophila*	35.7%-96.4%	Bandyopadhyay& Mohapatra (2009)
	B. circulans	Non	*A. hydrophila*	37.5%	Parthasarathy& Ravi (2011)
	B. megaterium	Non	*A. hydrophila*	56.3%	
	L. plantarum	Non	*A. hydrophila*	68.8%	
	Mixture: *B. megaterium* *L. plantarum,* *L. acidophilus.*	Non	*A. hydrophila*	NA	Patel *et al.* (2016)
Shabout (*Arabibarbus grypus*)	*L. casei*	Non	*A. hydrophila*	46.3%	Mohammadian *et al.* (2016)
	L. plantarum	Non	*A. hydrophila*	52.4%	
	L. bulgaricus	Non	*A. hydrophila*	66.7%	
Shabot (*Tor grypus*)	*L. casei*	Non	*A. hydrophila*	47.7%	Mohammadian *et al.* (2018)
	L. plantarum	Non	*A. hydrophila*	52.4%	
	L. bulgaricus	Non	*A. hydrophila*	66.7%	
	L. casei	Non	*A. hydrophila*	46.2%-69.2%	Mohammadian *et al.* (2019)
Perch (*Perca fluviatilis*)	*P. chlororaphis*	Non	*A. sobria*	9.3%	Gobeli *et al.* (2009)
Snakehead (*Channa striata*)	*S. cerevisiae*	Non	*A. hydrophila*	34.2%	Munir *et al.* (2018)
	L. acidophilus	Non	*A. hydrophila*	60.5%	
	S. cerevisiae	Non	*A. hydrophila*	58.5%	Talpur *et al.* (2014)
	L. acidophilus	Non	*A. hydrophila*	67.7%	
Hydrid catfish (*Clarias* spp.)	*L. plantarum*	Non	*A. hydrophila*	100%	Butprom *et al.* (2013)
Pla-mong catfish (*Pangasius bocourti*)	*B. aerius*	Non	*A. hydrophila*	64.2%	Meidong *et al.* (2018)
African catfish (*Clarias gariepinus*)	*B. thuringiensis*	Non	*A. hydrophila*	62.2%-73%	Reneshwary *et al.* (2011)
Basa fish (*Pangasius bocourti*)	*L. paraplantarum*	Non	*A. hydrophila*	56.3%	Meidong *et al.* (2021)
Pacific red snapper (*Sebastes rubernimus*)	*L. sakei*	Non	*A. veronii*	NA	Reyes-Becerril *et al.* (2012)

Pacu (*Piaractus brachypomus*)	*Mixture:* *B. subtilis,* *B. cereus.*	Non	*A. hydrophila*	75%-100%	Farias *et al.* (2016)
Leopard grouper (*Mycteroperca rosacea*)	*D. hansenii*	Non	*A. hydrophila*	NA	Reyes-Becerril *et al.* (2011)
Dabry's sturgeon (*Acipenser dabryanus*)	*B. subtilis*	Non	*A. hydrophila*	30%-65%	Di *et al.* (2019)

RPS: relative percent survival; * RPS = (1 - mortality in experiments / mortality in controls) * 100; NA: Not available;

JA: Jerusalem artichoke, GOS: galactoseoligosaccharides, MOS: fructoseoligosaccharides, MOS: mannan oligosaccharides



2.4.3 Plant-based approaches

Phytogenics, phytochemicals, or phytomedicines are natural bioactive compounds extracted from numerous kinds of plants. They are eco-friendly and safe for humans and animals, including aquatic organisms (Burdock & Carabin 2004; Kuebutornye & Abarike 2020). Based on their synthetic pathway, phytogenics can be divided into three major groups: terpenoids, phenolic metabolites and alkaloids and other nitrogen-containing metabolites (Harborne 1999). Phytochemicals can be extracted from many parts of fresh or dried plants such as roots, leaves, barks, flowers, fruits, and seeds.

In aquaculture, phytogenics have been used as a therapeutic and prophylactic treatment for many infectious diseases (Alsaid *et al.* 2010; Citarasu 2010; Manilal *et al.* 2012; Militz *et al.* 2014; Rattanachaikunsopon & Phumkhachorn 2009). They may act as an immunostimulant associated with disease prevention that enhances the host innate immune response (Sakai 1999). Consequently, the animal becomes more resistant to infections. Besides, phytochemicals as potential antioxidant agents can effectively reduce stress (Chander *et al.* 1992; Chander *et al.* 1998; Citarasu *et al.* 1999). Thus, the animals are in the optimal physiological state to adapt to disturbances in the aquatic environment or pathogens. On the other hand, plant-derived compounds can act as an alternative to chemotherapy with antibacterial, antiviral, antifungal, and antiparasitic ability (Citarasu 2010).

Many investigations have used phytochemicals to control MAS (Reverter *et al.* 2014). Phytochemicals are administrated into fish by oral feeding, immersion, or intramuscular and intraperitoneal injection. Amongst these, immersion is the least suitable method because bioactive compounds in the extract dilute quickly in the water, and it is not easy to apply them on an industrial scale. The application in the field requires a large volume of phytochemicals that could be costly. Hence, it is suitable for small stage fish or ornamental fish such as Siamese fighting fish, *Betta*

splendens (Purivirojkul 2012). Besides, some research used leaf extract of Chinese toon, *Toona sinensis* (Wu *et al.* 2010); Guduchi, *Tinospora cordifolia* (Alexander *et al.* 2010); Thuthuvalai, *Solanum trilobatum (Divyagnaneswari et al. 2007)*; and milky mangrove, *Excoecaria agallocha* (Dhayanithi *et al.* 2012) by injection method to control *A. hydrophila* with RPS value s of 47%, 87.9%, 70.84% and 66.7% respectively. Nonetheless, injection is a stressful and expensive method and not feasible for small fish, whereas oral feeding can be applied in all fish stages without considerable side effects on the animals and at an affordable cost. Thus, using the phytochemical extract as a dietary supplement is the most preferred method in aquaculture, and used in many investigations to control *A. hydrophila* causing MAS in a variety of freshwater fish species. For example, the feed supplemented with garlic powder can enhance immune parameters and increase the survival of Nile tilapia (Shalaby *et al.* 2006), rohu (Sahu *et al.* 2007), African catfish (Thanikachalam *et al.* 2010), or rainbow trout (Nya & Austin 2009b). The extract of *Zingiberaceae* families such as ginger, *Zingiber offcinale* or turmeric, *Curcuma longa* has been used in feed formulation to improve the health status of tilapia (Payung *et al.* 2017; Naliato *et al.* 2021), rohu (Sahu *et al.* 2008), common carp (Abdel-Tawwab & Abbass 2017) and rainbow trout (Nya & Austin 2009a) against *A. hydrophila* with RPS of 58.7%, 100%, 35.3% and 100%, respectively. In addition, root powder of American ginseng, *Panax quinquefolius* (Abdel-Tawwab 2012b), and Indian ginseng, *Withania somenifera* (Sharma *et al.* 2010; Zahran *et al.* 2018) are potential phytomedicines as immunity booster of fishes. While diet supplemented with American ginseng improved survival of Nile tilapia with RPS value of 35.3%, Indian ginseng showed the efficacy with RPS 42.9% and 71% on rohu and Nile tilapia, respectively. Root powder of licorice, *Glycyrrhiza glabra* L. was effective to improve the survival of Nile tilapia against *A. hydrophila* with RPS 100% compared to the control group (Abdel-Tawwab & El-Araby 2021). The plants with an acrid taste that are rich in phenolic metabolites such as green tea, *Camellia sinensis* (Abdel-Tawwab

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et al. 2010); false daisy, *Eclipta alba* (Christybapita *et al.* 2007); cinnamon, *Cinnamonum zeylanicum* (Ahmad *et al.* 2011); guava, *Psidium guajava* (Giri *et al.* 2015; Gobi *et al.* 2016); Korean mistletoe, *Viscum album coloratum* (Park & Choi 2012) could be extracted to apply in *A. hydrophila* management. The percentage mortality was significantly reduced as evidenced by high RPS values (65.55% - 89.9%) in the tested groups compared to control groups. Numerous others common vegetables, beans and traditional herbals have been studied to use as immunostimulants, and antipathogenic agents in aquaculture to control *Aeromonas* infections, such as holy basil, *Ocimum sanctum* (Das *et al.* 2015; Logambal *et al.* 2000), basil, *Ocimun basilicum* (Amirkhani & Firouzbakhsh 2015), moringa, *Moringa oleifera* (El-Gawad *et al.* 2020), velvet bean, *Mucuna pruriens* (Musthafa *et al.* 2018), Indian bael, *Aegle marmelos* (Pratheepa *et al.* 2010), scutch grass, *Cynodon dactylon* (Kaleeswaran *et al.* 2011), stinging nettle, *Urtica dioica* (Ngugi *et al.* 2015; Bilen *et al.* 2016), and green chiretta, *Adrographis paniculata* (Palanikani *et al.* 2020).

Apart from using a single phytobiotic, the combination of different plant-based ingredients has shown the potential to combat MAS. For example, the mixture of root extract from huangqi, *Astragalus membranaceus* and flower extract from Japanese honeysuckle, *Lonicera japonica* decreased 55% of Nile tilapia mortalities by oral administration compared to the group without medicinal herbs (Ardó *et al.* 2008). The fed diet supplemented with 5% root extract of huangqi and 5% powder of lingzhi mushroom, *Ganoderma lucidum* improved the survival of common carp challenged with *A. hydrophila* by 30%, compared to the control group (Yin *et al.* 2009). More recently, a mixture of lemon peel, *Citrus aurantifolia*, dry powder and probiotic *B. licheniformis* was used to protect common carp from *A. hydrophila* infection by improving fish immunity and antioxidative responses (Sadeghi *et al.* 2021). The majority of phytochemical research in aquaculture has focused on the crude or total extract

from different parts of the plant. Besides, several investigations have used essential oils containing bioactive compounds such as allicin, carvacrol, thymol or terpinen-4-ol to treat *A. hydrophila* infection (Baldissera *et al.* 2017; da Cunha *et al.* 2019; Dong *et al.* 2020; Liu *et al.* 2020; Nya *et al.* 2010; Souza *et al.* 2016).

Bioactive compounds have limited biological half-life resulting in low retention time in the bloodstream and tissue (Cui *et al.* 2009). Nanotechnology has offered the solution to overcome this limitation via scientific evidence related to the protection of nanoparticles from degradation. Simultaneously, nanoparticles may enhance the antibacterial activity of phytochemicals, especially essential oils due to the ability to combine with the outer membrane and increase membrane permeability (Hemmila *et al.* 2010). Based on these encouraging results, recent research focused on applying nanotechnology to synthesize phytomedicine for controlling *A. hydrophila* infection in aquaculture. For example, a diet supplemented with engineered silver nanoparticles of thumbai, *Leucas aspera* or four nano-encapsulated essential oils of oregano, Tasmanian blue gum, *Eucalytus globulus*; tea tree, *Melaleuca altermifolia*; and lavender, *Lavendula angustifolia* could treat *A. hydrophila* (Antony *et al.* 2013; Gholipourkanani *et al.* 2019). The results highlighted the potential of new formulations of phytochemicals to improve bacterial disease control in cultured fish. The fed diet supplemented with cinnamon and ginger nanoparticles generated by mechanical milling using a planetary ball mill was reported to enhance immune response and protect experimental fish against *A. hydrophila* (Abdel-Tawwab *et al.* 2018; Korni & Khalil 2017). The result revealed no mortality of Nile tilapia among tested groups fed by diets supplemented with nano-cinnamon, while the control groups showed 66.7% of mortality (Abdel-Tawwab *et al.* 2018). Besides, ginger nanoparticles were shown to be more effective than normal ginger in the prevention of MAS with the former

protecting 100% of *Cyprinus carpio* fingerlings (100% RPS), compared to only 80% of the fish fed by ginger diet with an RPS of 71.4% (Korni & Khalil 2017).

The effects of phytogenics to control MAS relate to immune response enhancement and antibacterial activity. They act as promising bio-antibiotics to block DNA and protein synthesis, lyse the bacterial cell wall, inhibit enzyme secretion, or intervene in the signalling mechanism of the quorum-sensing pathway (Citarasu 2010). Nonetheless, phytotherapy's efficacy is impacted by many factors such as constituent and quality of extract, dose, duration and administration. A suitable mode of administration should be considered first, followed by the type of phytochemicals, dose and duration, which will help increase their effectiveness in fish farms. Further investigations should discover novel phytochemical plants, novel combinations between plant-based and probiotic-based products, optimise the extraction process and standardize feed formulation, dose and duration. Simultaneously, the research themes related to the increase of long-term stability and enhancement of bioavailability of phytomedicines using encapsulation technology and nanotechnology are strongly recommended.

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2.4.4 Bacteriophage-based approaches

Bacteriophages or phages are unique viruses that can infect and kill bacterial cells in the case of lytic phages. The cycle of lytic phages goes through five steps to complete their replication process: absorption, penetration, replication, maturation and release (Fischetti 2005; Hogg 2013; Skurnik & Strauch 2006). At the end of the phage replication process, under conductor and protective proteins, the lysozyme and endolysin are used to hydrolyze peptidoglycan of the cell wall and release the new phages. Based on this characteristic, lytic phage therapies have been developed to control bacterial infections in animals and humans. The research of phages in aquaculture or biocontrol of MAS is mostly performed in the laboratory scale and

mainly focuses on *A. hydrophila*. In 2019, Proteon Pharmaceuticals became the first company that launched the phage-based product, namely BAFADOR (the cocktails contained seven phages: three phages 50AhydR13pp, 60AhydR15PP, 25AhydR2PP against *A. hydrophila* and four other phage trains against *Pseudomonas fluorescens*) in the aquaculture industry (Schulz *et al.* 2019).

Phages can be used as therapeutic or prophylactic agents based on the type of infection and target organisms (Anand *et al.* 2016; Cruz-Papa *et al.* 2014; Hoang *et al.* 2019; Pereira *et al.* 2011; Yuan *et al.* 2018). As a treatment, in an early report in 1981, phage AH1 was used to protect 100% experimental loach, *M. angillicaudatus* from *A. hydrophila* infection (Wu *et al.* 1981). Administration of phages using intraperitoneal injection is one of the most popular methods in aquaculture. In 2013, *Myoviridae*-phage pAh1-C and pAh6-C were administrated intraperitoneally to reduce loach mortality with *A. hydrophila* infection (Jun *et al.* 2013). This method also protected 100% Nile tilapia using *Caudovirales*-phage UP87 (Cruz-Papa *et al.* 2014) and striped catfish with *Myoviridae*-phage (Le *et al.* 2018). Recently, phage MJG was applied to control pathogenic *A. hydrophila* infection in rainbow trout, *O. mykiss* by injection, immersion and oral administration. The results showed that 100% of fish in the injection route survived while immersion and oral ones provided 80% and 70% survival, respectively (Cao *et al.* 2020). The immersion of phage was also used to control MAS in tilapia culture that reduced 50% mortality from 68% in the control group to 18% in the treatment group using *Podoviridae*-phage ϕZH1 (El-Araby *et al.* 2016). As a prophylactic agent, phages can be released directly to pond water as a water-borne administration to decrease the concentration of pathogens in a disease season or reduce the infection when the temperature changes abruptly (Pereira *et al.* 2011; Rong *et al.* 2014; Silva *et al.* 2014; Silva *et al.* 2016; Vinod *et al.* 2006). However, the preparation of many phages for use in commercial ponds or open aquaculture

system is a crucial obstacle. Because of this, hitherto, only a few researchers have focused on phages as a prophylactic agent. Although phages have been considered as an alternative to antibiotics for sustainable aquaculture, especially in controlling MAS, many disadvantages of phage therapy such as administration route, narrow host range, phage-resistant pathogens, and the transfer of critical genes hampered their application in the aquaculture ponds.

Regarding phage administration, most research related to phage therapy in *Aeromonas* biocontrol was conducted on a small scale. Future research should identify the targeted stage of fish to select optimal routes. For instance, both intraperitoneal and intravenous injection is impractical to larvae, fry, or fingerlings of fish, while an immersion route for large volumes of culture water or flow-through systems would not be feasible (Richards 2014).

The narrow host range and phage-resistant bacteria are two significant challenges in the widespread use of phages in aquaculture. Numhiteerous studies have revealed that phages could infect at many locations on the surface of bacterial cells, including receptors on the bacterial cell wall capsule, slime layers, appendages or flagella (Fehmel *et al.* 1975; Guerrero-Ferreira *et al.* 2011; Marti *et al.* 2013; Shin *et al.* 2012; Xia *et al.* 2011). Nonetheless, some phages can only recognize and bind specifically to a single receptor and are called monovalent phages. On the other hand, in nature, bacteria and phages exist parallelly, not only as enemies but also as co-evolution agents. Natural selection puts pressure on bacteria to make evolution against infection and create phage-resistant variants. The mechanisms of forming phage-resistant bacteria involve prevention of phage absorption (Stummeyer *et al.* 2006; Sutherland *et al.* 2004), prevention of phage DNA entry (Mahony *et al.* 2008; Moak & Molineux 2000; Sun *et al.* 2006), digestion of phage nucleic acids (Chaudhary 2018; Dupuis *et al.* 2013; Goldfarb *et al.* 2015; McGrath *et al.* 1999; Oliveira *et al.* 2014), inhibition of phage

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DNA replication (Barrangou & Van Der Oost 2015; Chaudhary 2018; Gordeeva *et al.* 2018), and interference with phage assembly (Carpena *et al.* 2016; Fillol Salom 2019; Fillol-Salom *et al.* 2018; O'Hara *et al.* 2017). Thus, further research about the strategies to overcome the narrow host range and phage-resistant variants has been reported to focus on three approaches: phage cocktails, engineered phages, and antimicrobial phage-derived proteins.

Hitherto, not much research about phage cocktails in aquaculture has been reported. Some of them focused on phage cocktails to control *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, or *A. salmonicida* (Chen *et al.* 2018; Kim *et al.* 2019b; Mateus *et al.* 2014; Ma *et al.* 2019c) and only one research to control MAS caused by *A. hydrophila* on rainbow trout (Schulz *et al.* 2019). Even though phage cocktails can expand the host range, bacteria can still resist. Hence, experimenting phage cocktails simultaneously with update collection with newly isolated phages is recommended. Along with phage cocktails, engineered phages were designed to enhance anti-bacterial activity and broaden the host range of phages using the anti-CRISPR-Cas data and the modifications of tailed-protein (Bhattarai *et al.* 2012; Dedrick *et al.* 2019; Lin *et al.* 2012; Mahichi *et al.* 2009; Pei & Lamas-Samanamud 2014). Unfortunately, in aquaculture, only natural phages were studied and tested until now, perhaps due to their limitations on the applicability in commercial culture, techniques and concerns with GMO. Another research related to phage-derived enzymes was focused on reducing phage-resistant variants such as endolysins (phage-encoded peptidoglycan hydrolase), holins (cell membrane disturbing proteins), VAPGH (virion-associated peptidoglycan hydrolase) and polysaccharide depolymerase (Bernhardt *et al.* 2002; Briers & Lavigne 2015; Maciejewska *et al.* 2018; Roach & Donovan 2015). The results from previous studies showed promise to control bacterial infection in the veterinary sector (Fan *et al.* 2016; Rodríguez-Rubio *et al.* 2016; Swift *et al.* 2015; Wang

et al. 2009). However, this research theme seems difficult in aquaculture applications due to industrial scale, cost-effectiveness, and administration.

During phage therapy, many phage-resistant variants were generated from the loss or the alteration of receptor reduced fitness (León & Bastías 2015; Oechslin 2018; Zahid *et al.* 2008; Azam & Tanji 2019). This suggests using phage-resistant variants as a material for vaccine development against *Staphylococcus aureus* (Capparelli *et al.* 2010), and *Yersenia pestis* (Filippov *et al.* 2011). In aquaculture, a few studies on phage-resistant pathogens such as *Flavobacterium psychrophylum* (Castillo *et al.* 2015), *Flavobacterium columnare* (Laanto *et al.* 2012), *Vibrio anguillarum* (León *et al.* 2019), and *A. hydrophila* (Jun *et al.* 2013) have been reported. However, no vaccine investigation have been done based on these materials so far. On the other hand, recent research indicated improved survival and enhanced immune parameters after injecting phage lysate into the fish (Schulz *et al.* 2019). The enhanced immune system is explained early by the interaction of the host immune system and antigens from phage lysate including extracellular proteins and lipopolysaccharides (Park *et al.* 2014; Weber-Dabrowska *et al.* 2000), suggesting a potential for novel vaccine development. Indeed, a phage lysate vaccine against *A. hydrophila* in common carp (*Cyrinus carpio*) has been studied. The results showed that phage lysate generated by phage pAh-6C for *A. hydrophila* JUNAH strain induces a robust immune response better than the formalin-killed vaccine because the phage lysate possessed some of the highly conserved antigens. In other words, using lytic phages minimized the deformities of antigenic proteins compared to formalin. Nonetheless, the untreated exotoxins and endotoxins in phage lysate preparation are limitations and should be a focus of further research (Yun *et al.* 2019). Phage lysate vaccines have been reported as potential vaccines in various fields of the veterinary sector to protect animals against equine salmonellosis, hemorrhagic septicemia developed from *Pasteurella*

multocida and bovine brucellosis (Kumar *et al.* 2018; Pushpa *et al.* 2017; Qureshi & Saxena 2019; Saxena & Raj 2018). Overall, future research should consider phage-resistant motile *Aeromonads* and phage lysate as potential candidates for inexpensive vaccine development against MAS in aquaculture.

The last challenge of phage therapy in aquaculture is related to the transfer of critical genes. Lysogenic phages as a vector for both generalized (when phage packaging accidentally incorporates) and specialized (when faulty excision of the prophage) transduction were indicated as a factor for horizontal transfer of antibiotic-resistance genes and virulence genes (Mahony *et al.* 2011; Pirnay *et al.* 2015; Wittebole *et al.* 2014). Although lytic phages are vulnerable to increased transformation due to the smash of bacterial cells in the last step of the infection cycle, they are an irreplaceable choice for phage therapy.

2.4.5 Omics-based approaches

Omics is a broad field of biological sciences ending with -omics, such as genomics, transcriptomics, proteomics, or metabolomics. Omics aims to identify, quantify, and characterize all biological molecules’ structure, function, and dynamics in a biological system (cell, tissue, organ, biological fluid or organism). The field of omics has been driven mostly by technological advances for high-throughput analysis of biological molecules and bioinformatics for data interpretation. That leads to a broad range of applications of omics technologies across all fields of life sciences. The recent emergence of omics applications in aquaculture study has demonstrated the power of this emerging novel technology in studies of disease in aquaculture (Rise *et al.* 2019; Nguyen & Alfaro 2020; Nguyen *et al.* 2019a; Nguyen 2020). There is a growing interest in omics studies of motile *Aeromonas* infections in aquaculture species, which cover different subjects, such as characterization of genome and other biological aspects of Aeromonads, responses of *Aeromonas* to antibiotics, and responses of fish to *Aeromonas* infections (Figure 2).

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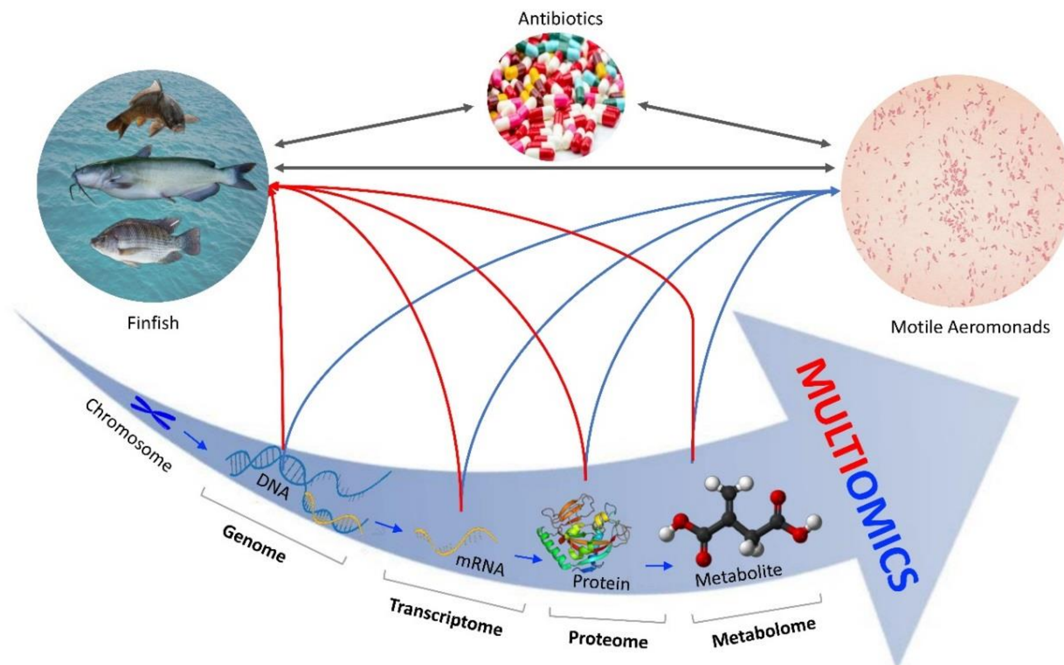

Figure 2. Omics studies of motile *Aeromonas* infections in aquaculture species

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Genome sequencing is a practical final step for pathogen confirmation and provides critical information to understand the virulence factors. Improvements in next-generation sequencing have resulted in an upsurge of genome sequences of *Aeromonas* species. The first genome of *Aeromonas* was reported on *A. hydrophila* ATCC 7966T (Seshadri *et al.* 2006). Subsequently, the draft genomes or complete genomes of other *A. hydrophila* strains and many other *Aeromonas* species have been sequenced (Reith *et al.* 2008; Li *et al.* 2011; Colston *et al.* 2014; Pang *et al.* 2015). This allows comparative genomic analyses and accurate species classification among *Aeromonas* species within a genus, critical for insights into the evolution and proper identification in clinical and veterinary diagnostic laboratories. Besides, the genomic information of *Aeromonas* can be helpful in the development of new drugs and vaccines. Such kind of applications have been investigated in other microorganisms in aquaculture such as *Streptococcus agalactiae* (Favero *et al.* 2020) and *Pseudomonas*

aeruginosa (Rashid *et al.* 2017). However, these approaches have not been reported for MAS, requiring future research to investigate and confirm their effectiveness for MAS.

In addition to genomes, other biological and physiological aspects of *Aeromonas* species have been characterized by omics technologies, including transcriptomics, proteomics and metabolomics. Proteomics approaches were successfully employed to identify extracellular/ outer membrane proteins associated with Aeromonads' pathogenicity and environmental adaptability, which could be used as potential vaccine candidates against *Aeromonas* infections in fish (Wang *et al.* 2017; Wang *et al.* 2019b; Yu *et al.* 2007). Proteomics was also combined with transcriptomics to reveal the iron transport factors of *A. hydrophila* under iron limitation (Teng *et al.* 2018). The expression of genes and proteins related to enterobactin synthesis and virulence establishment confirms that iron-limitation efficiently enhanced the virulence of *A. hydrophila*. Integration between proteomics and metabolomics revealed a crucial role of S-ribosylhomocysteine lyase in quorum sensing and metabolism of *A. hydrophila* (Yao *et al.* 2019). These studies demonstrated that proteomics is a powerful tool for characterizing virulence factors and finding potential candidates for developing appropriate diagnostics and non-antibiotic therapeutics for *Aeromonas*.

Understanding the mechanism of host defense against *Aeromonas* invasion is crucial for the development of disease control method. To this end, transcriptomics, proteomics and metabolomics have been employed to characterize the molecular pathway underlying the host response to pathogen and identify molecules of interest for biomarker discovery. There are abundant transcriptomics studies investigated on the gene expression of the host in response to *Aeromonas* species (Yang *et al.* 2016; Tran *et al.* 2015; Ling *et al.* 2019; Long *et al.* 2015). Similarly, proteomics techniques have been applied to characterize the expression of various proteins and identify

potential protein biomarkers associated with Atlantic salmon response to Aeromonads (Liu *et al.* 2017a). Many of these proteins could be considered as potential biomarkers for Atlantic salmon immune responses. Several workers have integrated omics to study the fish-*Aeromonas* interactions at both gene and protein levels (Chen *et al.* 2010; Long *et al.* 2015). The integration of transcriptomic and proteomic analyses was also developed to characterize the splenic immune mechanisms of rainbow trout infected by *A. salmonicida* subsp. *salmonicida* (Long *et al.* 2015). In a target omics approach, Chen *et al.* (2010) identified Beta2-microglobulin as an immune molecule of large yellow croaker to *A. hydrophila* at both gene and protein levels by the combination of differential proteomics with the use of expressed sequence tag (EST) resource.

The number of metabolomics studies on *Aeromonas* infections in fish species still remains limited. A few studies have highlighted the metabolite changes involved in fish's biochemical response against *Aeromonas,* which could determine the host health status (Solanky *et al.* 2005; Liu *et al.* 2016c). More investigations on fish metabolome in response to *Aeromonas* could better understand the host-pathogen relationship and identify biomarkers for diagnosing *Aeromonas* infection in fish. Recent metabolomics studies in bivalves identified itaconic acid as an internal metabolite of bivalves with an antimicrobial function (Nguyen *et al.* 2018; Nguyen & Alfaro 2019; Young *et al.* 2017). *Vibrio* growth inhibition by itaconic acid has been reported in an experimental challenge of *Vibrio* sp. with different concentrations of itaconic acid, suggesting that this metabolite could be developed as an antimicrobial compound in aquaculture for antibiotic-resistant bacteria (Nguyen *et al.* 2019b). The use of itaconic acid as a potential antimicrobial compound for *Aeromonas* should be tested in future studies. Besides, metabolomics could be used to study the effects of additive compounds in preventing diseases or improving the survival of aquatic animals against bacterial infections (Roques *et al.* 2020). Several studies have demonstrated the effects

of additive compounds in improving fish resistance against different bacterial species (Table 5), including *Aeromonas* ((Jiang *et al.* 2020). In an attempt to identify key metabolic biomarkers associated with neomycin sulfate resistance in *A. hydrophila* by a metabolomics approach, Zhao *et al.* (2018) observed that L-aspartate is the most important biomarker in neomycin sulfate-resistant *A. hydrophila*. Interestingly, L-aspartate enhanced the antibiotic sensitivity of neomycin sulfate-resistant *A. hydrophila,* and when injected or orally administrated, L-aspartate also reduced fish mortality after *A. hydrophila* challenge (Zhao *et al.* 2018). Jiang *et al.* (2020) demonstrated that exogenous injection of 600 μg maltose enhanced crucian carp survival by 30% compared to the control group. This remains an exciting side for future metabolomics to examine whether the other metabolites could be used to control Aeromonads as an alternative to antibiotics.

Overall, these omics applications in aquaculture, especially in *Aeromonas* infection control, have significantly improved our understanding of disease process and fish immunology, crucial for developing effective strategies to control bacteria and improve the host immune system. Furthermore, omics technologies are potent approaches for biomarker discoveries. Among the omics used for *Aeromonas*, information on metabolomics remains limited. Hence, there is a need for more metabolomics investigations for *Aeromonas*. Metabolomics profiles provide a snapshot of an organism's physiological state at a given time and the molecules of interest for drug discovery, such as antimicrobial metabolites and immune stimulants. More work on metabolomics and other omics will help link databases effectively from genotype to phenotype and support the multiple omics approaches. The increasing trend in applying omics technologies will continue in *Aeromonas* studies, which will provide an invaluable resource for the development of novel non-antibiotic control methods for MAS.

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Table 5. Sumary of some metabolites used to enhance survival of fish following experimental challenge with pathogenic bacteria

Metabolite	Analytical Platform	Administration route	Fish species	Challenged bacteria	RPS*	Reference
Glucose	GC-MS	Injection Oral	Tilapias	*E. tarda*	64% 35%	Peng *et al.* (2015)
Glucose	GC-MS	Injection	Tilapias	*E. tarda*	33%	Zeng *et al.* (2017)
L- aspartic acid	GC-MS	Injection	Zebrafish	*V. alginolyticus*	56%	Gong *et al.* (2020a)
L- leucine	GC-MS	Injection Oral	Tilapias	*S. iniae*	33% 23%	Ma *et al.* (2015)
L- leucine	GC-MS	Injection Oral	Tilapias	*S. iniae*	35% 51%	Du *et al.* (2017)
L- proline	GC-MS	Injection Oral	Tilapias	*S. agalactiae*	77% 55%	Zhao *et al.* (2015)
N - acetylglucosamine	GC-MS	Injection	Tilapias	*S. iniae*	75%	Cheng *et al.* (2014)
Phenylalanine	GC-MS	Injection	Zebrafish	*V. alginolyticus*	38%	Jiang *et al.* (2019)
Taurine	GC-MS	Injection	Zebrafish	*V. alginolyticus*	29%	Yang *et al.* (2020)
Tryptophan	GC-MS	Injection	Zebrafish	*V. alginolyticus*	47%	Gong *et al.* (2020b)

GC-MS: Gas chromatography – Mass spectrometry

RPS: relative percent survival; * RPS = (1 - mortality in experiments / mortality in controls) * 100

2.4.6 Nanobubble-based approaches

Nanobubbles are bubbles with a diameter <200 nm and have neutral buoyancy that enable them to have a long residence time in the solution (Agarwal *et al.* 2011; Tsuge 2014). They are generated commonly by decompression and gas-water circulation-type generator (Agarwal *et al.* 2011). Nanobubble technology has been applied in wastewater treatment, flotation, surface cleaning and defouling (Gurung *et al.* 2016; Temesgen *et al.* 2017). Oxygen-nanobubbles can increase the metabolic rate of cells and promote the growth of plants and animals (Ebina *et al.* 2013). Besides, the collapsing of micro-nanobubbles generates shock waves that lead to the release of hydroxyl radicals (Takahashi *et al.* 2007), which can powerfully oxidize and destroy membrane and the genetic material of microorganisms since they act as disinfectant agents (Beneš *et al.* 1999; Ikai *et al.* 2010).

In aquaculture, nanobubbles are considered an emerging technology for water treatment. Oxygen nanobubbles can increase dissolved oxygen in aquaculture system resulting in improved growth performance of Nile tilapia (Mahasri *et al.* 2018) and whiteleg shrimp, *L. vannamei* (Mauladani *et al.* 2020; Rahmawati *et al.* 2020). The efficiency of nanobubbles for disinfecting the pathogen is influenced by the concentration of free radicals released in water after their collapse. The effectiveness of prolonging the operation of a nanobubble generator to obtain enough hydroxyl radicals remains unknown. Therefore the long-time effects of nanobubbles on aquatic animal immunity and stress response have yet to be investigated.

Ozone is a powerful disinfectant to reduce pathogen concentration and improve water quality in both flow-through and recirculating aquaculture systems. However, the low ozone solubility and stability are the primary reasons that lead to low utilization efficiency. Increasing concentration and treatment time are standard solutions to overcome these problems. The misuse of direct ozonation can adversely

affect aquatic organisms, leading to behavioural abnormalities, physiology changes, tissue damage, and mortality (Powell & Scolding 2018). Nanobubbles that can enhance solubility and decrease the loss of ozone in water could mitigate these challenges. Besides, nanobubbles can promote rapid oxidation of organic substances and increase the concentration of hydroxyl radicals (Tekile *et al.* 2017). A few publications have reported the efficiency of ozone-nanobubbles to control parasites on planktonic crustaceans (Kurita *et al.* 2017) and *Vibrio parahaemolyticus* on whiteleg shrimp *L. vannamei* (Imaizumi *et al.* 2018). However, a high ozone concentration (3.5 mg/L ozone and 970 mV ORP) was toxic and caused mortalities for the experimental shrimps. Ozone-nanobubbles were proven to reduce more than 97% of *S. agalactiae* and *A. veronii* load in water following 10 minutes treatment (>800 mV ORP). Further, this treatment was safe for juvenile Nile tilapia in a controlled-laboratory experiment (Jhunkeaw *et al.* 2021). Recently, several studies have revealed that ozone nanobubbles (NB-O_3) show promise at reducing quantities of pathogenic bacteria and improving DO in water, as well as modulating the immune systems against bacterial infection (Nghia *et al.* 2021; Linh *et al.* 2021). Some publications showed that high ozone dosage could induce acute toxicity to aquatic animals. However, the level of toxicity depends heavily on the concentration of ozone, exposure time and the stage of animals (Reiser *et al.* 2010; Reiser *et al.* 2011; Schroeder *et al.* 2010).

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In summary, ozone-nanobubbles have potential applications to control motile *Aeromonads* infections in aquaculture, especially multidrug-resistant *Aeromonas,* because of their non-selective eradication. Nonetheless, applying this approach for prevention or treatment requires further in-depth investigations, such as exposure time and dose, specific to different stages of fish and shrimp, the effects of ozone-nanobubbles to environmental and intestinal microbiota, animal growth performance, immunity as well as cost-effectiveness.

2.5 Concluding remarks and future perspectives

In 2018, nearly 690 million people were undernourished and suffered from undernutrition globally, dominated by South Asian and Sub-Saharan Africa, representing 33.2% and 32.8%, respectively (Von Grebmer *et al.* 2020). In a crisis period such as COVID-19 pandemic, the people suffering from poverty and malnourished children are particularly vulnerable. Aquatic animals providing high-quality protein, beneficial fatty acids, and bioavailable vitamins and minerals are critical to prevent undernutrition. There is considerable evidence to demonstrate that aquaculture effectively contributed to poverty alleviation, economic growth and food and nutrition security in low-middle income countries (LMICs) (Béné *et al.* 2016; Gephart *et al.* 2020; Tacon *et al.* 2020). Three major groups of inland aquaculture including that of carps, tilapias and catfishes shared 35.85% of world aquaculture production in quantity of all species with more than 41 million tonnes of production, valued at US$ 83 billion. Besides, the global average unit value of freshwater carps, tilapias, and catfishes is 1.47, 2.01 and 1.16 US$/kg, respectively, which are correspondingly more affordable than the 7.70, 7.64. 8.40, and 6.36 US$/kg of shrimps and prawns, Atlantic salmon, Pacific salmon, and trouts, respectively (FAO 2020). Thus, the farming of carps, tilapias, catfishes, and other freshwater fish contributes great economic value for LMICs and entails enormous social impact on human welfare, benefitting the people suffering from poverty and malnourishment in Asia and Africa.

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Motile Aeromonas Septicemia (MAS) is one of the biggest challenges in sustainable freshwater finfish aquaculture worldwide that causes a significant loss in the production of major aquaculture species. The increasing use of antibiotics to combat Aeromonads has generated a negative consequence of antimicrobial-resistant Aeromonads and increased public health concerns, especially in the LMICs. During the last few decades, considerable efforts to search for suitable alternatives to antibiotics and develop non-

antibiotic approaches for tackling *Aeromonas* infections, such as vaccines, probiotics, phytochemicals, and bacteriophages, have been attempted. In the author's point of view, toward sustainable development of aquaculture, besides current non-antibiotic approaches, novel alternatives to antibiotics such as multivalent vaccines coupled with advanced nanotechnology to improve vaccine efficacy should be strategically targeted through practical, inexpensive oral and immersion delivery routes. Novel live attenuated vaccines, based on the selection of naturally bacteriophage-resistant variants, are highly potent immersion vaccines in aquaculture that do not involve GMO concerns. Prevailing unsupportive and inappropriate approval regulations for aquaculture vaccines are likely the most significant obstacles for their broader application in many countries in Asia. Most of these national regulations for antibiotics appear to be loosely enforced, causing tremendous antimicrobial resistance (AMR) and antibiotic residue consequences in aquaculture products. Therefore, switching the mindset on approval regulations between antibiotics and vaccines at national and multinational levels is an essential step to pave the way for a revision of regulations, aiming to limit antibiotic usage and promoting sustainable vaccination. Besides, the One Health platform should be integrated into national policies to reduce AMR.

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Aquaculture feed incorporated with microencapsulated probiotics, synbiotics, nano bioactive compounds or exogenous metabolites should be explored to develop the optimized feeding programs for strategic aquaculture species. Moreover, lytic bacteriophage cocktails as prophylactic and therapeutics are high-potential approaches for closed intensive, and super-intensive aquaculture systems. The novel non-antibiotic approach using ozone-nanobubbles for oxygenating water opens new frontiers to improve growth performance and reduce pathogen load in aquaculture systems. The development and application of omics technologies such as genomics, transcriptomics, proteomics, or metabolomics also offer powerful diagnostic and

therapeutic tools. Nonetheless, the complementarity of non-antibiotic approaches should be considered a strategic direction to combat MAS, including AMR Aeromonads and other bacterial pathogens in aquaculture.

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CHAPTER 3

Characterization and protective effects of lytic bacteriophage pAh6.2TG against a pathogenic multidrug-resistant *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*)

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3.1 Abstract

Bacteriophage is considered an alternative to antibiotics and environmentally friendly approach to tackle antimicrobial resistance (AMR) in aquaculture. Here, we reported isolation, morphology and genomic characterizations of a newly isolated lytic bacteriophage, designated pAh6.2TG. Host range and stability of pAh6.2TG in different environmental conditions, and protective efficacy against a pathogenic multidrug-resistant (MDR) *Aeromonas hydrophila* in Nile tilapia were subsequently evaluated. The results showed that pAh6.2TG is a member of the family *Myoviridae* which has genome size of 51,780 bp, encoding 65 putative open reading frames (ORFs), and is most closely related to *Aeromonas* phage PVN02 (99.33% nucleotide identity). The pAh6.2TG was highly specific to *A. hydrophila* and infected 83.3% tested strains of MDR *A. hydrophila* (10 out of 12) with relative stability at pH 7 - 9, temperature 0 - 40 °C and salinity 0 - 40 ppt. In experimental challenge, pAh6.2TG treatments significantly improved survivability of Nile tilapia exposed to a lethal dose of the pathogenic MDR *A. hydrophila*, with relative percent survival (RPS) of 73.3% and 50% for phage multiplicity of infection (MOI) 1.0 and 0.1, respectively. Significant reduction of bacterial counts in rearing water at 3 h (6.7 ± 0.5 to 18.1 ± 6.98 fold) and in fish liver at 48 h post-treatment (2.7 ± 0.24 to 34.08 ± 26.4 fold) was observed in phage treatment groups while opposite pattern for bacterial counts was observed in untreated control. Interestingly, the surviving fish provoked specific antibody (IgM) against the challenged *A. hydrophila*. These results might explain the higher survival in phage treatment groups. In summary, the findings suggested that the lytic bacteriophage pAh6.2TG is an effective alternative to antibiotics to control MDR *A. hydrophila* in tilapia and possibly other freshwater fish.

Keywords: *Aeromonas hydrophila*, alternative to antibiotics, antimicrobial resistance, aquaculture, bacteriophage, multidrug resistance

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3.2 Introduction

The farming of carps, tilapias, and catfishes accounts for 35.84% of world aquaculture production with revenue of 83 billion dollars in 2018. They contribute not only great economic value but also food and global nutrition security (FAO, 2020; Naylor et al., 2021). One of the challenges for sustainable aquaculture is production loss due to infectious diseases (Stentiford et al., 2020; Stentiford et al., 2017). *Aeromonas hydrophila* infection is considered one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry (da Silva et al., 2012; Hossain et al., 2014; Peterman & Posadas, 2019; Pridgeon & Klesius, 2012). The control of this disease still heavily relies on antibiotics, especially in low-middle income countries (LMICs). Consequently, a global issue of concern of multidrug-resistant (MDR) *A. hydrophila* is becoming increasingly ubiquitous (Guz & Kozinska, 2004; Patil et al., 2016; Stratev & Odeyemi, 2016). Non-antibiotic approaches can minimize the requirement for antimicrobials to tackle infectious diseases in both animals and human health (Hoelzer et al., 2018). In the battle to combat *A. hydrophila* infection in aquaculture system, bacteriophage is one of the environmentally friendly approaches which replace or complement chemotherapy to reduce the hazard of bacterial disease and antimicrobial resistance in aquatic animals.

Lytic bacteriophages (also called phages) are unique viruses that can infect and kill bacterial cells (Kutateladze & Adamia, 2010). Phage therapy is a viable option to control bacterial infections due to their unique advantages, including high host specificity, rapid self-proliferation, and low intrinsic toxicity (Cao et al., 2021). For instance, Luo et al. (2018) injected phage HN48 with multiplicity of infection (MOI) = 1 (MOI represents the ratio of the numbers of virus particles to the numbers of the host cells) against *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) with relative

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percent survival (RPS) of 60%. Feeding phage cocktails of PVHp5 and PVHp8 showed protective effectiveness in turbot (*Scophthalmus maximus*) against *Vibrio harveyi* infection with RPS from 38.6 to 79.5% (Cui et al., 2021). In addition, intraperitoneal injection of phages FpV4 and FPSV-D22 showed protection of rainbow trout (*Oncorhynchus mykiss*) to *Flavobacterium psychrophilum* with RPS of 53.8%, while feed-based and bath administrations were not effective (Donati et al., 2021). Previous studies have demonstrated that phages can be applied in aquaculture to combat *A. hydrophila* infection (Anand et al., 2016; Cao et al., 2020; Dang et al., 2021; Jun et al., 2013; Le et al., 2018). Hence, strategy using phages for biocontrol of *A. hydrophila* has become increasingly attractive. The earlier studies have analyzed phenotypic and genotypic characterization, and evaluated protective effect of phages against *A. hydrophila*, including *Myoviridae* pAh1-C and pAh6-C (Jun et al., 2013); *Podoviridae* Ahp1 (Wang et al., 2016); *Myoviridae* pAh-1 (Easwaran et al., 2017); *Myoviridae* CT45P and TG25P (Hoang et al., 2019); *Podoviridae* MJG (Cao et al., 2020), *Myoviridae* AHP-1 (Chandrarathna et al., 2020); *Siphoviridae* Akh-2 (Akmal et al., 2020), *Podoviridae* LAh1-LAh6, *Siphoviridae* LAh7, and *Myoviridae* LAh10 (Kabwe et al., 2020); *Myoviridae* PVN-02 (Tu et al., 2020); *Myoviridae* AhyVDH1 (Cheng et al., 2021). In this study, we isolated and characterized specific an *A. hydrophila* phage from water sources in Mekong Delta, Vietnam. Subsequently, we evaluated its protective effects for juvenile Nile tilapia challenged with a pathogenic MDR *A. hydrophila*.

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3.3 Materials and Methods

3.3.1 Bacterial isolates

All bacterial strains used in this study are listed in Table 1. The isolates of *Aeromonas*, *Streptococcus*, and *Edwardsiella* were cultured in Tryptic Soy Broth (TSB; Becton Dickerson, USA) at 28 °C while *Lactobacillus* isolates were cultured in De man, Rogosa, and Sharpe (MRS, HiMedia, India) broth at 37 °C. All laboratory isolates of

Aeromonas were previously isolated from diseased fish using selective medium, Rimler-Shotts agar (RS, HiMedia, India) supplemented with Novobiocin (Oxoid, UK), identified by PCR and sequencing of *gyr*B housekeeping gene (Navarro & Martínez-Murcia, 2018). Multidrug-resistant strains of *A. hydrophila* (Table S1) were identified based on the method proposed by Magiorakos et al. (2012).

3.3.2 Phage isolation and morphology

Preparation of host strain

The MDR *A. hydrophila* BT09 (Tables 1 and S1) was chosen as a bacterial host for phage isolation. Prior to phage isolation, prophage induction using Mitomycin C (Sigma-Aldrich, USA) was carried out as described by Walker et al. (2009) to ensure that the host cells do not contain prophage. Briefly, 100 μL of bacterial cells suspended in normal saline solution (OD_{600} = 0.6) was added into each of 10 mL of TSB supplemented with 250, 500, and 1,000 ng/mL of Mitomycin C. All cultures were incubated at 28 °C for 8 h. The induced phage production using Mitomycin C was evaluated by the Plaque Drop Assay (Adams, 1959).

Phage isolation

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Water samples were collected from striped catfish culture ponds in Tien Giang Province, Vietnam. The samples were enriched to increase phage concentration according to Van Twest and Kropinski (2009) and isolated by Plaque Assay method described by Jun et al. (2013). Briefly, the samples were centrifuged at 4,500 *x g*, 4 °C for 30 min, and the supernatant was filtered through a 0.2 μm filter (Merck Millipore, USA) to remove residual bacteria cells. Then, 10 mL filtrate was mixed with 10 mL of *A. hydrophila* BT09 in TSB supplemented with 1.0 mM $CaCl_2$ and 0.5 mM $MgSO_4$ (MTSB). The mixture was cultured at 28 °C for 24 h with 50 rpm shaking. The mixture was then centrifuged at 10,000 *x g*, 4 °C for 15 min, and the collected supernatant was serially

diluted (10^{-1} to 10^{-4}). A volume of 100 μL of each dilution was transferred to a tube containing 3.0 mL of TSA 0.5% agar supplemented with 1.0 mM $CaCl_2$ and 0.5 mM $MgSO_4$ (MTSA), together with 100 μL of *A. hydrophila*. The mixture was vortexed lightly and poured onto a plate of TSA 1.5% agar. The plates were incubated at 28 °C for 16 h and the growth of phages was observed (clear plaque on the plate). The individual clear plaque was picked and aseptically transferred to 200 μL of SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5). The mixture was vortexed vigorously and kept in 4 °C refrigerator overnight. The phages in SM buffer were obtained by filtering the supernatant through a 0.2 μm filter after centrifugation at 10,000 *x g* for 10 min. The filtrate was propagated four times continuously using the same protocol mentioned above for purification of the obtained phages. The isolated phages were stored in SM buffer supplemented with 30% glycerol at -80 °C until used.

3.3.3 Examination of phage morphology

The structure and size of the phage were determined by Transmission Electron Microscope (TEM). The specific procedure was as follows; the phage solution (3 mL) was centrifuged twice at 200,000 *x g* for 90 min. The pellets were resuspended in sterile distilled water. A volume of 50 μL of 1% glutaraldehyde (g/vol) was then added to immobilize the sample and rinsed with 0.1 M of cacodylate before proceeding with the dye. The samples were coated with 0.1% Poly-Lysine solution onto the surface of the 200-mesh carbon-coated grids to increase the adhesion of phages on the mesh. A volume of 10 μL of the phages was added to the grid and allowed to dry naturally for 5 min. The samples were dyed with 1% uranyl acetate sterilized with a 0.2 μm filter. The samples were washed with distilled water, allowed to dry for 5 min and imaged with a TEM-JEOL 1010 (Japan) with light projected through the grid for about 5 s at 80 kV. Phage morphology was classified according to the guideline of International Committee on Taxonomy of Viruses (ICTV) and Ackermann (2007).

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3.3.4 Host range and specificity

The host range of phage pAh6.2TG isolated in this study was conducted on the collection of 17 *A. hydrophila* isolates from diseased fish (Tables 1 and S1). In this study, the Plaque Drop Assay was performed as described by Adams (1959) with minor modifications. Briefly, double-layer agar plates containing tested bacterial cells were prepared. Then, 5 μL of phages (10^8 PFU/mL) was dropped on the surface of each plate, kept without moving for 30 min and incubated at 28 ^{o}C for 16 h. Normal saline solution was used as negative control. Phage susceptibility was indicated by a clear zone appearing at the location of the drops while no clear zone indicated unsusceptible host. Specificity test of phage pAh6.2TG to other common aquatic pathogens (*Aeromonas veronii*, *Aeromonas schubertii*, *Edwardsiella ictaluri*, *Streptococcus agalactiae*) and probiotic bacteria (*Lactobacillus fermentum*, *Lactobacillus plantarum*) (Table 1) was done in the same manner. All tests for host range and specificity were done in triplicates.

3.3.5 Phage stability in different environmental conditions

Stability of phage pAh6.2TG at different temperature (4, 25, 30, 35, and 40°C), pH (3, 5, 7, 9 and 11), salinity (0, 5, 10, 20, 40‰), and fish-rearing water (1, 3, 24, and 48 h) were investigated. These tests were carried out by incubating 100 μL of phage culture (approx. 10^9 PFU/mL) at the respective temperatures, pH, and salinity for 1 and 24 h in 10 mL of SM buffer. All the experiments were conducted in triplicates. The stability of phages in rearing water was performed in duplicates by adding 2 mL of phage pAh6.2TG (approx. 8.5×10^{10} PFU/mL) into 50 L of water (pH = 7.0 ± 1.0, 0% NaCl) containing 20 of Nile tilapia and maintained at 30 ± 1.0 ^{o}C. The concentration of viable phages was enumerated by plaque assay (Jun et al., 2013). Phage concentration (logPFU/mL) before incubation in different conditions was set to be 100%.

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3.3.6 Genome characterization

Phage genome extraction and next-generation sequencing

The phage particles prepared by liquid propagation in TSBM were desalted using Millipore Amicon ultracentrifugal filter 10,000 NMWL (Merck, United States) at 10,000 *x g*, 4 ^{o}C for 15 min and concentrated by ultracentrifugation at 300,000 *x g*, 4 ^{o}C for 3 h (Beckman Coulter, German). The pellets were resuspended in SM buffer. Phage genomic DNA was extracted using Phage DNA Extraction Kit (Cat. 46800, Norgen Biotek, Canada) following the manufacturer's protocol. Quality and concentration of DNA were measured by Nanodrop (Colibri, German) and Quibit 4.0 (Thermo Scientific, United States). Purified genomic DNA (3.15 ng/μL) was subjected to library preparation and sequencing using Next Generation Sequencing System with Illumina Novaseq 6000 platform (Pair-end, 150; library construction size, 350 bp; data output, 1.0 GB, data quality, Q30 > 80) at KTEST company, Vietnam.

Phage genome assembly and annotation

Raw reads were filtered using Fastp *v* 0.20.1 with the qualified phred score ≥ Q25 and 8 bases trimming from 5'/3' end (Chen et al., 2018). Host associated sequences were filtered out by mapping trimmed reads to the genome of *A. hydrophila* type strain (accession no. NZ_CP016990.1) using Bowtie2 *v* 2.3.4.3 (Langmead & Salzberg, 2012). Only unaligned reads were subjected to genome assembly using Unicycler *v* 0.4.8 (Wick et al., 2017) on the Galaxy web platform at usegalaxy.org (Afgan et al., 2016). Potential phage sequence was identified by submitting the assembled contigs to PHASTER web server (Arndt et al., 2016). The predicted phage sequence (assigned as 'pAh6.2TG' in this study) was annotated using Prokka *v* 1.14.6 with Viruses annotation mode (Seemann, 2014). The annotated phage genome was visualized using DNAplotter (Carver et al., 2009).

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Phage taxonomic identification and phylogenetic reconstruction

Identification of phage species was carried using VICTOR web service (Meier-Kolthoff & Göker, 2017). VICTOR is a tool that perform pairwise genome comparison of prokaryotic viruses and automatically constructs phylogenomic trees using Genome-BLAST Distance Phylogeny method (GBDP) with the formula D0. This tool also classifies the virus at the species, genus and family level with the taxon boundaries estimating by OPTSIL program (Göker et al., 2009). Herein, only the genomes of the viruses belonging to the family *Myoviridae* (*n* = 91) were included in this genome comparison since pAh6.2TG was predicted as an unknown *Myoviridae* by PHASTER tool described in the above section.

In addition to genome comparison, the phylogenetic analyses based on the terminase large subunit (terL) and major capsid protein (MCP) amino acid sequences of pAh6.2TG and other related species (predicted by VICTOR) were also performed via PhyloSuite v1.2.2 (Zhang et al., 2020). Amino acid sequences were aligned using MAFFT (Katoh & Standley, 2013) and the maximum-likelihood trees were constructed using IQ-TREE (Nguyen et al., 2015) with 5,000 ultrafast bootstraps and best-fit model (LG+G4) estimated by ModelFinder. Phylogenomic tree, terL- and MCP-based trees were visualized using Phandango (Hadfield et al., 2018) and iTOL web tools (Letunic & Bork, 2019). Lastly, the protein sequence similarities between pAh6.2TG and the closest viral taxa were determined using CoreGenes3.5 web server with Blastp threshold score at 75 (Turner et al., 2013).

3.3.7 Effect of phage on Nile tilapia challenged with MDR *A. hydrophila*

Experimental fish

Healthy Nile tilapia (10.5 ± 4.7 g) obtained from a commercial tilapia hatchery in Thailand were acclimated for 2 weeks in dechlorinated tap water with aeration at 28

± 1.0 °C before the experiments. The fish were fed with commercial tilapia feed (crude-protein 30%) at rate of about 3% of fish weight twice daily. Before starting the experiments, ten fish were randomly selected for bacterial isolation and found to be free of *A. hydrophila*. The experimental animal protocols were approved by Chulalongkorn University (Approval no. CU-IACUC 2031006).

Fish survivability and sample collection

This experiment aimed to investigate whether lytic phage treatment improves survivability of Nile tilapia challenged with a pathogenic MDR *A. hydrophila* BT14. A total of 258 fish were randomly divided into six groups with 2 replicate tanks per each group (Figure S1): Group 1 was exposed to culture medium without phage (no Ah + no phage); Group 2 was exposed to bacteria without phage (Ah + no phage); Group 3 was exposed to culture medium and phage pAh6.2TG at multiplicity of infection (MOI) = 0.1 (no Ah + phage 0.1); Group 4 was exposed to culture medium and treated with phage at MOI = 1.0 (no Ah + phage 1.0); Group 5 was challenged with *A. hydrophila* and treated with phage at MOI = 0.1 (Ah + phage 0.1); Group 6 was challenged with *A. hydrophila* and treated with phage at MOI = 1.0 (Ah + phage 1.0).

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In bacterial challenge groups (2, 5 and 6), 1 L of MDR *A. hydrophila* BT14 (approx. 8×10^8 CFU/mL) was added to 50 L water to reach a final concentration of approx. 2×10^7 CFU/mL. Groups 5 and 6 tanks had 2 and 20 mL of phage pAh6.2TG (approx. 8.5×10^{10} PFU/mL) added to reach a final concentration of approx. 2×10^6 and 2×10^7 PFU/mL, respectively. Group 2 tank had 20 mL of SM buffer without phage added. The mixtures in groups 2, 5 and 6 were maintained at 29 ± 1.0 °C with aeration for 3 h. In culture medium exposure groups (1, 3 and 4), 1 L of TSB was added to 50 L water. Groups 3 and 4 tanks had 2 and 20 mL of phage pAh6.2TG (approx. 8.5×10^{10} PFU/mL) added, respectively. After 3 h, the fish were transferred to all groups, maintained at 29 ± 1 °C with aeration for 14 days. In order to investigate the effect of phage on the

concentration of *A. hydrophila* in rearing water, a volume of 25 mL water from groups 2, 5 and 6 were sampled at 3, 24 and 48 h after exposure with phage. A volume of 1 mL water was centrifuged at 4 ^{o}C, 10.000 x *g*, for 5 min. The supernatant were collected and diluted in SM buffer to measure concentration of phage by Plaque Assay method (Jun et al., 2013). The pellet was washed 1 time and re-suspended in 1 mL of PBS buffer. Bacterial concentration was then enumerated by conventional plate count method using RS supplemented with Novobiocin (Harrigan & McCance, 2014). In order to investigate the effect of phage on the concentration of *A. hydrophila* in liver, two fish from groups 2, 5 and 6 were sampled at 24, 48 and 72 h after exposure with phage. The fish were necropsied, and 0.1 g of live tissue was collected and homogenized in a microtube containing 900 µL of SM buffer. The samples were then centrifuged at 10.000 x *g*, for 5 min. The supernatant and pellet were used for respective phage and bacterial enumeration same as above.

The remaining fish were observed daily for 14 days, and mortality was recorded. Representative moribund or freshly dead fish were collected for bacterial re-isolation using RS supplemented with Novobiocin as described above. The RPS was calculated according to the formula described by Ellis (1988): RPS = (1 - % mortality in challenge / % mortality in control) * 100.

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3.3.8 Determination of serum antibody by the enzyme-linked immunosorbent assay (ELISA)

For the comparison of specific antibody (IgM) levels against *A. hydrophila* between experimental groups, blood samples of 5 surviving fish in each tank (10 fish/group) were collected at the end of the experiment (day 14). Sera were collected after centrifugation at 8,000 x g for 15 min, stored at -20 ^{o}C until used. ELISA assay was carried out following the protocol described by Dien et al. (2021).

3.3.9 Statistical Analysis

Percent survival data from the challenge experiments was analyzed by the Kaplan-Meier method and differences among groups were tested using a log-rank test, *p*-values of 0.05 or less were considered to be statistically significant. Enumeration of *A. hydrophila* concentration and phage titer in rearing water and fish liver samples was analyzed by ANOVA. Dunnett post-hoc test was used to measure specific differences between pairs of mean. The OD_{450nm} readings from the indirect ELISA assay were analyzed using a Kruskal-Wallis test. Multiple comparison analyses were performed by Bonferroni test. All statistical analyses were performed using SPSS Software ver22.0 (IBM Corp., USA).

3.4 Results

3.4.1 Prophage induction, phage isolation, and morphology

Although three doses of Mitomycin C (250, 500, and 1,000 ng/mL) were used for prophage induction, no plaque was detected, indicating that *A. hydrophila* BT09 did not contain prophage and was suitable as a bacterial host for lytic phage isolation. Subsequently, a phage, designated pAh6.2TG, was isolated from a freshwater sample. Phage pAh6.2TG produced medium, clear, and round plaques with diameter of 1.3 - 1.8 mm (Figure 1A-B) after 16 h of incubation. TEM morphology examination showed that the phage had an icosahedral head with 59.6 ± 2.5 nm diameter (n = 3) and a contractile tail which was 137 ± 10.2 nm in length and 20.2 ± 2.7 nm in diameter (n = 3) (Figure 1C-D). Based on the morphological features, phage pAh6.2TG was initially classified to the *Myoviridae* family.

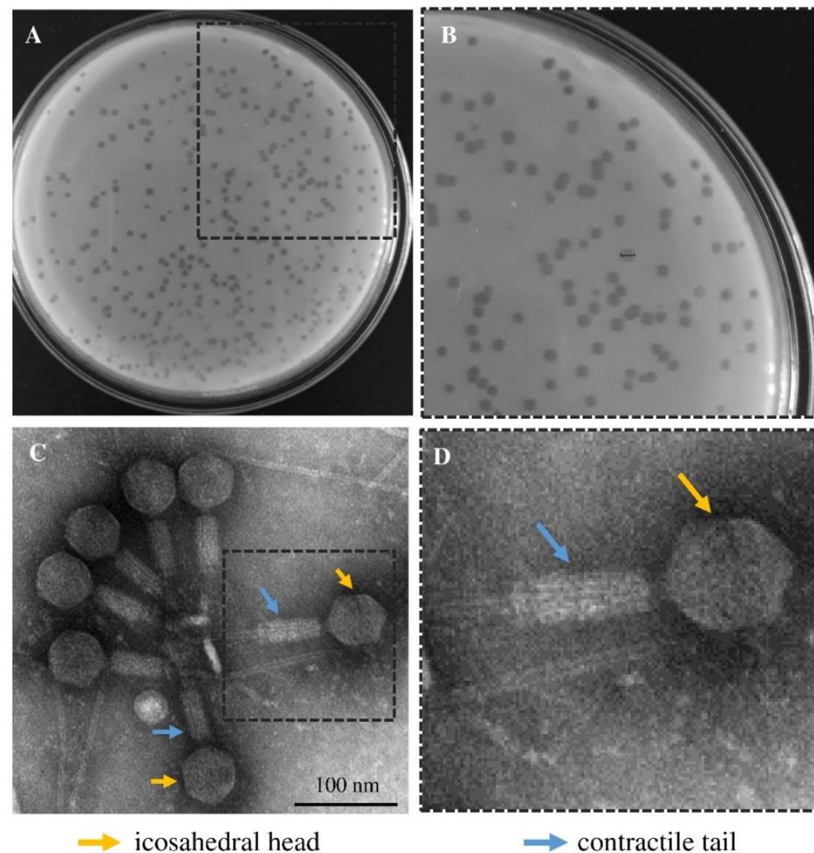

Figure 1. (A & B) Plaques of pAh6.2TG on double layer TSA. (C & D) Transmission electron micrographs of pAh6.2TG.

3.4.2 Host range and specificity of phage pAh6.2TG

Among all bacterial isolates tested, pAh6.2TG showed lytic activity against 10/17 *A. hydrophila* isolates (Table 1) of which 8 isolates were MDR (Table S1). In contrast, no lytic activity was observed against other fish bacterial pathogens including *A. veronii*, *A. schubertii, E. ictaluri*, *S. agalactiae* as well as two probiotic bacteria *L. fermentum*, and *L. plantarum* (Table 1).

Table 1. Bacterial strains used for determination of pAh6.2TG host range and specificity

Bacterial species	Strain	Location	Source	Year	pAh6.2TG specific	References
A. hydrophila	BT01	Ben Tre, Vietnam	Striped catfish	2018	-	Laboratory strain
	BT02	Ben Tre, Vietnam	Striped catfish	2018	-	Laboratory strain
	BT03	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT04	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT05	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT12	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT09[+]	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT13	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT14[#]	Ben Tre, Vietnam	Striped catfish	2018	+	Dien et al. (2021)
	BT22	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	TG26	Tien Giang, Vietnam	Striped catfish	2018	+	Laboratory strain
	TG35	Tien Giang, Vietnam	Striped catfish	2018	+	Laboratory strain
	CUVET02	Chonburi, Thailand	Asian seabass	2020	-	Laboratory strain
	CUVET21	Chonburi, Thailand	Walking catfish	2020	-	Laboratory strain
	CUVET46	Kanchanaburi, Thailand	Nile tilapia	2020	-	Laboratory strain
	CUVET52	Uttaradit, Thailand	Nile tilapia	2020	-	Laboratory strain
	CUVET92	Kanchanaburi, Thailand	Nile tilapia	2020	-	Laboratory strain
A. veronii	NK01	Nongkhai, Thailand	Nile tilapia	2014	-	Dong et al. (2015a)
	NK02	Nongkhai, Thailand	Nile tilapia	2014	-	Dong et al. (2015a)

	NT03	Pathum Thani, Thailand	Nile tilapia	2016		Dong et al. (2017)
A. schubertii	N1	Tra Vinh, Vietnam	Snakehead fish	2016	-	Laboratory strain
	N3	An Giang, Vietnam	Snakehead fish	2016	-	Laboratory strain
	N7	Dong Thap, Vietnam	Snakehead fish	2016	-	Laboratory strain
E. ictaluri	T1-1	Ratchaburi, Thailand	Striped catfish	2014	-	Dong et al. (2015b)
S. agalactiae	2809	Thailand	Nile tilapia	2018	-	Jhunkeaw et al. (2021)
L. fermentum	VTCC 11051	Vietnam	Pickles	2009	-	Vietnam Type Culture Collection
L. plantarum	VTCC 10890	Vietnam	Pickles	2009	-	Vietnam Type Culture Collection

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3.4.3 Stability of phage pAh6.2TG at different environmental conditions

Stability of pAh6.2TG at different temperatures (4 to 40 °C) is shown in Figure 2A. Similar percentages of viable phage were detected after 1 h (96 ± 0.55 – 99.6 ± 0.08%) and 24 h (93 ± 0.23 – 98.6 ± 0.17%) of incubation, indicating that pAh6.2TG is a relatively thermostable phage.

Phage pAh6.2TG was stable (93.5 ± 1.69 – 97 ± 0.87%) at pH 7, 9 and 11 (Figure 2B). However, the phage pAh6.2TG was not stable at low pH. At pH 5, 93 ± 0.24% phage remained viable after 1h, and decreased sharply to 32.7 ± 0.44% (from 7.88 to 2.58 ± 0.06 logPFU/mL) after 24 h. At pH 3, only 15.2 ± 1.47% (1.19 ± 0.2 logPFU/mL) of phage was still viable after 1 h and reduced to undetectable level at 24 h (Figure 2B).

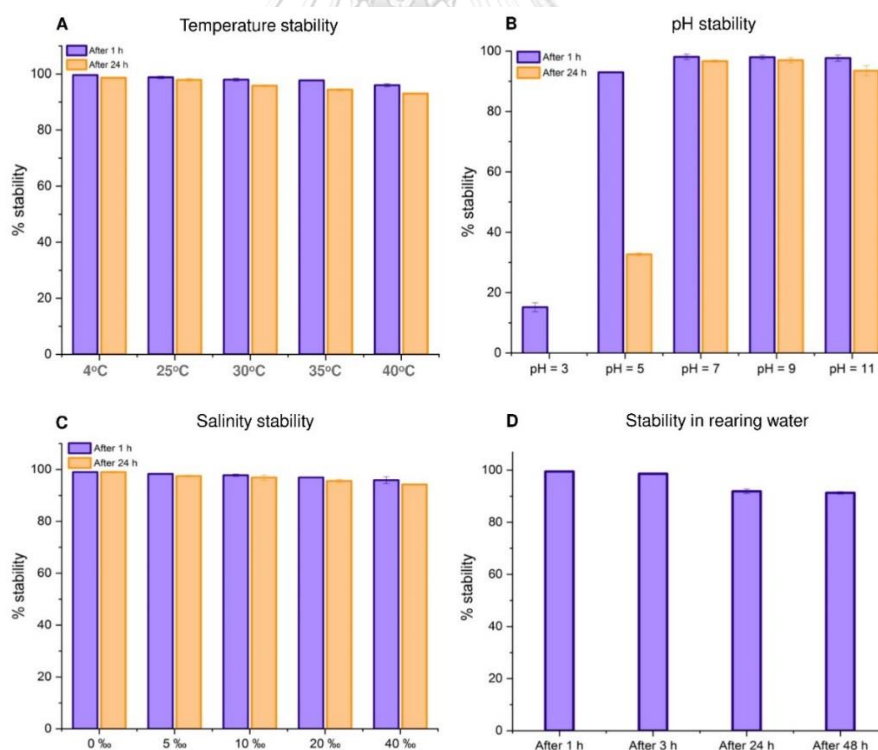

Figure 2. Stability of phage pAh6.2TG. (A) Temperature stability. (B) pH stability. (C) Salinity stability. (D) Stability in rearing water. Value of % stability are mean ± a standard error of the mean (SEM) bar (n = 3 in Figure A, B, C and n =2 in Figure D).

Phage pAh6.2TG was relatively stable at a wide range of salinity (0 – 40 ‰), with 95.9 ± 1.35 – 99 ± 0.19 % and 94.2 ± 0.29 – 99 ± 0.32 % viable after 1 and 24 h, respectively (Figure 2C). In fish-rearing water (30 ± 1 °C, pH 6.9, 0% NaCl) spiked with phages, percentage of stability at 1 and 3 h were 99.5 ± 0.15% and 98.6 ± 0.11%, respectively. After 24 and 48 h, phage titer decreased slightly to 91.9 ± 0.85% and 91.3 ± 0.5%, equivalent to 6.52 ± 0.07 and 6.47 ± 0.03 logPFU/mL, respectively.

3.4.4 Genome characterization of pAh6.2TG phage

Based on the assembly graph generated by Unicycler software, pAh6.2TG was predicted to contain a circular genome with a length of 51,780 bp, a GC content of 52.48%, encoding 65 putative open reading frames (ORFs) (Table S2) without tRNA genes (Figure 3). According to bioinformatics prediction, pAh6.2TG genome consists of three main functional modules: i) phage structure and DNA packaging (major capsid protein, baseplate protein, tail fiber protein, and terminase subunit), ii) DNA metabolism and replication (RNA polymerase, DNA polymerase, DNA helicase, 5’-3’ exonuclease, DNA ligase, and Ribonucleoside-diphosphate reductase large subunit), and iii) host lysis (cell wall hydrolase).

The closest phage taxonomic classification of pAh6.2TG toward other 91 *Myoviridae* phages in the public database revealed that *Aeromonas* phage pAh6.2TG and PVN02 (accession no. LR813619) were classified as the identical species with 99.33% identity. The result also showed total 64/65 ORFs were homologous between pAh6.2TG and PVN02 (97.3 - 100 % nt. identity), except for ORF03 that showed the highest homology (70%) to another *Aeromonas* phage pAh6-C (Table S2). Phylogenetic analysis based on whole genome (Figure 4A-B), major capsid protein sequence (Figure 4C), and terminase large subunit sequence (Figure 4D) confirmed high homology of phage pAh6.2TG and phage PVN02. In addition, pAh6.2TG was closely related to the *Aeromonas* phage pAh6-C (accession no. KJ858521), *Shewanella* phage Spp001 (accession no. NC023594), and *Shewanella* phage SppYZU05 (accession no. NC047824) (Figure 4).

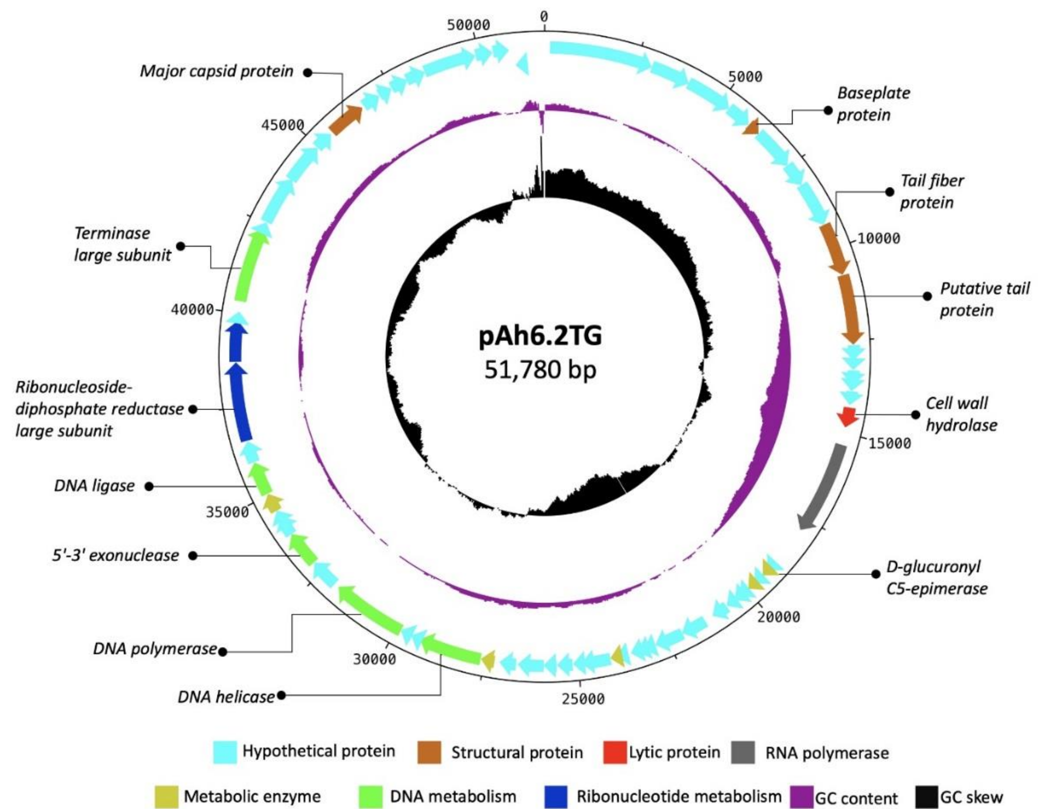

Figure 3. Genome map of phage pAh6.2TG. Arrows represent the annotated ORFs which are shown in various colors indicating their predicted protein function. Two inner rings represent the GC skew (in black) and GC content (in violet). Some genes of interest are marked.

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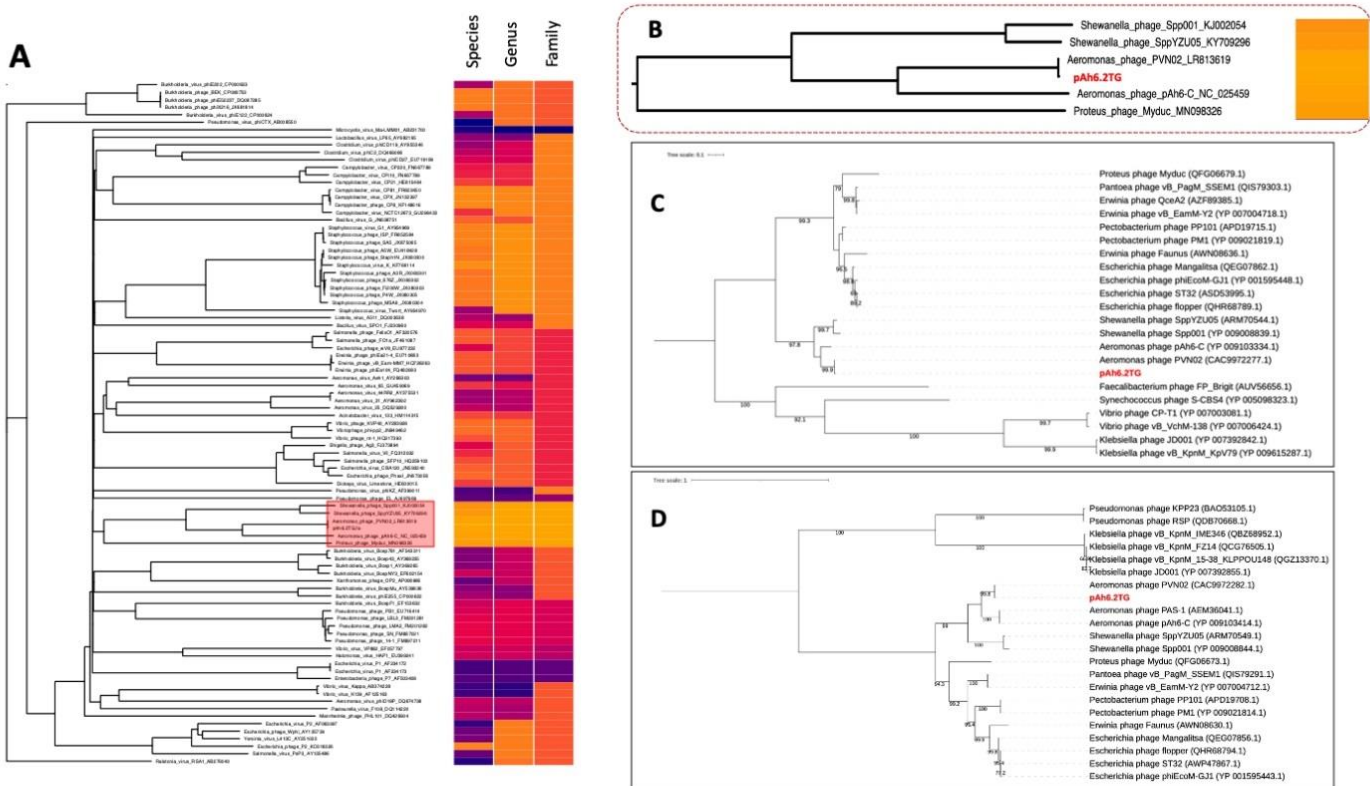
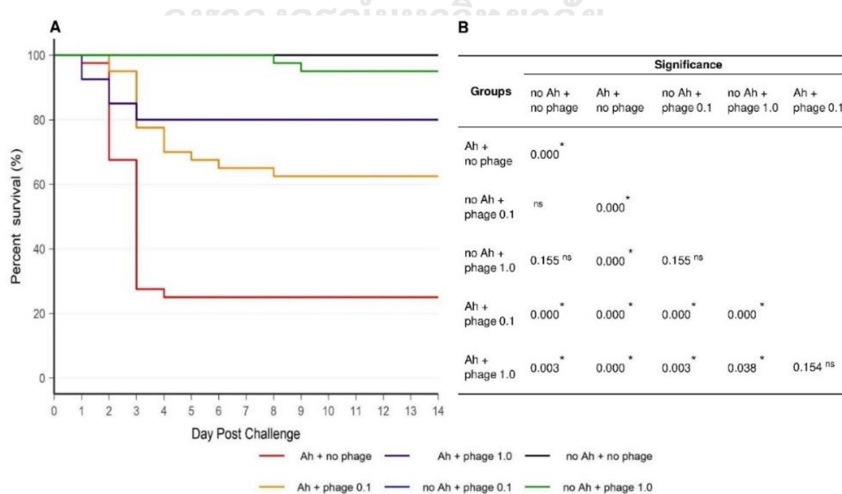


Figure 4. Phylogenetic analyses based on (A and B) whole genome, (C) major capsid protein sequence, and (D) terminase large subunit. (A) Comparative genomics (n = 91) was performed using VICTOR web server with settings recommended for prokaryotic viruses. Clustering of viruses at species, genus, and family level was determined automatically and allocated by color strips adjacent to the phylogenomic tree (same color representing identical taxonomic unit). Red box indicates phage pAh6.2TG and its monophyletic taxa which is magnified in panel B. Trees based on (C) major capsid protein and (D) terminase large subunit were constructed by the maximum-likelihood method with 5,000 ultrafast bootstrapping. Bootstrap value (in percentage) is shown at the node, whereas scale bar indicates amino acid substitution per site. Red taxon represents the phage of this study (pAh6.2TG).

3.4.5 In vivo challenge results

Phage pAh6.2TG improved survivability of Nile tilapia challenged with the MDR A. hydrophila

In vivo experiment showed that 100% fish in negative control group (no Ah + no phage) survived after 14 days, while only 25% survival was recorded in positive control group (Ah + no phage) (Figure 5). Interestingly, there was 62.5% and 80% survival in groups treated with pAh6.2TG with MOI = 0.1 (Ah + phage 0.1) and MOI = 1.0 (Ah + phage 1.0), respectively. These differences in percentage of survival of 2 phage treated groups were not statistically significant (p = 0.154) but statistically significant with positive control group (p = 0.000). The remaining two groups treated with phage without bacteria had 95 – 100% survival. The relative percent survival (RPS) of two treatments groups were 50% (MOI = 0.1) and 73.3% (MOI = 1), respectively. The moribund fish in challenge groups showed behavioral abnormalities (lethargy, loss of appetite, and surface swimming) and pale liver. Using selective medium, pure colonies with morphological characteristics of *A. hydrophila* were successfully isolated from representative dead fish (n=3).



Groups	Significance				
	no Ah + no phage	Ah + no phage	no Ah + phage 0.1	no Ah + phage 1.0	Ah + phage 0.1
Ah + no phage	0.000 *				
no Ah + phage 0.1	ns	0.000 *			
no Ah + phage 1.0	0.155 ns	0.000 *	0.155 ns		
Ah + phage 0.1	0.000 *	0.000 *	0.000 *	0.000 *	
Ah + phage 1.0	0.003 *	0.000 *	0.003 *	0.038 *	0.154 ns

Figure 5. Kaplan - Meier analysis of percentage survival of Nile tilapia (n = 40) challenged with MDR *A. hydrophila* BT14 (A). Differences between groups were tested using log-rank test (B). “*” denotes significant difference ($p < 0.05$), and “ns” means not significant.

Phage pAh6.2TG suppressed bacterial concentration in water and fish tissue

Fluctuation of bacterial concentration and phage titer in water and fish liver are shown in Figure 6 and Table S3. In rearing water, after 3 h of bacteria and phages exposure, bacterial concentration reduced 6.7 ± 0.5 fold in group treated with phage MOI = 0.1, and 18.1 ± 6.98 fold in group treated with phage MOI = 1.0 (Figure 6A). The calculation of fold changes is displayed in Table S3. In contrast, after 3 h, bacterial concentration increased 10.2 ± 3.15 fold in Ah + no phage group. Simultaneously, phage titer in groups treated with phage MOI = 0.1 and 1.0 after 3 h increased 51.04 ± 5.16 fold, and 20.98 ± 1.03 fold, respectively (Figure 6B). Phage was absent in Ah + no phage control group. At 24 h post-challenge, bacterial concentration in three groups was increased, while phage concentration in water slightly decreased. Besides, slight reduction of bacterial and phage concentration was observed in all groups at 48 h post-treatment (Figure 6A – B).

Moreover, in fish liver, bacterial concentrations of 5.8 ± 0.14, 5.52 ± 0.06, 5.51 ± 0.24 logCFU/g were recorded in Ah + no phage, Ah + phage 0.1, and Ah + phage 1.0 groups, respectively (Figure 6C). In Ah + phage 0.1 and Ah + phage1.0 groups, phage titers were 4.55 ± 0.2 and 4.75 ± 0.12 logPFU/g, respectively (Figure 6D). Similar pattern of phage concentration in rearing water was observed at 48 h post-challenge, while bacterial load decreased in all groups. In fish liver, compared to 24 h post-treatment, bacterial concentration in Ah + no phage groups increased 10.69 ± 3.85 fold, while in Ah + phage 0.1 and Ah + phage 1.0 groups, bacteria decreased 2.7 ± 0.24 and 34.08 ± 26.4 fold, respectively (Table S3).

The bacterial load in fish liver of Ah + no phage group decreased 3.8 ± 0.64 fold, from $6.58 \times 10^6 \pm 3.18 \times 10^5$ at 24 h post-challenge to $1.75 \times 10^6 \pm 2.12 \times 10^5$ CFU/g at 72 h post-challenge (Table S3). The same pattern was recorded in Ah+ phage 0.1 and Ah + phage 1.0 groups with 4.03 ± 0.83 and 2.18 ± 0.96 fold-reduction, respectively

(Table S3). At 72 h post-challenge, phage titer in fish liver decreased 15.13 ± 3.35 and 13.96 ± 3.95 fold in groups treated with phage 0.1 and 1.0 at 24 h post-challenge, respectively (Table S3).

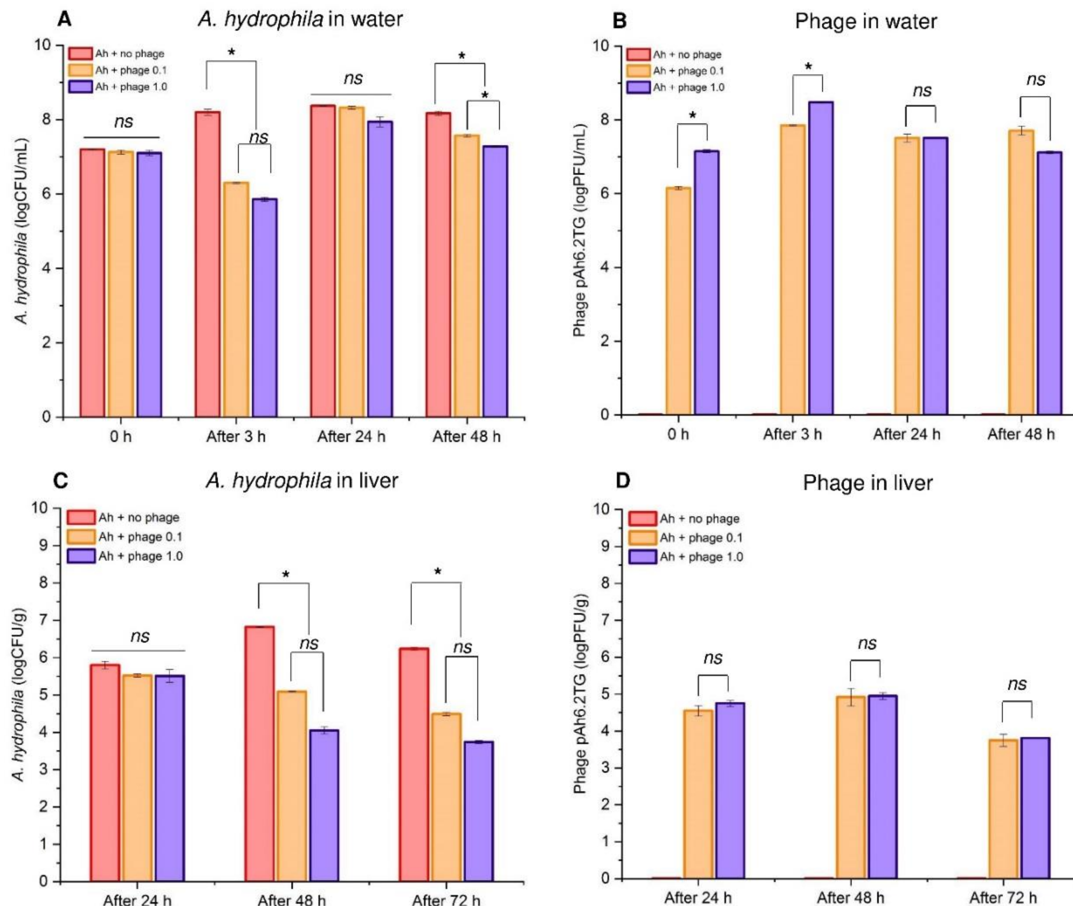


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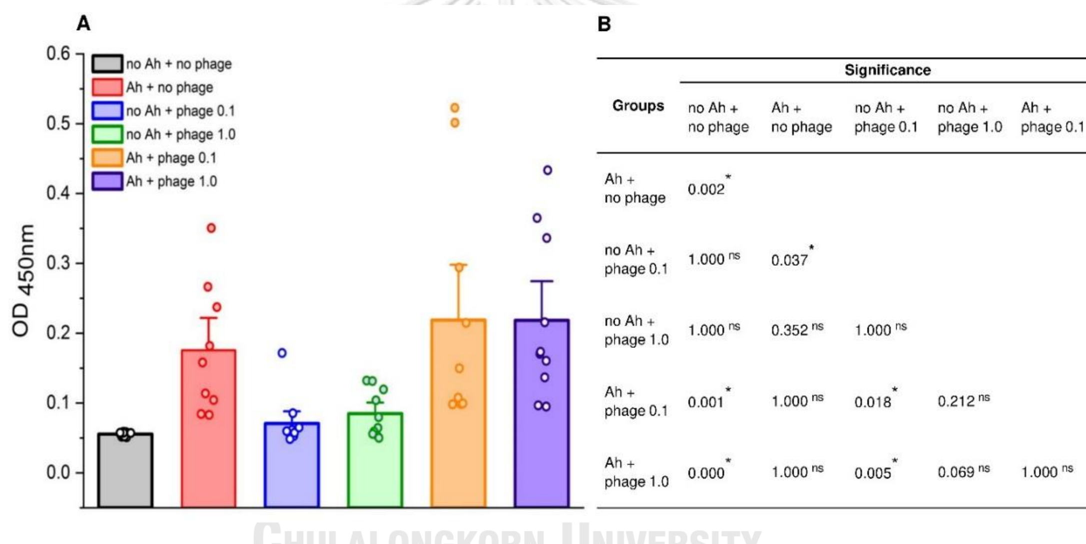
Figure 6. Enumeration of *A. hydrophila* concentration and phage titer in rearing water and fish liver samples. (A) *A. hydrophila* concentration in rearing water (logCFU/mL). (B) Phage pAh6.2TG titer in rearing water (logPFU/mL). (C) *A. hydrophila* concentration in fish liver (logCFU/g). (D) Phage pAh6.2TG titer in fish liver (logPFU/g). Value of *A. hydrophila* concentration and phage titer are mean ± a standard error of the mean (SEM) bar (n = 2) and "*" above the bar indicates significant difference between groups ($p < 0.05$), "*ns*" means not significant.

Surviving fish developed specific IgM against MDR A. hydrophila

All surviving fish in three groups challenged with MDR *A. hydrophila* had significantly higher levels of specific antibody (IgM) compared to the three unchallenged groups ($p < 0.05$) as measured by indirect ELISA, Kruskal - Wallis test: H (5) = 35.218, $p = 0.000$ (Figure 7). The serum from fish in the Ah + no phage, Ah + phage 0.1, and Ah + phage 1.0 groups had OD readings of 0.18 ± 0.09, 0.22 ± 0.17, and 0.22 ± 0.12, respectively. The IgM level was slightly higher in 2 phage treated groups but not statistically significant difference. In contrast, the low level of OD_{450} readings were recorded in the remaining groups (0.06 ± 0.003 to 0.08 ± 0.03) (Figure 7).



Groups	no Ah + no phage	Ah + no phage	no Ah + phage 0.1	no Ah + phage 1.0	Ah + phage 0.1
Ah + no phage	0.002*				
no Ah + phage 0.1	1.000 ns	0.037*			
no Ah + phage 1.0	1.000 ns	0.352 ns	1.000 ns		
Ah + phage 0.1	0.001*	1.000 ns	0.018*	0.212 ns	
Ah + phage 1.0	0.000*	1.000 ns	0.005*	0.069 ns	1.000 ns

Figure 7. Indirect ELISA analysis of *A. hydrophila* specific IgM antibody. Fish sera were collected on day 14 and dilutions with 1:256 were used to test for antigen specific IgM. Data were expressed as mean absorbance at OD_{450nm} with a SEM bar (A). One dot represents one biological replicate (n = 9 in group Ah + no phage, n = 10 in other groups). Differences between groups were tested using log-rank test (B). "*" denotes significant difference ($p < 0.05$), and "ns" means not significant.

3.5 Discussion

The *Myoviridae* phages specific to *A. hydrophila* are highly diverse in nature (Chandrarathna et al., 2020; Cheng et al., 2021; Jun et al., 2013). The lytic pAh6.2TG isolated in this study had genome characteristics most closely related to phage PVN02 (99.33% nt. identity) in the GenBank database, previously isolated from Vietnam (Tu et al., 2020). The origins of two phages from the closed geographical area of Mekong basin, although from different rivers, may explain the high genomic similarity of pAh6.2TG and PVN02. Compared to previously reported *A. hydrophila*-specific phages, pAh6.2TG (51,780 bp) had similar genome size with the phage PVN02 (51,668 bp) from Vietnam (Tu et al., 2020), and pAh6-C (53,744 bp) from Korea (Jun et al., 2015), but is larger than phage AhyVDH1 (39,175 bp) from China (Cheng et al., 2021), and smaller than phage LAh10 (260,310 bp) from Australia. The latter is the largest known phage infecting *A. hydrophila* (Kabwe et al., 2020). Genome analysis indicated that pAh6.2TG does not contain potential virulent genes or antimicrobial resistant genes, suggesting it is highly relevant as a biocontrol agent in aquaculture systems without concern of antimicrobial-resistant gene transmission.

Climate change has affected aquaculture environments by perturbing chemical and physical properties of water, particularly in the increase of water temperature and salinization (Maulu et al., 2021; Seggel & De Young, 2016). The stability of pAh6.2TG under a wide range of temperatures (4 – 40 °C) and salinity (0 – 40 ppt) might be important characteristics for its wider application in diverse aquaculture environments. Relatively high stability of pAh6.2TG in fish-rearing water suggests that immersion route is practical. However, low viability of pAh6.2TG at pH 3 – 5 suggests that oral administration might not be applicable due to the low pH in gastrointestinal tract of aquatic animals, e.g. pH in Nile tilapia stomach range from 1.4 – 2.0 (Moriarty, 1973).

One of the major limitations of phage application is its narrow host range and geographical specificity (Culot et al., 2019; Pérez-Sánchez et al., 2018; Ross et al., 2016). Although the newly isolated phage pAh6.2TG could lyse multiple isolates of MDR *A. hydrophila* from Vietnam, however, it does not lyse the isolates from Thailand and other bacterial species from the same or different genera. Therefore, to expand wider application of phage in aquaculture, a cocktail of multiple phage strains from different geographical locations might be the better approach to tackle not only AMR *A. hydrophila* but also other important bacterial pathogens in freshwater fishes. In addition, the specific infection of pAh6.2TG to *A. hydrophila* and not probiotic bacteria suggest the potential combination of phage therapy and probiotics to combat MDR *A. hydrophila* infection in aquaculture.

Carps, tilapias, and catfishes are crucial inland freshwater fish that play a vital role for food system transformation to tackle micronutrient deficiencies in LMICs (FAO, 2020; Hicks et al., 2019). *A. hydrophila* infection is one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry (Hossain et al., 2014; Peterman & Posadas, 2019; Pridgeon & Klesius, 2012). Increasing prevalence of pathogenic MDR *A. hydrophila* in aquaculture poses the high risk for serious uncontrollable disease outbreaks and public health concern due to spread of AMR. Non-antibiotic approach using lytic phages, therefore, was explored to control disease caused by MDR *A. hydrophila* in aquaculture systems. In this study, we provided *in vivo* evidences for the efficacy of phage application in rearing water which is effective at suppressing bacterial concentration in water as well as reducing the bacterial load in fish liver. The presence of phages in the fish liver also suggests that immersion administration could deliver considerably large number of phages into the fish tissue. These factors may contribute to improvement of survivability (RPS = 50 – 75%) of tilapia. Importantly, not only was there higher survival

in phage treated groups, but all surviving fish also developed specific IgM against *A. hydrophila*. This suggests that phages possibly weakened the bacteria which allowed the fish immune system to respond more effectively and saved the fish from death. Similarly, there were several studies using phages as therapeutic agent to control *A. hydrophila* infection. Le et al. (2018) used phage cocktails (ϕ2 and ϕ5) with MOI = 0.01, 1.0, and 100 to control *A. hydrophila* infection in striped catfish (*Pangasianodon hypophthalmus*) by injection administration and obtained RPS of 16.33%, 44.9%, and 100%, respectively. Immersion treatment of 1 x 10^8 PFU/mL phage Akh-2 improved survivability of Nile tilapia with RPS of 41.1% (Akmal et al., 2020). Cao et al. (2020) applied phage MJG by injection, immersion, and oral administration to control a pathogenic *A. hydrophila* in rainbow trout and the fish gained RPS of 100%, 66.7%, and 50%, respectively. Dang et al. (2021) showed protective efficacy of phage PVN02-sprayed feed against *A. hydrophila* 4.4T in striped catfish with RPS from 75.6 – 87.8%.

The findings in this study suggest a potential approach using phage as prophylactic agent that was effective in protecting Nile tilapia from a MDR *A. hydrophila*. This approach provided comparable RPS to other promising alternatives to antibiotics, such as probiotic-based or plant-based products (Dawood et al., 2020; Kuebutornye et al., 2020; Naliato et al., 2021; Neamat-Allah et al., 2021). Apart from tilapia, pAh6.2TG has great potential to be applied in catfish aquaculture industry due to the lytic activity of pAh6.2TG against multiple MDR *A. hydrophila* strains isolated from diseased striped catfish.

In summary, this study reported a newly isolated lytic phage pAh6.2TG that infects several isolates of MDR *A. hydrophila*. The phage was classified as a member of *Myoviridae* based on a combination of morphology and genomic characterization. *In vitro* tests showed that pAh6.2TG was relatively stable at different environmental conditions. Using this phage as prophylactic agent was successful at reducing mortality

in Nile tilapia. Phage pAh6.2TG application in rearing water not only suppressed MDR *A. hydrophila* loads in the rearing water and colonization of the bacteria in fish liver, but also improved fish survivability. These findings supported that pAh6.2TG could be used in rearing water for biocontrol of MDR *A. hydrophila* infection towards sustainable aquaculture.

3.6 Nucleotide sequence data

Phage pAh6.2TG sequence data has been submitted to the GeneBank databases under accession number MZ336020.

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CHAPTER 4

Ozone nanobubble treatments improve survivability of Nile tilapia (*Oreochromis niloticus*) challenged with a pathogenic multidrug-resistant *Aeromonas hydrophila*

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4.1. Abstract

Multidrug-resistant (MDR) bacteria has rapidly increased in aquaculture, which highlights the risk of production losses due to diseases and potential public health concerns. Previously, we reported that ozone nanobubbles (NB-O_3) were effective at reducing concentrations of pathogenic bacteria in water and modulating fish immunity against pathogens; however, multiple treatments with direct NB-O_3 exposures caused alterations to the gills of exposed-fish. Here, we set up a modified recirculation system (MRS) assembled with an NB-O_3 device (MRS-NB-O_3) to investigate whether MRS-NB-O_3 were 1) safe for tilapia (*Oreochromis niloticus*), 2) effective at reducing bacterial load in rearing water, and 3) improved survivability of Nile tilapia following an immersion challenge with a lethal dose of MDR *Aeromonas hydrophila*. The results indicated no behavioral abnormalities or mortality of Nile tilapia during the 14 day study using the MRS-NB-O_3 system. In the immersion challenge, although high bacterial concentration (~2×10^7 CFU/mL) was used, multiple NB-O_3 treatments in the first two days reduced the bacteria between 15.9% to 35.6% of bacterial load in water while bacterial concentration increased 13.1% to 27.9% in the untreated control. There was slight up-regulation of non-specific immune-related genes in the gills of the fish receiving NB-O_3 treatments. Most importantly, this treatment significantly improved survivability of Nile tilapia with relative percent survival (RPS) of 64.7 - 66.7% in treated fish and surviving fish developed specific antibody against MDR *A. hydrophila*. In summary, the result suggests that NB-O_3 is a promising non-antibiotic approach to control bacterial diseases, including MDR bacteria, and has high potential for application in recirculation aquaculture system (RAS).

Keywords: *Aeromonas hydrophila*, antimicrobial resistance, multidrug resistance, non-antibiotic approach, ozone nanobubbles

4.2 Introduction

Motile *Aeromonas* septicemia (MAS) is one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry (da Silva et al., 2012; Hossain et al., 2014; Peterman & Posadas, 2019; Pridgeon & Klesius, 2012). The control of bacterial diseases still depends heavily on antibiotics. In recent years, a global issue of concern is the increase in antimicrobial resistant (AMR) bacteria as the consequence of misuse of antibiotics (Cabello, 2006; Cantas & Suer, 2014; Malik & Bhattacharyya, 2019). The high levels of AMR in the aquatic environment and aquaculture products pose a negative impact to not only aquaculture production, but also public health and international trade, especially in low- and middle-income countries (LMICs) where aquaculture is highly concentrated (Ben et al., 2019; Heuer et al., 2009; Okocha et al., 2018; Reverter et al., 2020). Currently, there is a high proportion of pathogenic multidrug-resistant (MDR) bacteria strains causing diseases in aquaculture (Santos & Ramos, 2018). In the battle to combat AMR, apart from alternatives to antibiotics, there are efforts to explore novel approaches for reducing the risk of bacterial diseases in aquaculture systems e.g. bacteriophage and nanobubble technology.

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Nanobubbles (NBs) are bubbles less than 200 nm in diameter filled with chosen gases, neutral buoyancy, and having long residence time in the liquid solutions (Agarwal et al., 2011; Tsuge, 2014). Oxygen nanobubbles (NB-O_2) have been used for improving dissolved oxygen (DO) in aquaculture systems, and promoting growth of Nile tilapia (*O. niloticus*) (Mahasri et al., 2018) and whiteleg shrimp (*Penaeus vannamei*) (Mauladani et al., 2020; Rahmawati et al., 2020). Recently, several studies have revealed that ozone nanobubbles (NB-O_3) show promise at reducing quantities of pathogenic bacteria and improving DO in water, as well as modulating the immune

systems against bacterial infections (Imaizumi et al., 2018; Jhunkeaw et al., 2021; Linh et al., 2021; Nghia et al., 2021).

Ozone is a powerful disinfectant that has been used to reduce concentrations of pathogens and improve water quality in both flow-through and recirculating aquaculture systems for many years (Powell & Scolding, 2018). However, low ozone solubility and poor stability are major reasons for low utilization efficiency. In addition, misuse of direct ozonation can critically impact aquatic organisms, resulting in behavioral abnormalities, changes in physiology, tissue damage, and mortality (Powell & Scolding, 2018). However, NBs technology has been reported to improve gas dissolvability in water and promote rapid oxidation of organic substances (Gurung et al., 2016). Hence, NB-O_3 may enhance the solubility, stability, and efficacy of ozone in aquaculture systems (Fan et al., 2020). Kurita et al. (2017) reported that NB-O_3 treatment significantly reduced planktonic crustacean parasites (63%) in juvenile sea cucumbers (*Apostichopus japonicas*) and sea urchins (*Strongylocentrotus intermedius*). In another study, NB-O_3 demonstrated good disinfection of *Vibrio parahaemolyticus* in water, and prevention of acute hepatopancreatic necrosis disease (AHPND) in whiteleg shrimp (Imaizumi et al., 2018). We found that NB-O_3 treatment (1-2 × 10^7 bubbles/mL) reduced the level of *Streptococcus agalactiae* and *Aeromonas veronii* in water by more than 97% and made it relatively safe for juvenile Nile tilapia (Jhunkeaw et al., 2021). Most recently, we also reported that NB-O_3 treatment modulated the innate immune defense system of Nile tilapia, and that pre-treatment of NB-O_3 improved survivability of fish challenged with *S. agalactiae* (relative percent of survival of 60 - 70%) (Linh et al., 2021). This finding suggests that NB-O_3 may be a promising non-antibiotic treatment to control pathogenic MDR bacteria in aquaculture.

The limitations of direct application of NB-O_3 with high level of ozone (3.5 mg/L, 970 mV ORP (oxidation reduction potential) is the tissue damage that this gas can cause

to animals. Toxicity resulting in mortalities were reported for experimental shrimp in a study by Imaizumi et al. (2018). In our previous study on tilapia, we did not observe fish mortality but the fish gill morphology was damaged when fish were exposed directly to multiple NB-O_3 treatments with an ORP range between 860 ± 42 and 885 ± 15 mV (Jhunkeaw et al., 2021). In this study, we set up a modified recirculation system coupled with ozone nanobubbles (MRS-NB-O_3). Subsequently, we evaluated the system to determine if it was effective at suppressing pathogenic MDR *A. hydrophila* and the survivability of juvenile Nile tilapia.

4.3 Materials and methods

4.3.1 Bacterial strain and culture conditions

A laboratory strain of multidrug resistant *A. hydrophila* BT14, isolated from an outbreak of MAS in 2018, was used in this study. Briefly, this bacterial strain was identified by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and PCR-sequencing using *gyrB* housekeeping gene, following previous studies (Anand et al., 2016; Navarro & Martínez-Murcia, 2018). Based on the method proposed by Magiorakos et al. (2012), *A. hydrophila* BT14 was identified as a multidrug-resistant bacterium due to the fact that it resisted at least three classes of antimicrobials, including Ampicillin 10 μg (Penicillins), Tetracycline 30 μg (Tetracyclines), and Sulfamethoxazole-Trimethoprim 23.75 - 1.25 μg (Folate pathway inhibitors) (Table S1). For the bacterial challenge test, MDR *A. hydrophila* BT14 was propagated in 1 L of Tryptic Soy Broth, TSB (Becton Dickerson, USA) at 28 °C with 18 h shaking-culture at 150 rpm. The bacterial concentration was determined by conventional plate count method (Harrigan & McCance, 2014).

4.3.2 Experimental fish

Healthy Nile tilapia (3.92 ± 1.01 g) from a commercial tilapia hatchery in Thailand were acclimated in dechlorinated tap water for 2 weeks at 29 ± 1.0 °C before the experiments. Fish were fed with commercial tilapia feed (crude-protein 30%) at rate of

about 3% of fish weight twice daily. Before starting the experiments, ten fish were randomly selected for bacterial isolation and found to be free of *A. hydrophila*. The experiments on animals were conducted with permission of Thai Institutional Animal Care and Use Committee (Approval no. MUSC62-039-503).

4.3.3 MRS-NB-O3 system setup and water parameter measurement

The ozone nanobubble system consisted of an oxygen concentrator (Model: VH5-B, Shenyang Canta Medical Technology Company Limited, Liaoning, China) connected to an ozone generator (Model: CCba15D, Coco Technology Company Limited, Chonburi, Thailand) and a nanobubble generator (Model: aQua+075MO, AquaPro Solutions Private Limited Company, Singapore). The NB-O_3 system was attached to a modified recirculation system (MRS) which contained two 100 L-fiberglass tanks (50 L dechlorinated tap water in each tank) that exchanged water by water pumps. One tank received the NB-O_3, the other tank housed the fish (Figure 1). All water quality parameters were measured in triplicate in the MRS-NB-O_3. Water temperature, pH, dissolved oxygen (DO), and oxidation reduction potential (ORP) were measured and compared from both tanks using a multi-parameter meter (YSI Professional Plus, YSI Incorporated, USA). During the application of the NBs, water samples were collected at 0 min, 5 min, 10 min of NB-O_3 treatment and 30 min post-treatment for measurement of dissolved ozone (ppm-mg/L) using K-7434 Ozone Vacu-vials Kit (Oxidation Technologies, USA).

4.3.4 Effect of MRS-NB-O3 on fish safety

To evaluate the safety of Nile tilapia juveniles cultured in MRS-NB-O_3 system, 136 fish were divided into four tanks (50L dechlorinated tap water per tank) consisting of two replicate groups (controls and MRS-NB-O_3) with 34 fish per tank. The treatment group was treated with NB-O_3 (oxygen input 2 L per min) 7 times (10 min/time) at 1, 12, 24, 36, 48, 60, and 72 h from the start time of the experiment. Aeration was

provided one hour after each treatment. The control group was treated with normal aeration instead of NB-O_3. Fish were observed every 12 h for behavioral abnormality and mortality over a 14-day period. The water parameters including temperature, pH, DO, and ORP were measured before and during treatment. After every treatment, two fish in each tank were randomly collected and preserved for gill histology examination. Formalin preserved samples (n = 28) were subjected to routine histology. The histopathological changes were observed under the Leica DM1000 digital microscope equipped with a digital camera DFC450 (Leica, Singapore).

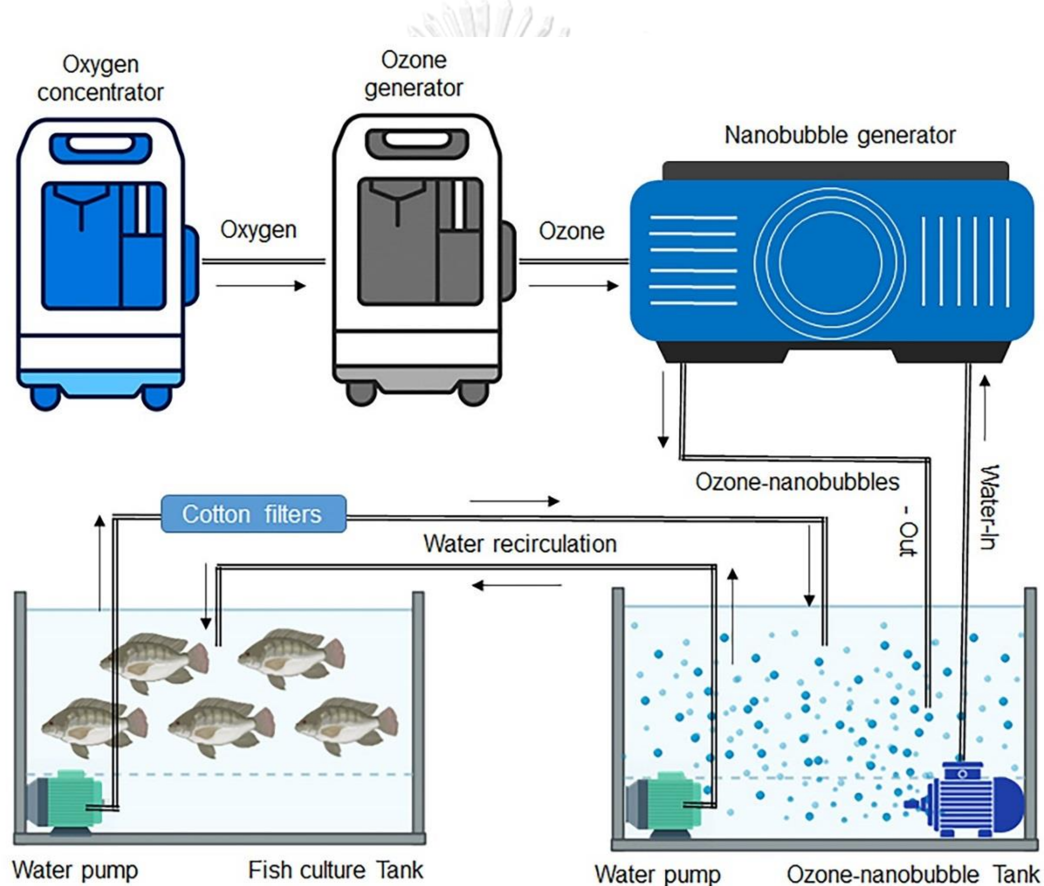

Figure 1. Experimental set-up of MRS-NB-O_3. Oxygen concentrator releases oxygen as a material to synthesize ozone using ozone generator. Ozone was lead to nanobubble generator. Inside the system, ozone was diffused in nanobubble water and released to ozone-nanobubble tank. Thereafter, NB-O_3 water was pumped to fish culture tank. The rearing water were recirculated between NB-O_3 tank and fish culture tank via a pump system assembled to cotton filter box to absorb fish feces and leftover feed.

4.3.5 Immersion challenge trial for MDR A. hydrophila BT14

To establish the immersion challenge dose, 80 fish were divided into four 50 L tanks, each tank containing 20 fish. Three tanks were challenged with MDR *A. hydrophila* BT14 by adding 1 L of bacterial culture (approx. 8×10^6, 8×10^7, and 8×10^8 CFU/mL) to each tank to reach the final concentrations of 2×10^5, 2×10^6, and 2×10^7 CFU/mL, respectively. A total 1 L of culture medium without bacteria was added to a negative control tank. Air-stones were used in all tanks for air supply and approximate 50% of the water was changed after 48 h. Clinical signs of MAS and mortalities were recorded every 12 h for 14 days. The representative dead or moribund fish were subjected to bacterial re-isolation using selective medium Rimler Shotts (RS, Himedia, India) supplemented with Novobiocin (Oxoid, UK).

4.3.6 Effect of multiple NB-O3 treatments in MRS on Nile tilapia challenged with MDR *A. hydrophila*

Fish survivability, gill collection, and water collection

Two trials were conducted to test the effect of our MRS NB-O_3 treatments. In the first trial, 128 fish were randomly divided into four groups (32 fish per tank): Group 1 was exposed to culture medium without NB-O_3 treatment (no Ah + no NB-O_3); Group 2 was exposed to bacteria without NB-O_3 (Ah + no NB-O_3); Group 3 was exposed to culture media only and treated with NB-O_3 (no Ah + NB-O_3); Group 4 was challenged with *A. hydrophila* and treated with NB-O_3 (Ah + NB-O_3). In bacterial challenge groups 2 and 4, 1 L of MDR *A. hydrophila* BT14 (approx. 8×10^8 CFU/mL) was added to 50 L water to reach a final concentration of approx. 2×10^7 CFU/mL. The fish were maintained at 29 ± 1 °C with aeration for 3 h. Afterwards, fish in groups 3 and 4 were treated for 10 min with NB-O_3 at 1, 12, 24, 36, and 48 h post-challenge, while group 1 and group 2 were treated with normal aeration. In order to investigate the effect of NB-O_3 treatments on the fish immune response in our MRS, the gills from 4 fish were

randomly sampled at 3 h after the 1st, 2nd, and 3rd NB-O_3 treatments and preserved in 200 µL of Trizol reagent (Invitrogen, USA) for immune genes analysis. The remaining fish were observed daily for 14 days and mortality was recorded. Representative moribund or freshly dead fish were collected for bacterial re-isolation using Rimler Shotts (RS) medium plus Novobiocin as described above. The relative percent survival (RPS) was calculated according to the formula described by Ellis (1988): RPS = [1 - (% mortality in challenge/ % mortality in control)] × 100. In parallel, water samples from groups 2 and 4 (challenged with *A. hydrophila*) were evaluated for bacterial enumeration using conventional plate count method (Harrigan & McCance, 2014). The percentage of bacterial fluctuation was calculated based on bacterial concentration (CFU/mL) before and after NB-O_3 treatment.

In the second trial, the experiment was repeated in the same manner as the first with the exception that 20 fish were used for each group and this experiment focused mainly on monitoring survival rate and bacterial enumeration. This experiment was repeated to confirm our initial survival results in the first trial.

Visualization of live and dead bacteria before and after treatment with NB-O3

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A volume of 25 mL water in group 4 (Ah + NB-O_3) was sampled before and after the first NB-O_3 treatment for assessment of the viability of *A. hydrophila*. A bacterial suspension was prepared and stained following the protocol of LIVE/DEAD *Bac*light Bacterial Viability Kit (Cat. No. L7012, Thermo-Fisher Scientific, USA). In brief, the bacterial suspensions were centrifuged at 10,000 x *g* for 10 min at 4°C. The pellets were collected and re-suspended in 2 mL of sterile normal saline buffer, incubated at room temperature for 1 h, mixing every 15 min. Bacteria were washed two times by centrifugation at 10,000 x *g* for 10 min at 4°C and pellet resuspension was done in 20 mL and 10 mL of sterile normal saline buffer for the first and second time of washing. Staining processes were conducted by mixing 1.5 µL of SYTO®9, 1.5 µL of Propidium

Iodine (PI), and 1 mL of bacterial suspension in a microtube. The mixture was incubated at room temperature in the dark for 15 min. After that, 5 µL mixtures were pipetted onto glass slides, covered with a coverslip and examined under a confocal laser scanning microscope CLSM (Model: DM1000, Leica Microsystem Private Limited Company, Singapore) assembled with incident light fluorescence to visualize live and dead bacteria. Five random fields from each slide were imaged. Fluorescence signals were counted in ImageJ software based-on Watershed algorithm.

Expressions of innate immune-related genes

Due to similar immune response patterns of six immune-related genes of tilapia were observed in our previous study (Linh et al., 2021), three representative genes (*LYZ*, *HSP90*, and *TNF-α*) involved in different immune pathways were chosen in this study to evaluate whether the MRS-NB-O_3 system had an impact on fish immunity. To investigate expression of innate immune-related genes, total RNA of gill samples was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The first complementary DNA (cDNA) strand was synthesized from 2.0 µg of the total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad, USA) according to the procedure described in the product manual. Quantitative real-time PCR (qPCR) using SYBR green reagent (iTaq™ Universal SYBR™ green Supermix, Bio-Rad, Hercules, CA, USA) was carried out using primers specific for 3 immune genes (Table 1). The qPCR amplification cycles were performed using a CFX Connect™ Real-time System (Bio-Rad, USA). Cycling conditions were 94 °C for 15 s, 40 cycles of denaturation at 95 °C for 30 s, annealing at the optimal temperature of each primer as indicated for 30 s, and a final extension at 72 °C for 30 s. Melting curves were obtained in the 55 to 85°C range with 0.1 °C increments per second to evaluate for the specificity of all qPCR products. The qPCR data will be analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001). The transcript levels of each target gene were obtained as C_q values and normalized to that the *EF-1α* as an internal reference.

Table 1. Primers used to quantify relative gene expression in this study

Target gene	Oligo sequence (5ʹ -3ʹ)	Genbank Accession No.	Product size	Annealing temperature	References
TNF-α	F: CTTCCCATAGACTCTGAGTAGCG R: GAGGCCAACAAAATCATCATCCC	NM_001279533	161 bp	60 °C	Liu et al. (2011)
HSP90	F: ATTGCTCAGCTGATGTCCCT R: GTGGGATCCGTCAAGCTTTC	XM_003440645.5	128 bp	56 °C	Linh et al. (2021)
LYZ	F: AAGGGAAGCAGCAGCAGTTGTG R: CGTCCATGCCGTTAGCCTTGAG	XM_003460550.2	151 bp	63 °C	Qiang et al. (2016)
EF-1α	F: CTACAGCCAGGCTCGTTTCG R: CTTGTCACTGGTCTCCAGCA	AB075952	139 bp	60 °C	Velázquez et al. (2018)

F: Forward primer, R: Reverse primers, bp: base pair

Determination of serum antibody by the enzyme-linked immunosorbent assay (ELISA)

In order to determine whether surviving fish at day 14 post challenge develop specific antibodies (IgM) against *A. hydrophila*, blood samples were collected from fish in the first trial (four from Ah + no NB-O_3 group and five from each of the other groups). Blood samples were kept at room temperature for 1 h before being centrifuged at 8,000 x g for 15 min. The collected fish sera were stored at -20°C until used. An ELISA was carried out following the protocol described by Linh et al. (2021) with minor modification. In brief, 96 well EIA/RIA plates (Costar®, Corning Inc., USA) were coated with formalin-killed *A. hydrophila* whole-cell antigen (OD_{600nm} = 1.0). Fish sera (dilution 1:256), anti-tilapia IgM secondary antibody (1:200) (Soonthonsrima et al., 2019), and commercial goat anti mouse antibody horseradish peroxidase (HRP) conjugate (1:3,000) were used for the ELISA assay in this study and samples were read at an absorbance of 450 nm using a SpectraMax® iD5 Multi-Mode Microplate Reader (Molecular Devices, USA).

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4.3.7 Statistical analysis

Cumulative mortality and percent survival data from the challenge experiments were analyzed by the Kaplan-Meier method and differences among groups were tested using a log-rank test, *p*-values of 0.05 or less were considered statistically significant. Fish innate immune-related gene expression was analyzed by ANOVA, *p*-values of 0.05 or less were considered statistically significant. Duncan's post-hoc test was used to measure specific differences between pairs of mean. The OD_{450nm} readings from our indirect ELISA assay were analyzed using a Kruskal-Wallis test, *p*-values of 0.05 or less were considered statistically significant. Multiple comparison analyses were performed by Bonferroni test. All statistical analyses were performed using SPSS Software ver22.0 (IBM Corp., USA).

4.4 Results

4.4.1 Effect of MRS-NB-O_3 on water parameters

For the 10 min NB-O_3 treatment in the MRS, the change of water parameters, including temperature, pH, DO, and ORP, are displayed in Figure 2. Temperature and pH values appeared stable over time in both the NB-O_3 treated tank and the culture tank (which did not have fish for this investigation). The DO increased significantly after 10 min NB-O_3 treatments in both tanks. The DO level in the culture tank increased from 5.07 ± 1.61 to 13.97 ± 0.84 mg/L (increase of 8.9 mg/L), while there was a higher increase in NB-O_3 tank (from 6.84 ± 1.08 to 19.74 ± 1.28 mg/L). The significantly different trend of ORP value was observed in the NB-O_3 treated tank and culture tank. The ORP decreased slightly from 424.9 ± 24 to 396 ± 61.9 mV in fish culture tank, whereas the ORP in NB-O_3 tank increased rapidly from 417.7 ± 23.6 to 791.7 ± 71.5 mV after 5 min NB-O_3 treatment and reached 870.1 ± 12.4 mV after 10 min. During NB-O_3 treatment, dissolved ozone concentration at 0 min, 5 min, and 10 min in treated tank were 0.02, 1.16, and 1.37 mg/L respectively, whereas significantly lower values, 0.03, 0.06, and 0.14 mg/L were recorded in system's fish culture tank at the same time points. At 30

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min post-treatment, dissolved ozone concentration in NB-O_3 treated and fish culture tanks decreased to 0.05 and 0.03 mg/L, respectively.

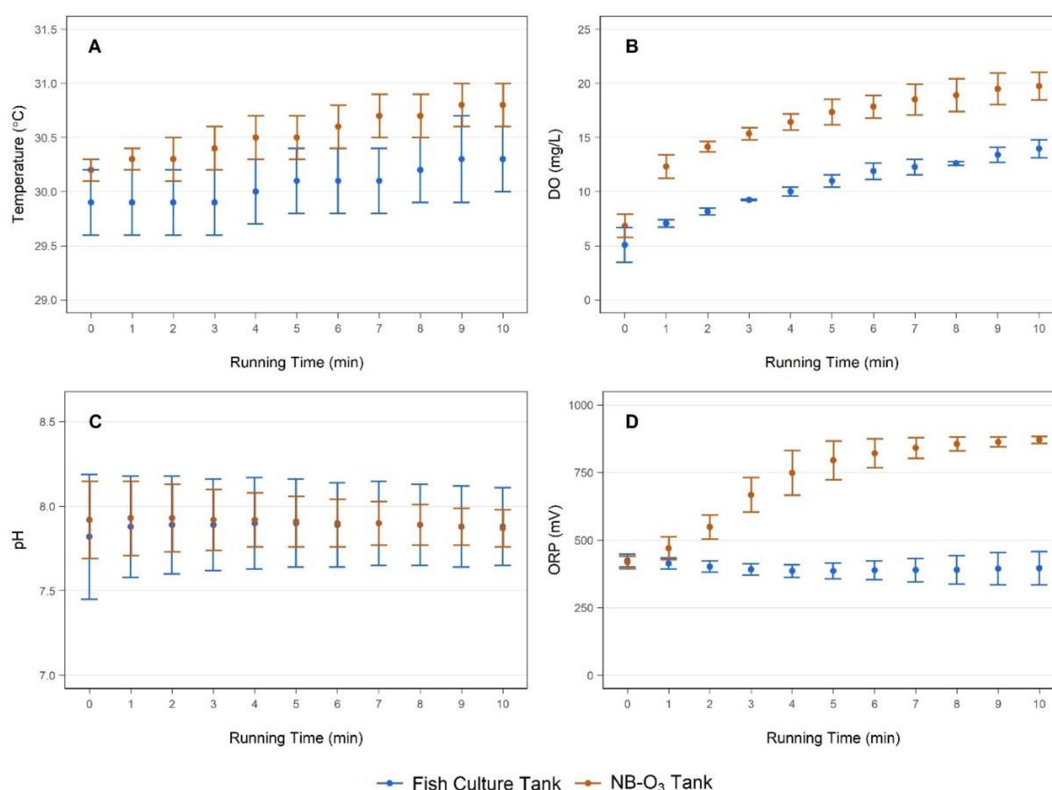

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Figure 2. Measurement of water parameters including temperature (**A**), DO (**B**), pH (**C**), and ORP (**D**) during 10 min NB-O_3 treatment with 2 L/min oxygen input in MRS. Value of water parameters are mean ± SD (n = 3).

4.4.2 Effect of MRS-NB-O_3 on fish safety

No mortality or behavioral abnormalities in fish were observed in either the control and NB-O_3 treated groups during and after treatments. All fish survived the 14 day study period. Histologically, there were no differences in gill morphology in control and treatment groups after five NB-O_3 treatments. However, alterations were observed in the gill filaments after the 6th and 7th treatments (Figure S1). The fluctuation of water parameters was consistently similar during every treatment (Table S2), and similar to

the trend in the previous experiment without fish (Figure 2). Temperature and pH increased slightly in both groups during treatment. Dissolved oxygen in the fish culture tanks of the MRS-NB-O_3 increased significantly from 4.98 - 6.97 mg/L (before each treatment) to 12.26 - 15.33 mg/L (at each 10 min of treatment) and dropped to 9.28 - 12.69 mg/L after the 10 min treatment. ORP values in fish culture tanks did not increase and remained relatively stable in control and NB-O_3 treated groups.

4.4.3 Immersion challenge trial for MDR *A. hydrophila* BT14

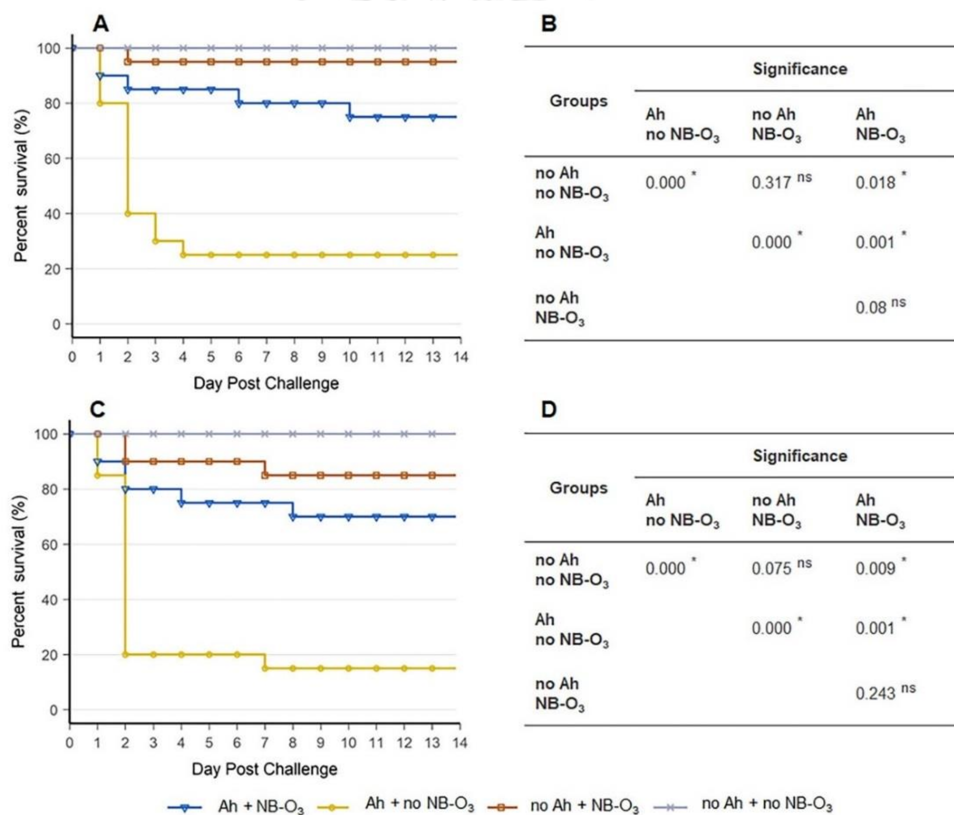
The cumulative mortality of Nile tilapia challenged with three different doses of MDR *A. hydrophila* BT14 by immersion was dose-dependent (Figure S2). The fish challenged with 2×10^7 CFU/mL (high dose) had a 75% mortality rate, and death occurred mainly in the first 4 days of the experiment. In the 10-fold lower dose, there was only 25% mortality and most fish died from days 4 to 9. There was no mortality in the group challenged with 2×10^5 CFU/mL or the control group (Figure S2). The clinically sick fish showed lethargy, loss of appetite, and tended to swim at the surface, but did not reveal significant external or internal symptoms except pale livers. Bacterial isolation from representative dead fish (n = 5) revealed dominant colonies of bacteria, morphologically resembling *A. hydrophila* on selective medium. From this result, the dose of 2×10^7 CFU/mL was used for subsequent challenge assays.

4.4.4 MRS-NB-O_3 improved survivability of Nile tilapia challenged with the MDR *A. hydrophila* BT14

The results of the challenge tests were consistent between replicates (Figure 3). The group challenged with *A. hydrophila* followed by NB-O_3 treatments (Ah + NB-O_3) had 70 and 75% survival compared to 15 and 25% in the group challenged with bacteria receiving no NB-O_3 treatment (Ah + no NB-O_3). This difference was statistically significant (p = 0.001) in both trials. No mortality was observed in the negative control group (no Ah + no NB-O_3) during the 14 day study period. However, there were 5 and

15 % mortality in the control groups treated with NB-O_3 without a precedent bacterial challenge (no Ah + NB-O_3). However, this was not statistically significant to the negative control group in either trials (p = 0.317 in trial 1 and p = 0.075 in trial 2 (Figure 3). The relative percent survival (RPS) of NB-O_3 treatments in the 2 replicate treatment groups were 64.7 and 66.7%, respectively.

The moribund fish in challenge groups showed pale liver and behavioral abnormalities, including lethargy, loss of appetite, and surface swimming. The typical colonies of *A. hydrophila* were consistently recovered from internal organs (i.e. liver, kidney) of representative dead fish using RS medium supplemented with Novobiocin.



B

Groups	Significance		
	Ah no NB-O_3	no Ah NB-O_3	Ah NB-O_3
no Ah no NB-O_3	0.000 *	0.317 ns	0.018 *
Ah no NB-O_3		0.000 *	0.001 *
no Ah NB-O_3			0.08 ns

D

Groups	Significance		
	Ah no NB-O_3	no Ah NB-O_3	Ah NB-O_3
no Ah no NB-O_3	0.000 *	0.075 ns	0.009 *
Ah no NB-O_3		0.000 *	0.001 *
no Ah NB-O_3			0.243 ns

Figure 3. Kaplan - Meier analysis of percentage survival of Nile tilapia (n = 20) challenged with MDR *A. hydrophila* BT14. The experiment was done in two independent trials, trial 1 (A) and trial 2 (C). Differences between groups in each trial were tested using log-rank test shown in (B) and (D) respectively. "*" denotes significant difference ($p < 0.05$), "ns" means not significant.

In parallel, bacterial concentration in the water column was monitored in two groups challenged with *A. hydrophila*. In the group Ah + NB-O_3, bacterial load in fish culture tanks after the 1st, 2nd and 3rd treatments were reduced by 35.6, 23.3, and 20.2%, respectively in the first trial, and by 23.9, 21.1, and 15.9%, respectively in the second trial (Figure 4). By contrast, bacterial load in the Ah + no NB-O_3 increased by 13.4, 13.1, and 27.1% in the first trial, and by 15.6%, 19.8, and 27.9 % during the same time period in the second trial. Representative photomicrographs of comparative visualization of live and dead bacteria before and after treatment with NB-O_3 are illustrated in Figure 5. Before NB-O_3 treatment, the majority of bacterial cells appeared to be alive (i.e. stained fluorescent green), with very few dead cells (i.e. red color) (Figure 5A-C). However, after 10 min NB-O_3 treatment, the density of dead cells (red staining cells) increased considerably (17.45%) per microscopic field.

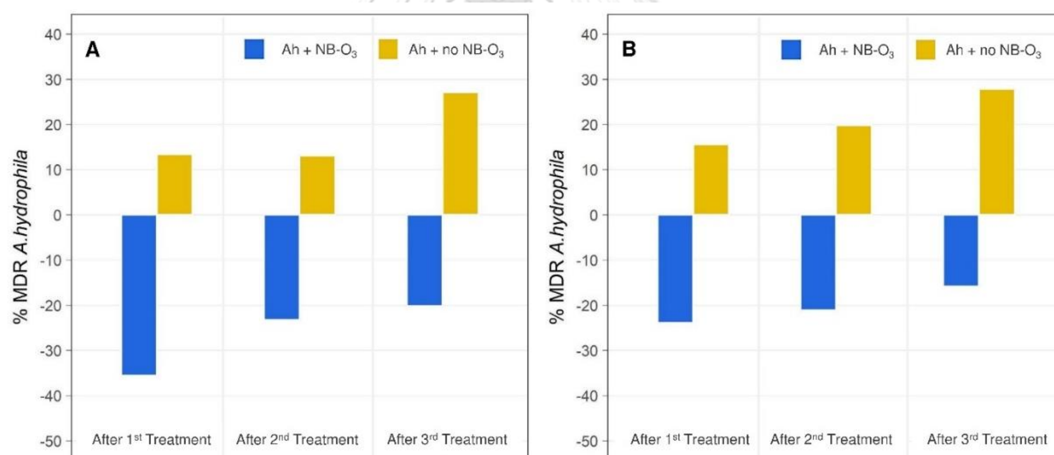


Figure 4. Concentration of MDR *A. hydrophila* BT14 in rearing water between un-treated and treated by 10 min NB-O_3 groups after the 1st, 2nd, and 3rd treatment. A, trial 1; B, trial 2.

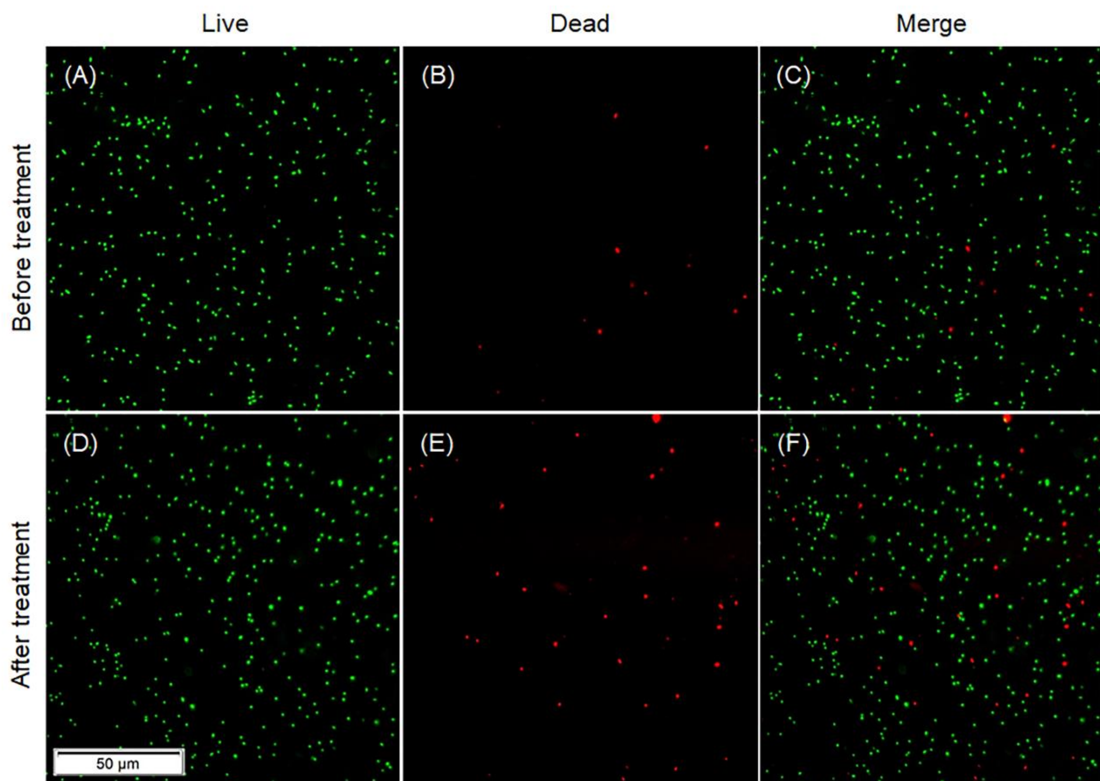


Figure 5. Confocal scanning laser microscope image of MDR *A. hydrophila* BT14 viability following the 1st treatment with 10 min NB-O_3 (A-C: before 1st treatment and D-F: after 1st treatment). Figure C is merged by A and B whereas figure F is merged by D and E. Green fluorescent indicates live bacterial cells and red fluorescent indicates dead bacterial cells using LIVE/DEAD *Bac*light Bacterial Viability Kit with two staining reagents SYTO®9 and PI.

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4.4.5 Expressions of innate immune-related genes

The expression levels of innate immune genes from different groups after each NB-O_3 treatment are shown in Figure 6. Although not statistically significant, the overall expression levels of immune genes *LYZ, HSP90,* and *TNF-a* in the gills of the fish exposed to NB-O_3 treatments tended to be slightly higher than that of the untreated control, except for the first treatment. Specifically, the trends included *LYZ* expression in treated group with or without *A. hydrophila* challenge which rose after the 2nd and 3rd treatment compared to that in the negative control group. The highest expression level (approx. 2.2 fold) was recorded in NB-O_3 treated group with *A. hydrophila* at the 3rd treatment. Expression of *HSP90* had different patterns for different experiments.

The expressions in NB-O_3 treated group with or without *A. hydrophila* challenge increased at the 2nd treatment but decreased similar to the levels in the control group for the 3rd treatment. The relative transcription level of *TNF-a* increased slightly (1.4 fold) with the highest expression level in NB-O_3 treated group.

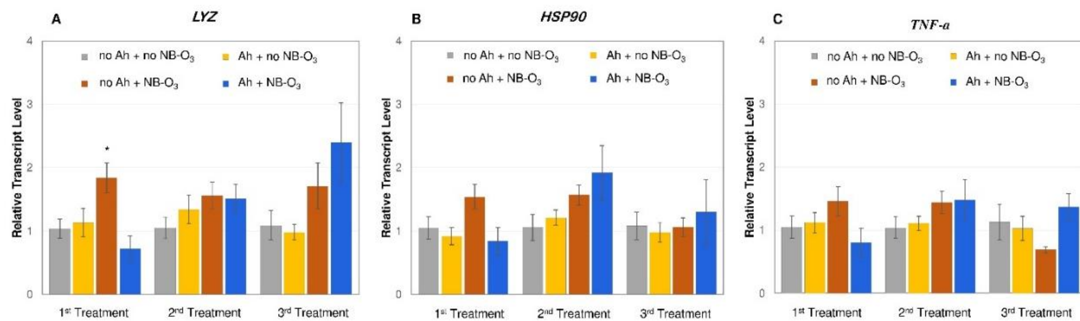

Figure 6. Relative expression of *LYZ* (**A**), *HSP90* (**B**) and *TNF-a* (**C**) in fish gills in 4 groups: no Ah + no NB-O_3, Ah + no NB-O_3, no Ah + NB-O_3 and Ah + NB-O_3 after 1st, 2nd and 3rd treatment with NB-O_3. The expression of target genes was normalized using *EF-1a*. Value of relative transcript level are mean ± a standard error of the mean (SEM) bar (n = 4) and "*" above the bar indicates significant difference between groups ($p < 0.05$).

4.4.6 Specific antibody (IgM) response post-challenge

All surviving fish in both groups challenged with MDR *A. hydrophila* had significantly higher levels of specific antibody (IgM) compared to the two unchallenged control groups ($p < 0.05$) as measured by indirect ELISA (Kruskal-Wallis test: H (3) = 15.542, p = 0.001). The serum from fish in the Ah + NB-O_3 group had the highest OD_{450} readings (0.44 ± 0.076), followed by OD readings of serum in Ah + no NB-O_3 group (0.42 ± 0.06). In contrast, the lowest level (0.06 ± 0.004) was recorded in the negative control (no Ah + no NB-O_3). A higher level but not statistically significant difference with negative control was shown in group no Ah + NB-O_3 (0.1 ± 0.013) (Figure 7).

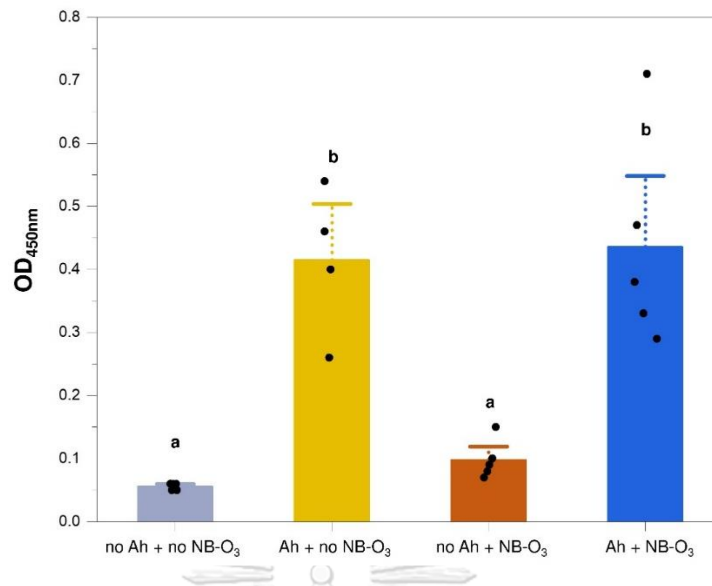

Figure 7. Indirect ELISA analysis of *A. hydrophila* specific IgM antibody. Fish sera were collected on day 14 and 1:256 dilutions were used to test for antigen specific IgM. Data were expressed as mean absorbance at OD_{450nm} with a SEM bar. One dot represents one biological replicate (n = 4 in group Ah + no NB-O_3, n = 5 in other groups). Different letters above the bar indicate significant difference between groups ($p < 0.05$).

4.5 Disscussion

Several studies have reported potential applications of NB-O_3 for pathogen disinfection in aquaculture water to reduce the risk of infectious diseases in both fish and shrimp (Imaizumi et al., 2018; Jhunkeaw et al., 2021; Kurita et al., 2017). We recently reported an additional benefit of NB-O_3 in modulating of the innate immune defense system in Nile tilapia to fight against *S. agalactiae* (Linh et al., 2021). However, all the precedent studies exposed the animals directly to NB-O_3 (NB-O_3 was exposed directly into the tank containing fish or shrimp) and this resulted in mild to severe health impacts on the exposed animals. High dose of ozone (960 mV ORP) were toxic to shrimp (Imaizumi et al., 2018), or caused gills alteration in tilapia after repeated exposures to NB-O_3 (~860 mV ORP) (Jhunkeaw et al., 2021). Therefore, we modify a NB-O_3 system on a laboratory scale to better understand this technology and overcome this drawback.

Ozone is an unstable molecule, even in the form of nanobubbles, which degrades relatively quickly (Jhunkeaw et al., 2021). Based on this characteristic, we set up a modified recirculation system coupled with NB-O_3 technology (MRS-NB-O_3), which separated the NB-O_3 treatment tank from the culture tank containing fish to reduce direct exposure of the fish to high level of ozone. Interestingly, during treatment, ozone level increased rapidly in the NB-O_3 treatment tank but did not increase in the fish culture tank, as indicated by ORP values (870.1 ± 12.4 vs. 396 ± 61.9 mV ORP) and dissolved ozone concentrations (1.37 vs. 0.14 mg/L). Several studies suggested that ORP levels in the range from 300 to 425 mV ORP were safe for fish, crustaceans, and molluscs (Li et al., 2014; Powell & Scolding, 2018; Stiller et al., 2020). In the MRS-NB-O_3 set up, multiple treatments (up to seven 10 min treatments) in this study appeared to be relatively safe for juvenile Nile tilapia, with no mortality over a 14 day period. We also noticed that the MRS-NB-O_3 system could avoid excess DO level in the culture tank that commonly occurred when the NB-O_3 treatments were applied directly to the fish tanks (Jhunkeaw et al., 2021).

This study revealed that multiple NB-O_3 treatments in our MRS-NB-O_3 system improved survivability of Nile tilapia (*O. niloticus*) challenged with a pathogenic multidrug-resistant *A. hydrophila*. Motile Aeromonads have been reported as one of the most common pathogens in freshwater aquaculture (Hayatgheib et al., 2020). *A. hydrophila* can cause between 35-100% mortality during disease outbreaks (Baumgartner et al., 2018; Pridgeon & Klesius, 2011; Rasmussen-Ivey et al., 2016). Under experimental conditions, *A. hydrophila* can cause between 50 to 80% mortality in Nile tilapia (Abass et al., 2018; Dawood et al., 2020; Suprayudi et al., 2017). In the present study, relatively high mortality (75 - 85%) was observed in immersion challenges with a MDR *A. hydrophila*. Interestingly, multiple NB-O_3 treatments were effective with RPS of 64.7 - 66.7%. The RPS value in this study was similar or higher than several studies

using antibiotics for Aeromonads control in Nile tilapia e.g. RPS of 60% in orally administered with Oxytetracycline 4g/kg/feed per day (Abraham et al., 2017) or RPS 25.9 % in orally fed with Oxytetracycline 60 mg/kg biomass per day (Julinta et al., 2017).

Compared to other non-antibiotic approaches, NB-O_3 offered comparable protective efficacy to some probiotic-based products against *Aeromonas* sp. AC9804 infection such as *Lactobacillus rhamnosus,* which reported RPS values of 66.7% (Ngamkala et al., 2010) and *L. plantarum* with an RPS of 64% (Dawood et al., 2020). The results of this study were also comparable to some plant-based products used to control *A. hydrophila*, with reported RPS around 71% for Indian ginseng, *Withania somnifera* powder (Zahran et al., 2018), 35.3% for American ginseng, *Panax quinquefolius* (Abdel-Tawwab, 2012), and 58.7% for ginger, *Zingiber offcinale* (Payung et al., 2017). The differences in RPS may also have been from other factors such as different bacterial strains, different exposure doses of *A. hydrophila*, different fish species, fish sizes, and fish sources. However, our finding suggests that NB-O_3 treatments could be considered a potential non-antibiotic approach to control bacterial disease in aquaculture.

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Ozone is among the most powerful oxidant known with oxidative potential of 2.07 volts, nearly twice of chlorine (Hugo et al., 1999). Further, aqueous ozone can generate hydroxyl radicals (OH^-) with higher oxidative potential (2.83 volts) than ozone (Qingshi et al., 1989). Ozone ruptures cells by destroying the glycoproteins and glycolipids on the cell membranes. Moreover, ozone attacks the sulfhydryl groups of enzymes results in disruption of normal cellular enzymatic activity and loss of function. Lastly, ozone can directly damage the purine and pyrimidine bases of nucleic acids (Megahed et al., 2018). When NBs collapse, they generate shock waves that consequently lead to the

formation of hydroxyl radicals (Fan et al., 2020; Takahashi et al., 2007). Thus, NB-O_3 may enhance the disinfectant efficacy of ozone in aquaculture systems.

Although the differences in bacterial concentration in the Ah + NB-O_3 group were only 1.0 to 1.6 fold lower than the Ah + no NB-O_3 group after each treatment, clear differences in survivability of the fish were observed in these groups. It is also possible although not statistically significant on an individual basis the overall upregulation of innate immune genes and stimulation of humoral immune response for fish in the NB-O_3 treatment group partially contributed to better survival rates after bacterial challenges. This has been reported by others as well (Linh et al., 2021). The stimulation of innate immunity is the first line of defense against invading pathogens and leads to improvements in health conditions and resistance to pathogens of fish (Magnadóttir, 2006). Pro-inflammatory cytokines, particularly *TNF-α* is an important macrophage-activating factor produced by leukocytes (Whyte, 2007), while lysozyme is a vital defense molecule of fish immune system due to make the demolition of bacterial cell wall (Saurabh & Sahoo, 2008). In addition, heat-shock proteins have a function in the development of specific and non-specific immune response to infections (Roberts et al., 2010).

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Another factor which may also have improved survival of fish in this experiment was the DO in treated groups. Higher level of DO in NB-O_3 treated groups during and after treatments may improve fish health by maintaining or improving normal physiological functions. Previous studies suggested that high level of oxygen improved the immunocompetence in fish (Bowden, 2008; Cecchini & Saroglia, 2002). Romano et al. (2017) revealed that 12 - 13 mg/L oxygen increased immune response performance of sea bass (*Dicentrarchus labrax*). It is also possible that the increased survivability of Nile tilapia exposed to NB-O_3 treatment in this study was from a combination of

synergistic effects of bacterial reduction, increased DO, and stimulation of the fish immune response.

One of the limitations of this study was our small sample size which could account for the non- significant difference in the up-regulation of innate immune genes between groups. Further, we were unable to compare effectiveness of different forms of ozone bubbles (macro-, micro- and nanobubbles) in reducing bacterial loads and improving fish survival rate upon bacterial infection. Further studies should explore these issues to gain better understanding of this promising technology. In addition, the MRS-NB-O_3 system need to be scaled up to be utilizable in aquaculture systems.

Despite these limitations, this study reported a MRS coupled with NB-O_3 technology was successful at reducing mortality in fish and not exposing fish to high levels of ozone. It may be possible to scale this system up for use in hatcheries and commercial farms that use RAS systems. Our MRS-NB-O_3 allowed multiple NB-O_3 treatments without obvious negative impacts on the fish. This system not only suppressed MDR bacterial loads in the culture tanks, but also improved fish survivability. Application of NB-O_3 may be a promising non-antibiotic method of reducing the risk of infectious diseases caused by bacteria, including MDR bacterial strains.

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Chapter 5

Impacts of oxygen and ozone nanobubbles on bacteriophage in aquaculture system

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5.1 Abstract

Injection of gas nanobubbles into water reduces bacterial load, improves dissolved oxygen, and modulates the fish innate immune system. Little is known about the effect that nanobubble treatment has on the concentration of viruses in water. This study, investigated the disinfection impact of oxygen and ozone nanobubbles (NB-O_2 and NB-O_3) on an *Aeromonas hydrophila*-specific phage, pAh6.2TG, a virus lab model. After 5-, 10- and 15-min treatment with NB-O_2, the concentration of phage remained at the same level, while the same treatment with NB-O_3 eradicated 99.99 to 100% of the phage in the water. Since this lytic phage has been tested and has been shown to control bacterial infections, we further investigated whether NB-O_2 improved adherence of the phage on fish body surface (skin, mucus, and gills) and phage penetration into fish internal organs, specifically the liver. Nile tilapia, *Oreochromis niloticus*) were used as experimental fish in this study. The results showed that the number of phages adhered to the skin mucus and gills in NB-O_2 treatment group was 1.07 to 15.0 times higher than in the untreated control group without gas bubbles. The number of phage uptake into fish liver after NB-O_2 treatment increased 1.29 to 4.75 fold compared to untreated control. These findings suggested a plausible application of NB-O_2 treatment for improving efficacy of phage therapy in aquaculture. On the other hand, NB-O_3 application may be useful for disinfection of harmful viruses in culture water, but the treatment would need to be omitted during phage treatment. This study provided preliminary information on potential applications of nanobubble technology in aquaculture to reduce viral loads in the water and potentially reduce the magnitude of viral outbreaks.

Keywords: aquatic viral diseases, adherence, bacteriophage, nanobubble, virus model

5.2 Introduction

Aquaculture is one of the fastest-growing food industries, and it plays a crucial role in global food security and nutrition, particularly in low- and middle-income countries (LMICs) (FAO, 2020; Hicks et al., 2019; Naylor et al., 2021; Webb et al., 2020). However, aquaculture sectors have faced increasing challenges with infectious diseases including antimicrobial resistance (AMR) microorganisms (Stentiford et al., 2020; Stentiford et al., 2017). Thus, research efforts on non-chemical approaches, such as nanobubbles (NBs) for treating pathogens have increased in recent years to reduce the risk of AMR and address production losses caused by the emergence of pathogenic AMR bacterial strains (Hoelzer et al., 2018; Reverter et al., 2020; Watts et al., 2017).

Nanobubbles are bubbles less than 100 nm in diameter. They can be created with different gases, and have neutral buoyancy, which enables them to have a lengthy residence time in water (Agarwal et al., 2011; Tsuge, 2014). In aquaculture, oxygen nanobubbles (NB-O_2) are commonly used for improving dissolved oxygen (DO) and promoting the growth of aquatic animals (Mahasri et al., 2018; Mauladani et al., 2020; Rahmawati et al., 2020). Recent studies have indicated that ozone nanobubbles (NB-O_3) have potential to reduce pathogenic bacteria, improve DO in water, and modulate the immune systems against bacterial infection (Dien et al., 2021b; Imaizumi et al., 2018; Jhunkeaw et al., 2021; Linh et al., 2021; Nghia et al., 2021). Hitherto, the effect of NB-O_2 and/or NB-O_3 on aquatic viruses remains uninvestigated.

Lytic bacteriophages (also known as phages) are viruses that infect and kill bacteria (Kutateladze and Adamia, 2010). Due to the high similarity to animal virus properties, phages have been considered as models for studies related to the survival of viruses under different environmental conditions and to evaluate the efficacy of disinfection methods (Grabow, 2001; Pinon and Vialette, 2018). On the other hand, phage therapy could also be used as a natural strategy to control bacteria to replace or supplement

chemotherapy in aquaculture (Angulo et al., 2019; Culot et al., 2019; Rao and Lalitha, 2015; Richards, 2014). Several studies have revealed that the increase of phage binding to the mucosal layer of the host improved protection against bacterial infections (Almeida et al., 2019; Barr et al., 2013; Barr et al., 2015; Dabrowska et al., 2005). Given the properties of nanobubbles we hypothesized that depending on the gas used this technology could either destroy phages or enhance their uptake into fish, which might improve their therapeutic function against bacterial diseases.

The effects of NB-O_2 and NB-O_3 treatments on phage concentration in water were explored in this study. Subsequently, we investigated whether NB-O_2 treatment could improve adherence of phages to fish body surfaces and their uptake into the fish.

5.3 Materials and Methods

5.3.1 Bacteria, phage, and nanobubble system

The bacterial isolate, *Aeromonas hydrophila* BT14 and the *Myoviridae* phage pAh6.2TG used in this study were isolated and characterized in our previous studies (Dien et al., 2021a; Dien et al., 2021b). Prior to phage propagation, *A. hydrophila* BT14 was cultured in 15 mL of Tryptic Soy Broth (TSB; Becton Dickerson, USA) at 28 °C for 24 h. Then, 100 µL of phage (10^8 PFU/mL) was mixed with 100 µL of bacteria in 3 mL of TSB supplemented with 0.5% agar. The mixture was propagated on Tryptic Soy Agar (TSA; Becton Dickerson, USA) incubated at 28 °C for 16 h. Subsequently, a total of 3 mL of SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5) was added to each plate and kept at 4°C for 24 h. The solution of phage in SM was then collected and centrifuged at 10,000 x g, 4°C for 15 min. The supernatant was filtered through a 0.2 µm filter. The filtrate was harvested and enumerated by a phage plaque assay as indicated previously (Dien et al., 2021a). Nanobubble system (Model: aQua+075MO, AquaPro Solutions Private Limited Company, Singapore) was set up as previously described (Jhunkeaw et al., 2021) with oxygen input of 2 L/min.

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5.3.2 Effect of oxygen and ozone nanobubbles on phage concentration in water

To evaluate the effect of oxygen (NB-O_2) and ozone (NB-O_3) nanobubbles on phage concentration in water, three groups with 2 replicates each were used (Fig. 1A): group 1 (control group) used normal aeration, while groups 2 and 3 were exposed to NB-O_2 and NB-O_3, respectively for 15 min prior to phage addition. Each group used 100 L-fiberglass tanks contained 50 L dechlorinated tap water. A total of 5 mL of phage pAh6.2TG (approx. 10^{11} PFU/mL) was added to each experimental tank to get a final concentration of 10^6 PFU/mL. A volume of 25 mL of water (a mixture of 5 mL from each tank corners and 5 mL from the middle of the tank) was sampled at 0, 5, 10, and 15 min. One mL of each collected water sample was centrifuged at 4 ^{o}C, 10,000 *x g*, for 5 min. The supernatant was used for phage enumeration using a serial dilution plaque assay method as described in Jun et al. (2013). Water parameters including temperature, pH, DO, and oxidation reduction potential (ORP) were measured before and during treatment using a multi-parameter meter (YSI Professional Plus, YSI Incorporated, USA) (Fig. S1). Ozone (mg/L) was measured at a few times points (0, 2, 4, 6, 8, 10 min) during the study using a K-7434 Ozone Vacu-vials Kit (Oxidation Technologies, USA). We plotted the dissolved ozone concentration vs. ORP measurements (Fig. S2) to convert ORP values (mV) to dissolved ozone concentration (mg/L) to extrapolate the concentration of O_3 when it could not be measured due to time restraints.

5.3.3 Effects of oxygen nanobubbles on adherence and uptake of phage to fish

Experimental fish

Healthy Nile tilapia (4.68 ± 0.67 g) obtained from a tilapia hatchery (Department of Fisheries, Thailand) with no history of disease outbreaks. Fish were acclimated for 14 days at 28 ± 1.0 ^{o}C, and fed twice daily with commercial tilapia feed (30% crude-protein) at the rate of about 3% of their body weight. Before starting the experiments, ten fish were randomly selected for bacterial isolation and confirmed to be free of *A.*

hydrophila and phage pAh6.2TG. The experimental animal protocols used in this study were approved by Chulalongkorn University (no. CU-IACUC 2031006)

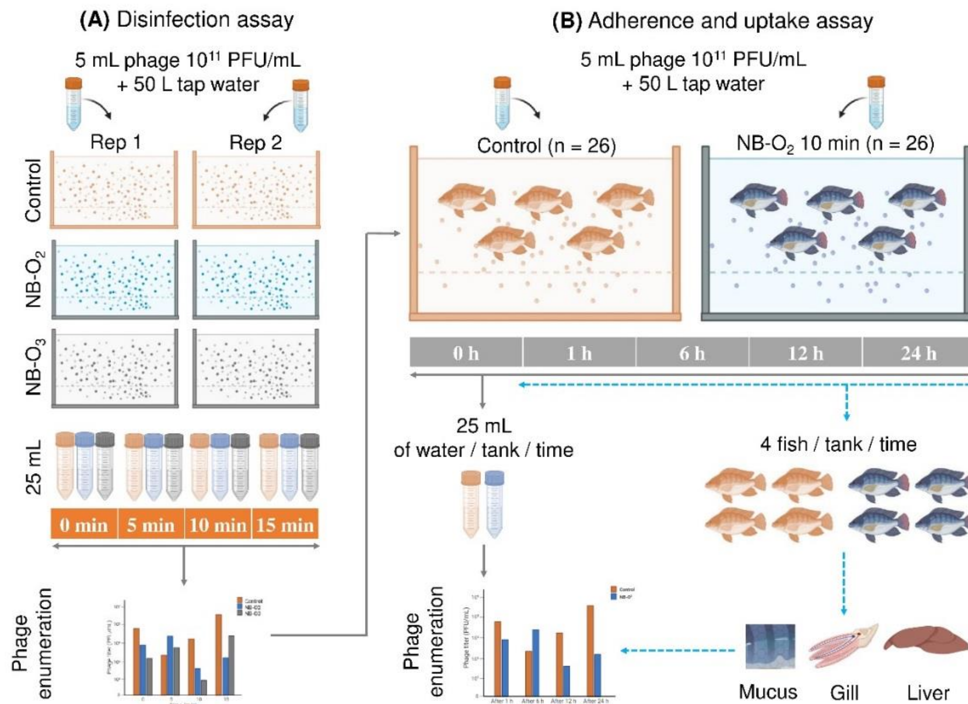

Figure 1. Experimental design of disinfection assay (A) and adherence and uptake assay (B).

Adherence and uptake of phage to fish

To investigate the effects of NB-O_2 on adherence and uptake of phage to fish, a total of 52 Nile tilapia were randomly divided into two groups (Fig. 1B): group 1 (control group) was exposed to normal aeration while group 2 was treated with NB-O_2 for 10 min and later supplied with normal aeration throughout the remainder of the experimental period (24 h). Phage pAh6.2TG (5 ml) was added to each tank after their treatment with aeration or nanobubbles to make a final concentration of phage of approx.10^6 PFU/mL. In order to investigate the effect of NB-O_2 treatment on the concentration of phage in rearing water, a volume of 25 mL water (was collected from both group was sampled at 0 min, 1, 6, 12, and 24 h post-treatment for phage enumeration.

At 1, 6, 12, and 24 h post NB-O_2 treatment, four fish from each group were collected for phage enumeration in their mucus, gills, and liver (Fig. 1B). In order to collect skin mucus, no anesthesia was given to the fish and the fish were placed in tanks with phage-free dechlorinated tap water for 10 min before being transferred to a plastic bag containing 1 mL of SM buffer and gently rubbed for 30 s. The fish were removed from the bags and mucus was collected into 1.5 mL microtubes. The mucus samples were then centrifuged at 4,000 *x g* for 15 min. The supernatant from the mucus samples was then aliquoted into sterile tubes and used for phage enumeration by the method described in Jun et al. (2013).

After the mucus collection the fish were euthanized by immersion in clove oil (1 g/L water) prior to necropsy. A total of 0.1 g of the gills and liver were collected from each fish, transferred into 1.5 mL microtubes containing 0.2 mL of SM buffer. Individual fish tissues were homogenized by tissue grinder with suitable pestles. The samples were then combined with 700 µL of SM buffer and centrifuged at 10,000 x *g*, for 5 min. The obtained supernatant was used for phage enumeration as described above. The phage adherence was measured using phage titers in mucus (PFU/mL) and gill (PFU/g), while phage uptake was evaluated by comparing phage titer (PFU/g) in the liver. Behavioral abnormality and mortality were recorded over a 7 day period for the remaining fish (10 fish/tank) to assess the safety of NB-O_2 treatment.

5.3.4 Statistical analysis

A pair of phage titers in the water of the control group, NB-O_2, and NB-O_3 treatment groups were analyzed by Student unpaired *t*-Test and Mann Whitney U test, *p*-values of 0.05 or less were considered statistically significant levels. The phage titers between control and NB-O_2 treatment groups in mucus, gill, and liver were analyzed using a Mann Whitney U test, *p*-values of 0.05 or less were considered statistically significant levels. All statistical analyses were performed using SPSS Software ver22.0 (IBM Corp., USA).

5.4 Results

5.4.1 Ozone nanobubbles kill bacteriophage while oxygen nanobubbles do not

The change of the phage concentration in the control and nanobubble treatment groups are shown in Fig. 2. Before treatment (0 min), concentration of phage pAh6.2TG in control, NB-O_2, and NB-O_3 treatment groups were not significantly different at 2.63 $\times 10^6 \pm 2.75 \times 10^5$, $2.53 \times 10^6 \pm 2.75 \times 10^5$, and $2.45 \times 10^6 \pm 1.50 \times 10^5$ PFU/mL, respectively. However, after 5 min of treatment, phage titers in control and NB-O_2 treatment groups remained at the same level, while 99.99% of phages in the NB-O_3 treatment group were destroyed (from $2.45 \times 10^6 \pm 1.50 \times 10^5$ to 8.75 ± 1.25 PFU/mL). A slight reduction was recorded in the control and NB-O_2 treatment groups at 10 and 15 min post-treatment (Fig. 2). However, this was not statistically significant compared to control group (*p=0.439* and *p=0.121*, respectively). By contrast, phages pAh6.2TG could not be detected after 10 and 15 min of NB-O_3 treatment (Fig. 2).

With respect to water parameters, temperature was slightly increased (Fig. S1). In the control group, the temperature was 33 ± 1.34 °C at 0 min to 32.8 ± 1.27 °C at 15 min, while the values of 32.7 ± 0.85 to 34.5 ± 0.42 °C and 31.4 ± 0.07 to 33.6 ± 0.07 °C were recorded in NB-O_2 and NB-O_3 treatments, respectively (Fig. S1A).

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The DO in NB-O_2 and NB-O_3 treatment groups also increased steadily from 3.45 ± 0.84 and 3. 5 ± 0.36 mg/L at 0 min to 27.68 ± 0.12 and 23.84 ± 1.1 mg/L at 15 min, respectively. DO in the control group remained at the same level during treatment period (Fig. 3A). ORP in the control and NB-O_2 treatment groups were stable during the treatments, while this value in the NB-O_3 treatment group increased from 310.45 ± 32.31 mV at 0 min to 829.9 ± 60.53 mV at 5 min, and 941.55 ± 11.1 mV at 15 min. Fluctuation of ORP levels in NB-O_3 treatment group and its conversion to concentrations of dissolved ozone is shown in Fig. 3B-C. The pH values at 0 min in the control, NB-O_2, and NB-O_3 groups were 7.38 ± 0.63, 7.37 ± 0.09, and 7.43 ± 0.72,

respectively. At 15 min, these values were 7.77 ± 0.21, 7.27 ± 0.05, and 7.69 ± 0.08, respectively (Fig. S1B).

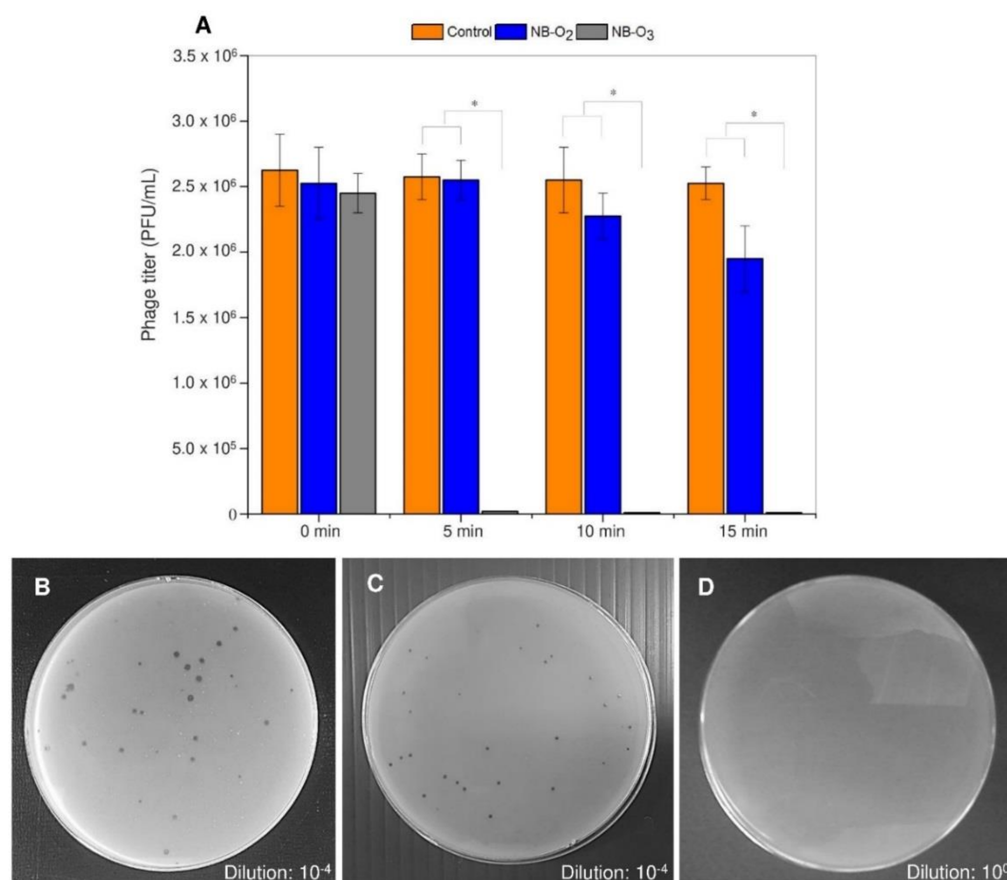
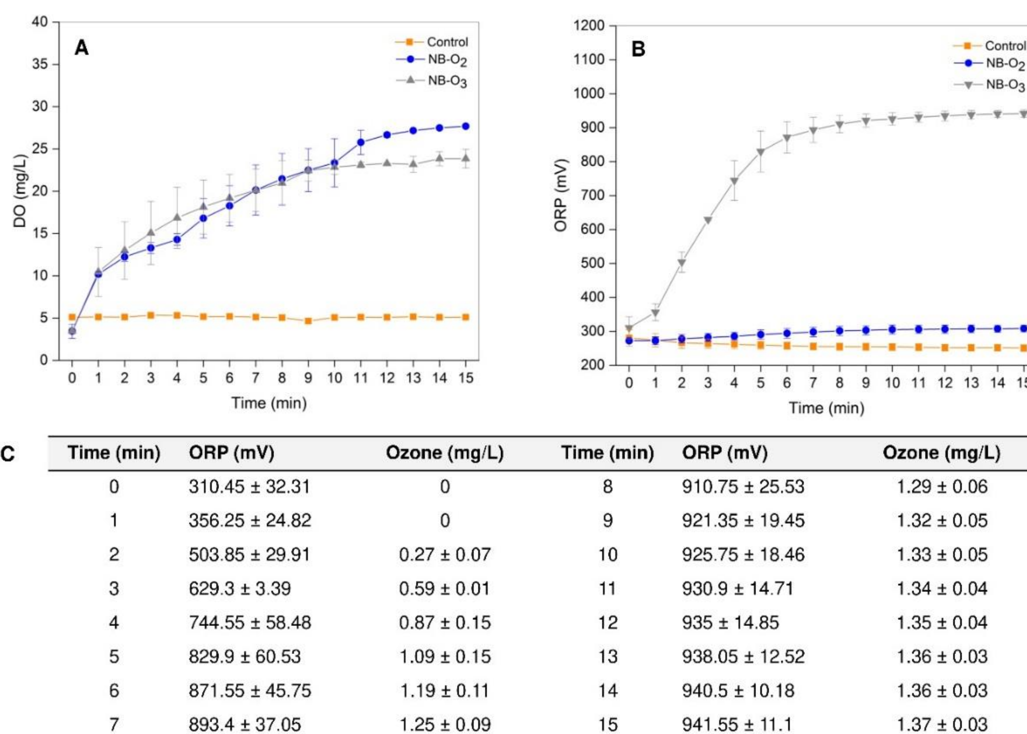


Figure 2. Effect of oxygen ($NB\text{-}O_2$) and ozone ($NB\text{-}O_3$) nanobubbles on phage titer in water (**A**). Values of pAh6.2TG phage titer (PFU/mL) are mean ± a standard error of the mean (SEM) bar (n = 2) and "*" above the bar indicates statistically significant difference between groups ($p < 0.05$). Representative graphs of phage pAh6.2TG in control (**B**), $NB\text{-}O_2$ (**C**), and $NB\text{-}O_3$ (**D**) after 10 min of treatment.



Time (min)	ORP (mV)	Ozone (mg/L)	Time (min)	ORP (mV)	Ozone (mg/L)
0	310.45 ± 32.31	0	8	910.75 ± 25.53	1.29 ± 0.06
1	356.25 ± 24.82	0	9	921.35 ± 19.45	1.32 ± 0.05
2	503.85 ± 29.91	0.27 ± 0.07	10	925.75 ± 18.46	1.33 ± 0.05
3	629.3 ± 3.39	0.59 ± 0.01	11	930.9 ± 14.71	1.34 ± 0.04
4	744.55 ± 58.48	0.87 ± 0.15	12	935 ± 14.85	1.35 ± 0.04
5	829.9 ± 60.53	1.09 ± 0.15	13	938.05 ± 12.52	1.36 ± 0.03
6	871.55 ± 45.75	1.19 ± 0.11	14	940.5 ± 10.18	1.36 ± 0.03
7	893.4 ± 37.05	1.25 ± 0.09	15	941.55 ± 11.1	1.37 ± 0.03

Figure 3. Measurement of DO (**A**), ORP (**B**) during 15 min treatment of NB-O_2, NB-O_3 with 2 L/min oxygen input in tap water and conversation of dissoleved ozone concentration (mg/l) from ORP (**C**). Value of water parameters are mean ± standard deviation (SD) bar (n = 2).

5.4.2 Oxygen nanobubbles improve adherence of bacteriophage on fish and uptake into fish

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The number of phages adhered to fish mucus, and gills at 1, 6, 12 and 24 h post treatment in both NB-O_2 and control groups are shown in Table 1. Overall, the concentration of phage titers from fish mucus and gills were higher in NB-O_2 treatment group. In mucus, phage adherence was 1.25, 1.88 and 2.36 folds higher than that of control after 6, 12 and 24 h post treatment, respectively. In fish gills, phage titers in NB-O_2 treatment group were 1.07 to 1.76 folds higher than in the control group at the first 12 h post treatment, but after 24 h, there was a 15 fold higher level of phage

adhered to the gill in NB-O_2 treatment group (7,500 ± 4,402 PFU/g) compared to the control group (500 ± 204 PFU/g) with p = 0.028 (Table 1).

The concentration of phages in the fish liver of NB-O_2 and control treated groups is illustrated in Table 1. After 1 h exposure to NB-O_2, phage the titer in liver of the treatment group was 212 ± 90 PFU/g and no phage was detected in the control group (p = 0.014). At 6 h post-treatment, phage titers in the liver were 512 ± 356 and 662 ± 313 PFU/g in control and NB-O_2 treatment groups (p = 0.663), respectively (1.29 fold difference). However, phage titer in NB-O_2 treatment group after 12 h and 24 h were 4.75 and 4.0 folds higher than that of the control group, respectively (Table 1). The differences in phage concentration were statistically significant at 12 and 24 h post-treatment (p = 0.027 and p = 0.034, respectively).

No fish mortality or behavioral abnormalities were observed during and 7-day post-treatment in either the control or the NB-O_2 groups.

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Table 1. Concentration and fold change of phage pAh6.2TG in mucus, gill, and liver (n = 4)

Organ	Experimental group	Concentration (PFU/g ± SE)			
		1 h	6 h	12 h	24 h
Mucus	Control	26,875 ± 10,166	33,500 ± 7,309	68,750 ± 16,007	25,375 ± 9,562
	$NB-O_2$	25,125 ± 16,085	41,750 ± 15,682	74,500 ± 19,002	59,875 ± 22,935
	Fold change[+]	-1.07	+1.25	+1.08	+2.36
Gill	Control	5,025 ± 1,546	6,375 ± 5,047	4,375 ± 2,164	500 ± 204
	$NB-O_2$	5,362 ± 2,416	11,250 ± 4,191	5,500 ± ,1568	7,500 ± 4,402*
	Fold change[+]	+1.07	+1.76	+1.26	+15
Liver	Control	0	512 ± 356	50 ± 20	62 ± 12
	$NB-O_2$	212 ± 90*	662 ± 313	237 ± 51*	250 ± 2134*
	Fold change[+]	NC	+1.29	+4.75	+4.0

NC: Not calculate; Fold change[+] fold change in $NB-O_2$ treatment group compared to control group

Comparision of phage titer between control and $NB-O_2$ treatment groups was performed by Mann Whitney U Test, *p*-values of 0.05 or less were considered statistically significant. "*" indicates statistically significant difference between groups ($p < 0.05$), without "*" means not significant.

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5.5 Discussion

Disinfection of NB-O_3 against pathogenic bacteria has previously been investigated in both marine and freshwater. Imaizumi et al. (2018) reported that most *Vibrio parahaemolyticus* were killed after 1 min incubation in NB-O_3 seawater. Nghia et al (2021) also demonstrated that treatment with NB-O_3 for 6 min inactivated 100% of the *V. parahaemolyticus* in laboratory experiments. In addition, Jhunkeaw et al. (2021) demonstrated that 10 min of NB-O_3 treatment in freshwater reduced 96.11 and 97.92% of *Streptococcus agalactiae* and *Aeromonas veronii*, respectively. However, there has not been any study on the effects of nanobubbles (either NB-O_2 or NB-O_3) on the concentration of viruses in water. In this study, we discovered that NB-O_3 treatment effectively eradicated phages in water after 5 to 10 min treatments, with corresponding ORP levels ranging from 829.9 ± 60.53 to 925.75 ± 18.46 mV, which was equivalent to dissolve ozone levels of 1.09 ± 0.15 to 1.33 ± 0.55 mg/L (Fig. 3C). Although we did not evaluate pathogenic fish viruses specifically, disinfection of bacteriophage with NB- O_3 implied that this technology may be effective for fish viruses commonly infecting tilapia aquaculture systems such as tilapia lake virus (Jansen et al., 2019), infectious spleen and kidney necrosis virus (Machimbirike et al., 2019; Ramírez-Paredes et al., 2020), and nervous necrosis virus (Keawcharoen et al., 2015; Machimbirike et al., 2019). Nevertheless, further investigation with these pathogenic viruses may be required to gain basic understanding of the effect of NB-O_3 on these specific viral pathogens.

The findings in this study also suggests that NB-O_3 should not be used during phage therapy due to disinfection property of ozone. However, phage treatment could be considered after NB-O_3 treatment since ozone degraded relatively quickly in our tanks.

After identifying that 10 min NB-O_2 treatment is not harmful to bacteriophages, we then explored NB-O_2 technology to improve adherence and uptake of phage on the mucosal surface and internal organs of fish. Increased concentration of phage

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pAh6.2TG in mucus, gills, and liver indicated that NB-O_2 technology could be combined with bacteriophage therapy to improve efficacy or uptake of phages. Barr et al. (2013) revealed the increased concentration of the lytic phage on mucosal surfaces provided an antimicrobial defense that limited mucosal bacteria. The sub-diffusive motion of phage on the mucosal surface enhanced bacterial encounter rates for phages, especially when bacterial concentration is low (Barr et al., 2015). Another study by Almeida et al. (2019) also showed that the binding of phage FLC-2 and T4 on the rainbow trout (*Oncorhynchus mykiss*) skin mucosal provided protection against *Flavobacterium columnare* infection. Pretreatment with a single dose of phage cocktails (FLC-2 and T4) for 1 day before *F. columnare* challenge delayed the disease onset and improved 25% of fish survival.

Phages have been investigated for treating acute infections. They have been found to reduce the densities bacteria sufficiently to enable the fish immune system to fight off infections (Levin and Bull, 2004). However, phages must reach the sites of infection to be effective. Nakai and Park (2002) suggested that phages could penetrate into the fish body, via the skin and gills. The persistence of phage and phage titer in particular organs of fish strongly rely on the absence or presence of bacterial host (Dabrowska et al., 2005). Thus, the uptake of phage in internal organs is a critical factor that contributes to efficacy of phage therapy.

The enhancement of phage-mucin protein interaction and the improvement of phage diffusion across the mucus by NB-O_2 activity possibly explains the higher adherence and uptake of phage in fish compared to control group. Most phages have an overall negative charge (Anany et al., 2011; Hosseinidoust et al., 2014; Van Voorthuizen et al., 2001), which permits it to adhere to the glycan component of mucin through weak binding interactions with the Hoc capsid proteins (Barr, 2017). However, mucus also contains a high density of negatively charged glycoproteins and

oligosaccharides (Crater and Carrier, 2010; Kitiyodom et al., 2019). Consequently, the binding of phages on the mucus layer may be less effective due to the electrostatic repulsion between negative charge components (Esteban et al., 2016). NB-O_2 also have a negative charge with zeta potential about -34 to -45 mV (Ushida et al., 2012; Ushikubo et al., 2010). It is possible that the electrostatic repulsion between NB-O_2 and negative charge glycoproteins and oligosaccharides may facilitate the binding of phages and glycans. Moreover, free-adherence phages with negative zeta potential can move easily within the mucus layer (Crater and Carrier, 2010; Pangua et al., 2021). The electrostatic repulsive forces between phage and NB-O_2 may facilitate free-phage diffusion across the mucus layer. It is also possible that the NB-O_2 treatment increases the permeability of the gills facilitating the entry of phages into the host.

In this study, the higher uptake of phage pAh6.2TG in the fish liver after NB-O_2 treatment compared to the control group may improve efficacy of phage therapy against bacterial infections in fish. This should be evaluated with laboratory challenge tests.

Although this study revealed the potential application of NB-O_2 in improving phage adherence and uptake into the liver, one of the limitations of this study was the small sample size and short duration of the phage study. There is a necessity for further studies to investigate the mechanism of NB-O_2 to enhance phage adherence to mucus and uptake of phages into fish organs and potential benefits of this promising technology for disease prevention and mitigation.

Herein, using phage as a virus model, this study demonstrated that NB-O_3 treatment was effective at eradicating viruses in water. This opens a novel application for this technology as one of the biosecurity measures that could be used to prevent viral diseases in aquaculture. In addition, this study also discovered that NB-O_2 treatment

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improved the adherence and uptake of phage in fish, which may improve the success of phage therapy in aquaculture.

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Chapter 6
GENERAL CONCLUSION

6.1 Conclusion

Sustainable production of healthy, safe, and nutritious diets in the required quantity is an irrefutable urgency for realizing global food system transformation. Aquatic animals providing high-quality protein, beneficial fatty acids, and bioavailable vitamins and minerals are critical to prevent undernutrition. There is considerable evidence to demonstrate that aquaculture effectively contributed to poverty alleviation, economic growth and food and nutrition security in low-middle income countries (LMICs). The farming of carps, tilapias, catfishes, and other freshwater fish contributes great economic value for LMICs and entails enormous social impact on human welfare, benefitting the people suffering from poverty and malnourishment in Asia and Africa. Motile Aeromonas Septicemia (MAS) is one of the biggest challenges in sustainable freshwater finfish aquaculture worldwide that causes a significant loss in the production of major aquaculture species. At least eight pathogenic motile *Aeromonas* species (*A. hydrophila*, *A. veronii*, *A. jandaei*, *A. caviae*, *A. sobria*, *A. bestiarum*, *A. dhakensis* and *A. schubertii*) have been reported in aquaculture; some causing up to 100% mortalities during disease outbreaks. Among motile *Aeromonas* species, *A. hydrophila* has been reported as the most common pathogen in at least fifteen freshwater fishes. The control of these bacterial infections still heavily rely on disinfectants and antibiotics. The increasing use of antibiotics to combat *A. hydrophila* has generated a negative consequence of antimicrobial-resistant *A. hydrophila* and increased public health concerns, especially in the LMICs.

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Chapter 1 is designed to give an overview on importance and rationale of this study as well as specific objectives. In chapter 2, we provide a systemic review on Motile Aeromonas Septicemia (MAS) in freshwater aquaculture and current stage of knowledge and future perspective on non-antibiotic approaches to combat MAS in

aquaculture. The review focus on vaccines, probiotics, phytochemicals, and bacteriophages, and nanobubble technology. In the author's point of view, toward sustainable development of aquaculture, besides current non-antibiotic approaches, novel alternatives to antibiotics such as multivalent vaccines coupled with advanced nanotechnology to improve vaccine efficacy should be strategically targeted through practical, inexpensive oral and immersion delivery routes. In addition, aquaculture feed incorporated with microencapsulated probiotics, synbiotics, nano bioactive compounds or exogenous metabolites should be explored to develop the optimized feeding programs for strategic aquaculture species. Lytic bacteriophage cocktails as prophylactic and therapeutics are high-potential approaches for recirculation aquaculture system or in hatchery. The novel non-antibiotic approach using ozone-nanobubbles for oxygenating water opens new frontiers to improve growth performance and reduce pathogen load in aquaculture systems. Nonetheless, the complementarity of non-antibiotic approaches should be considered a strategic direction to combat MAS, including AMR Aeromonads and other bacterial pathogens in aquaculture.

In the battle to combat *A. hydrophila* infection in aquaculture system, bacteriophage is one of the environmentally friendly approaches which replace or complement chemotherapy to reduce the hazard of bacterial disease and antimicrobial resistance in aquatic animals. In chapter 3, a lytic phage namely pAh6.2TG specific to multidrug-resistant (MDR) *Aeromonas hydrophila* isolates was isolated, identified and characterized. Phage pAh6.2TG was classified as a member of the family *Myoviridae* which has genome size of 51,780 bp, encoding 65 putative open reading frames (ORFs) pAh6.2TG was highly stable at pH = 7 - 9, temperature from 4 to 40 ^{o}C, and salinity from 0 to 40 ppt. The stability of pAh6.2TG under different environmental conditions might be important characteristics for its wider application in diverse

aquaculture environments, especially in global issue of climate change. In addition, the combination of immersion phage therapy using pAh6.2TG and probiotics to combat MDR *A. hydrophila* infection in aquaculture is potential. Phage pAh6.2TG was effective at suppressing bacterial concentration in water as well as reducing the bacterial load in fish liver. Application of this phage as prophylactic agent significantly improved survivability of Nile tilapia challenged with the pathogenic MDR *A. hydrophila* with RPS of 50 - 73.3%. All surviving fish developed specific antibody IgM against *A. hydrophila*. This suggests that phages possibly weakened the bacteria which allowed the fish immune system to respond more effectively and saved the fish from death. These findings support that pAh6.2TG could be used in rearing water for biocontrol of MDR *A. hydrophila* infection towards sustainable aquaculture.

Ozone is a powerful disinfectant that has been used to reduce concentrations of pathogens and improve water quality in both flow-through and recirculating aquaculture systems. However, low ozone solubility and poor stability are major reasons for low utilization efficiency. Moreover, Nanobubbles (NBs) are bubbles less than 200 nm in diameter filled with chosen gases, neutral buoyancy, and having long residence time in the liquid solutions. NBs technology has been reported to improve gas dissolvability in water and promote rapid oxidation of organic substances. Hence, NB-O_3 may enhance the solubility, stability, and efficacy of ozone in aquaculture systems. Previous studies reported that ozone nanobubbles (NB-O_3) were effective at reducing concentrations of pathogenic bacteria in water and modulating fish immunity against pathogens; however, multiple treatments in long period with direct NB-O_3 exposures caused alterations to the gills of exposed-fish. Therefore, this study modify a NB-O_3 system on a laboratory scale to better understand this technology and overcome this drawback. In chapter 4, multiple treatments of NB-O_3 in a modified recirculation system (MRS) were reported relatively safe for juvenile Nile tilapia. NB-O_3

treatments in MRS significantly improved survivability of Nile tilapia challenged with MDR *A. hydrophila* with RPS of 64.7 - 66.7%. The concentration of MDR *A. hydrophila* in MRS was reduced by 15.9 to 35.6% following each NB-O_3 treatment, and increased by 13.1 to 27.9 % in untreated control. There was slight up-regulation of non-specific immune-related genes in the gills of the fish receiving NB-O_3 treatments. In addition, all surviving fish developed specific antibody IgM against MDR *A. hydrophila.* It is also possible that the increased survivability of Nile tilapia exposed to NB-O_3 treatment in this study was from a combination of synergistic effects of bacterial reduction, increased DO, and stimulation of the fish immune response. These findings suggest that NB-O_3 is a promising non-antibiotic approach to control diseases caused by MDR *A. hydrophila* in freshwater fish aquaculture industry.

Single application of phages and ozone nanobubbles were effective to control MDR *A. hydrophila*. In chapter 5, the potential combination of phage and nanobubble-based technology was investigated. The results showed that NB-O_3 killed 99.99 and 100% bacteriophage in water after 5- and 10-min treatment, respectively. This finding suggests that a combination of NB-O_3 treatment and phage therapy are not feasible to control bacterial infections in aquaculture. However, NB-O_3 might be a promising approach to viral disinfection method to combat fish viruses commonly infecting tilapia aquaculture systems such as tilapia lake virus, infectious spleen and kidney necrosis virus, or nervous necrosis virus, but the treatment would need to be omitted during phage treatment. Further investigation with these pathogenic viruses may be required to gain basic understanding of the effect of NB-O_3 on these specific viral pathogens. After identifying that 10 min NB-O_2 treatment is not harmful to bacteriophages, this study then explored NB-O_2 technology to improve adherence and uptake of phage on the mucosal surface and internal organs of fish. The result revealed that 10 min of NB-O_2 treatment improved the adherence of phage pAh6.2TG on fish body surface and

uptake into fish liver. The uptake of phage in internal organs is a critical factor that contributes to efficacy of phage therapy. The enhancement of phage-mucin protein interaction and the improvement of phage diffusion across the mucus by NB-O_2 activity possibly explains the higher adherence and uptake of phage in fish compared to control group. Moreover, it is also possible that the NB-O_2 treatment increases the permeability of the gills facilitating the entry of phages into the host. In summary, NB-O_2 treatment might be potential method to improve efficacy of phage therapy against bacterial infections in aquaculture system.

6.2 Suggestions for further studies

- Application of lytic phage pAh6.2TG and MRS-NB-O_3 system should be scaled up to be utilizable in aquaculture systems.
- More experiments should be performed to gain a better understanding mechanism on how NB-O_2 technology improve phage adherence on and uptake into the fish.
- Further investigation on combination of NB-O_2 treatment and phage therapy against bacterial diseases are needed.

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APPENDIX

Supplementary data for Chapter 3

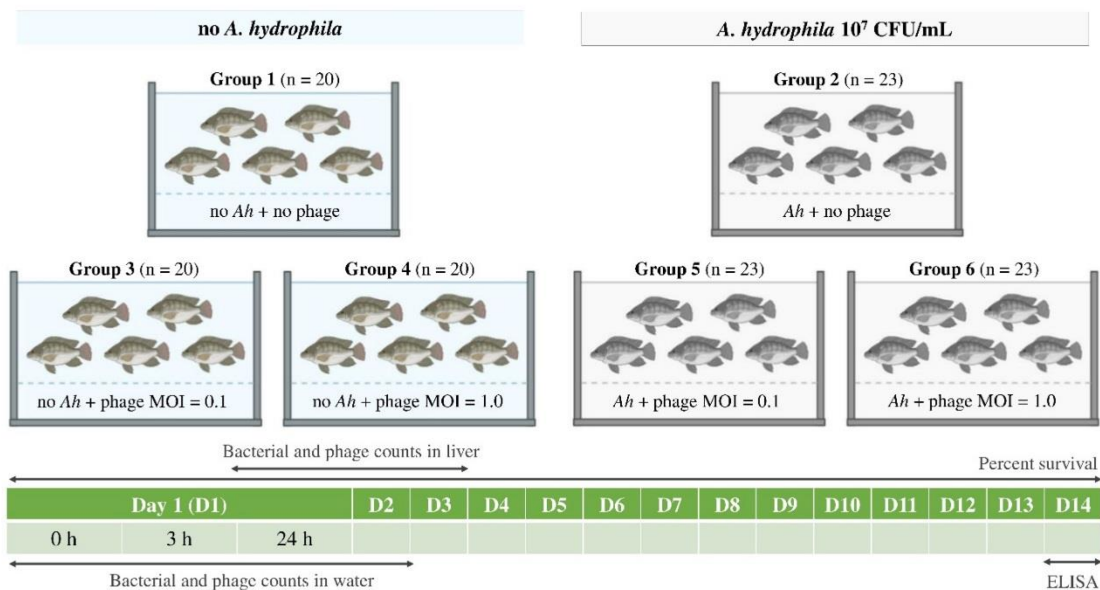

Figure S1. Experimental design to investigate effect of phage on Nile tilapia challenged with MDR *A. hydrophila.*

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Table S1. Antimicrobial susceptibility testing of *A. hydrophila* isolates

No.	*A. hydrophila* isolates	Antimicrobials						Multidrug-resistance
	Class	Penicillins	Tetracyclines	Folate pathway inhibitors	Quinolones	Aminoglycosides	Phenicols	
	Name	AMP CIA (10 μg)	TET HIA (30 μg)	SXT HIA (23.75/1.25 μg)	CIP CIA (5 μg)	GEN CIA (10 μg)	CHL HIA (30 μg)	
1	BT01	R	R	R	R	S	R	+
2	BT02	R	R	R	S	S	I	+
3	BT03	R	R	R	I	S	I	+
4	BT04	R	R	R	S	S	S	+
5	BT05	R	R	R	R	S	I	+
6	BT09	R	R	R	S	S	I	+
7	BT12	R	R	R	S	S	S	+
8	BT13	R	R	R	S	S	S	+
9	BT14	R	R	R	S	S	I	+
10	BT22	R	R	R	S	S	S	+
11	TG26	R	R	R	S	S	I	+
12	TG35	R	R	R	S	S	I	+
13	CUVET02	R	S	S	S	S	S	-
14	CUVET21	R	S	S	S	S	S	-
15	CUVET46	R	S	S	S	S	S	-
16	CUVET55	R	S	R	S	S	S	-
17	CUVET92	R	S	S	S	S	S	-
Standard protocol		CLSI M100	CLSI M45-A3	CLSI M45-A3	CLSI M45-A3	CLSI M45-A3	CLSI M45-A3	Magiorakos et al. (2012)

R: Resistant; I: Intermediate; S: Susceptible

AMP: Ampicillin; TET: Tetracycline; SXT: Sulfamethoxazole/Trimethoprim; CIP: Ciprofloxacin; GEN: Gentamicin; CHL: Chloramphenicol

CIA: Critically important antimicrobials for human medicine; HIA: Highly important antimicrobials for human medicine

Multi-drug resistant identification: resist at least 3 classes of antimicrobials

Table S2. Annotation of predictive ORFs based on Panzer2 rapid annotation and % nucleotide identity to the database sequences

ORF	Length (aa)	Gene name	Species	Identity (%)	Description
01	924	PVN02_00002	*Aeromonas* phage PVN02	99.8	Hypothetical protein
02	357	PVN02_00001	*Aeromonas* phage PVN02	98.0	Hypothetical protein
03	148	AH6C_014	*Aeromonas* phage pAh6-C	70.0	Hypothetical protein
04	223	PVN02_00064	*Aeromonas* phage PVN02	99.6	Gp138_N domain-containing protein
05	115	PVN02_00063	*Aeromonas phage* PVN02	99.1	Hypothetical protein
06	380	PVN02_00062	*Aeromonas phage* PVN02	98.9	Hypothetical protein
07	213	PVN02_00061	*Aeromonas phage* PVN02	99.5	Pf11041 family protein
08	406	PVN02_00060	*Aeromonas phage* PVN02	95.8	Hypothetical protein
09	474	PVN02_00059	*Aeromonas phage* PVN02	97.5	Tail fibers protein
10	626	PVN02_00058	*Aeromonas phage* PVN02	94.6	Hypothetical protein
11	133	PVN02_00057	*Aeromonas phage* PVN02	99.3	Putative tail protein
12	76	PVN02_00056	*Aeromonas phage* PVN02	100	Hypothetical protein
13	127	PVN02_00055	*Aeromonas phage* PVN02	98.4	Hypothetical protein (integral component of membrane)
14	55	PVN02_00054	*Aeromonas phage* PVN02	100	Hypothetical protein
15	121	PVN02_00053	*Aeromonas phage* PVN02	100	Peptidase m15a
16	178	PVN02_00052	*Aeromonas phage* PVN02	98.9	Cell wall hydrolase
17	843	PVN02_00051	*Aeromonas phage* PVN02	99.3	RNA polymerase
18	57	PVN02_00050	*Aeromonas phage* PVN02	100	TPR_REGION domain-containing protein
19	58	PVN02_00049	*Aeromonas phage* PVN02	100	D-glucuronyl C5-epimerase
20	60	PVN02_00048	*Aeromonas phage* PVN02	98.4	Hypothetical protein
21	74	PVN02_00047	*Aeromonas phage* PVN02	97.3	ATP-binding cassette domain-containing protein
22	74	PVN02_00046	*Aeromonas phage* PVN02	100	Hypothetical protein
23	83	PVN02_00045	*Aeromonas phage* PVN02	100	Hypothetical protein
24	148	PVN02_00044	*Aeromonas phage* PVN02	100	Hypothetical protein
25	230	PVN02_00043	*Aeromonas phage* PVN02	98.3	Hypothetical protein
26	258	PVN02_00042	*Aeromonas phage* PVN02	99.6	Hypothetical protein
27	85	PVN02_00041	*Aeromonas phage* PVN02	98.8	Hypothetical protein

28	47	PVN02_00040	*Aeromonas phage* PVN02	100	Hypothetical protein
29	58	PVN02_00039	*Aeromonas phage* PVN02	100	Hypothetical protein
30	59	PVN02_00038	*Aeromonas phage* PVN02	100	Hypothetical protein
31	56	PVN02_00037	*Aeromonas phage* PVN02	98.2	SesA domain-containing protein
32	257	PVN02_00036	*Aeromonas phage* PVN02	98.4	Hypothetical protein
33	45	PVN02_00035	*Aeromonas phage* PVN02	100	DUF4974 domain-containing protein
34	136	PVN02_00034	*Aeromonas phage* PVN02	99.3	Hypothetical protein
35	113	PVN02_00033	*Aeromonas phage* PVN02	98.2	Hypothetical protein
36	231	PVN02_00032	*Aeromonas phage* PVN02	99.6	Hypothetical protein
37	145	PVN02_00031	*Aeromonas phage* PVN02	100	Hypothetical protein
38	121	PVN02_00030	*Aeromonas phage* PVN02	99.2	Amino acid adenylation domain-containing protein
39	564	4	*Aeromonas phage* PVN02	100	DNA primase/helicase
40	79	PVN02_00028	*Aeromonas phage* PVN02	97.5	Hypothetical protein
41	103	PVN02_00027	*Aeromonas phage* PVN02	95.2	Hypothetical protein
42	674	PVN02_00026	*Aeromonas phage* PVN02	99.7	DNA polymerase
43	259	PVN02_00025	*Aeromonas phage* PVN02	98.5	Hypothetical protein
44	317	PVN02_00024	*Aeromonas phage* PVN02	100	5'-3' exonuclease
45	180	PVN02_00023	*Aeromonas phage* PVN02	99.4	Hypothetical protein
46	77	PVN02_00022	*Aeromonas phage* PVN02	98.7	Hypothetical protein
47	167	PVN02_00021	*Aeromonas phage* PVN02	99.4	HD domain-containing protein
48	300	PVN02_00020	*Aeromonas phage* PVN02	100	DNA ligase
49	193	PVN02_00019	*Aeromonas phage* PVN02	100	Hypothetical protein
50	705	I4L	*Aeromonas phage* PVN02	99.7	Ribonucleoside-diphosphate reductase
51	366	PVN02_00017	*Aeromonas phage* PVN02	100	Ribonucleoside-diphosphate reductase
52	52	PVN02_00016	*Aeromonas phage* PVN02	98.1	Peptidase M20
53	674	PVN02_00015	*Aeromonas phage* PVN02	99.9	Terminase large subunit
54	59	PVN02_00014	*Aeromonas phage* PVN02	100	Hypothetical protein
55	440	PVN02_00013	*Aeromonas phage* PVN02	99.8	Hypothetical protein
56	365	PVN02_00012	*Aeromonas phage* PVN02	100	Hypothetical protein
57	168	PVN02_00011	*Aeromonas phage* PVN02	98.8	Hypothetical protein
58	343	PVN02_00010	*Aeromonas phage* PVN02	99.4	Major capsid protein
59	162	PVN02_00009	*Aeromonas phage* PVN02	99.4	Hypothetical protein

60	122	PVN02_00008	*Aeromonas phage* PVN02	100	Hypothetical protein
61	153	PVN02_00007	*Aeromonas phage* PVN02	98.7	Hypothetical protein
62	174	PVN02_00006	*Aeromonas phage* PVN02	98.9	Hypothetical protein
63	471	PVN02_00005	*Aeromonas phage* PVN02	97.9	Hypothetical protein
64	150	PVN02_00004	*Aeromonas phage* PVN02	99.3	Hypothetical protein
65	141	PVN02_00003	*Aeromonas phage* PVN02	99.3	Hypothetical protein

Table S3. Concentration and fold change of *A. hydrophila* and phage pAh6.2TG in rearing water and fish liver

Parameter	Experimental group	Concentration (CFU or PFU/mL ± SD)					Fold change in CFU or PFU/mL ± SD			
		0 h	3 h	24 h	48 h	72 h	3h	24 h	48 h	72 h
Ah in water	Ah + no phage	1.58×10^{7} $\pm 9.9 \times 10^{5}$	1.60×10^{8} $\pm 3.96 \times 10^{7}$	2.34×10^{8} $\pm 8.84 \times 10^{6}$	1.50×10^{8} $\pm 2.33 \times 10^{7}$	ND	+10.2 ± 3.15	+14.84 ± 1.49	+9.43 ± 0.89	ND
	Ah + phage 0.1	1.35×10^{7} $\pm 2.35 \times 10^{6}$	2.0×10^{6} $\pm 1.56 \times 10^{5}$	2.08×10^{8} $\pm 2.93 \times 10^{7}$	3.7×10^{7} $\pm 3.54 \times 10^{6}$	ND	-6.7 ± 0.50	+15.8 ± 4.59	+2.8 ± 0.69	ND
	Ah + phage 1.0	1.28×10^{7} $\pm 3.0 \times 10^{6}$	7.33×10^{5} $\pm 1.17 \times 10^{5}$	9.18×10^{7} $\pm 3.64 \times 10^{7}$	1.9×10^{7} $\pm 1.41 \times 10^{6}$	ND	-18.1 ± 6.98	+7.7 ± 4.64	+1.54 ± 0.47	ND
Ah in liver	Ah + no phage	ND	ND	6.52×10^{5} $\pm 2.05 \times 10^{5}$	6.58×10^{6} $\pm 3.18 \times 10^{5}$	1.75×10^{6} $\pm 2.12 \times 10^{5}$	ND	ND	+10.69 ± 3.85	+2.77 ± 0.55
	Ah + phage 0.1	ND	ND	3.35×10^{5} $\pm 4.24 \times 10^{5}$	1.24×10^{5} $\pm 4.6 \times 10^{3}$	3.13×10^{4} $\pm 5.3 \times 10^{3}$	ND	ND	-2.7 ± 0.24	-10.99 ± 3.22
	Ah + phage 1.0	ND	ND	3.49×10^{5} $\pm 1.85 \times 10^{5}$	1.16×10^{4} $\pm 3.57 \times 10^{3}$	5.5×10^{3} $\pm 7.79 \times 10^{2}$	ND	ND	-34.08 ± 26.4	-61.68 ± 24.95
Phage in water	Ah + no phage	0	0	0	0	ND	0	0	0	ND
	Ah + phage 0.1	1.4×10^{6} $\pm 1.77 \times 10^{5}$	7.13×10^{7} $\pm 1.77 \times 10^{6}$	3.38×10^{7} $\pm 1.17 \times 10^{7}$	5.33×10^{7} $\pm 2.02 \times 10^{7}$	ND	+51.04 ± 5.16	+23.69 ± 5.52	+37.29 ± 9.65	ND
	Ah + phage 1.0	1.44×10^{7} $\pm 2.05 \times 10^{6}$	3.0×10^{8} $\pm 2.83 \times 10^{7}$	3.23×10^{7} $\pm 3.54 \times 10^{6}$	1.31×10^{7} $\pm 1.13 \times 10^{6}$	ND	+20.98 ± 1.03	+2.27 ± 0.35	+0.92 ± 0.05	ND
Phage in liver	Ah + no phage	ND	ND	0	0	0	ND	ND	0	0
	Ah + phage 0.1	ND	ND	3.7×10^{5} $\pm 1.63 \times 10^{5}$	9.6×10^{5} $\pm 6.72 \times 10^{5}$	6.0×10^{4} $\pm 3.11 \times 10^{4}$	ND	ND	+2.43 ± 0.75	-6.31 ± 0.56
	Ah + phage 1.0	ND	ND	5.78×10^{4} $\pm 1.52 \times 10^{4}$	9.08×10^{4} $\pm 2.58 \times 10^{4}$	6.5×10^{3} ± 0.0	ND	ND	+1.57 ± 0.03	-8.88 ± 2.34

Ah: *Aeromonas hydrophila*, ND: Not done

Supplementary data for Chapter 4

Table S1. Identification and antibiogram of *A. hydrophila* BT14

Identification using MALDI-TOF MS

Sample name	Organism (best match)	Score value	Organism (second-best match)	Score value	Identification
BT14	*Aeromonas hydrophila*	2.15	*Aeromonas hydrophila*	2.08	*Aeromonas hydrophila* BT14

DNA gyrase subunit B (*gyrB*) sequence (1030 bp) of *A. hydrophila* BT14 (99.03% identity to *A. hydrophila* 2TS54 strain (accession number MT371989.1)

ATCAGGGTGCCCACCTCCTGGGAGGAGATCATCTTGTCGAAACGGGCCTTCTCCACGTTCAGGATCTTGCCCTTGAGCGGCAGGATGGCCTGGTTCTTCCGGTT
GCGACCCTGCTTGGCGGAACCGCCAGCAGAGTCCCCTTCCACTATGTAGAGTTCGGAGAGCGCCGGGTCTTTTTCCTGACAGTCGGCCAGCTTGCCGGGCAGAC
CGGCAATATCCAGCGCGCCTTTGCGGCGGGTCAGTTCGCGAGCCTTGCGGGCCGCTTCACGGGCACGGGCCGCATCGATGATCTTGTTGACCACGATCTTGGCA
TCGCCCGGGTTTTCCAGCAGGAAGTCGGCCAGCTTCTCGCCCATCGCCTGTTCGACTGCGGTCTTCACTTCGGAAGAGACCAGCTTGTCCTTGGTCTGGGAGGA
GAACTTAGGGTCAGGCACCTTGACGGAGATAACGGCAATCAGACCTTCACGCACGTCGTCGCCACTGGCGGCAGACTTGGCCTTCTTGCTGTAGTCCTCTTTGT
CCATGTAGGAGTTGAGGGTACGGGTCAGCGCGGTACGGAAGCCCACCAGGTGGGTGCCGCCATCACGCTGGGGAATGTTGTTGGTGAAGCAGTAGACCCCTTC
CTGATAGGCGTCGTTCCACTGCATCGCCACTTCGACGCCAATGCCGTCCTGCTCGGTGGTGAAGTGGAACACCTTCGGGTGGATCGGGGTCTTGTTCTGGTTCA
GGTACTCGACGAACGCCTTAATGCCGCCTTCGTAGCAAAAGTGCGCCTCGCGGCCGTCACGCTCGTCCATCAGACGGATGGAGACGCCGGAATTGAGGAAGGA
GAGCTCGCGCAGACACTTGGCCAGGATCTCGTAGTGGAACAGGGTGTCGCTGAAGATGGTCGGGCTCGGCCAGAAACGCACTTCGGTCCCGGTGGTGGTGGCA
TCGCCCATCTGCTTGAGCGGCGCCTGCGGCTCACCCAGGTGATAGGTCTGCTCGTAAAAATGACCGTTGCCACGGAAGGTCAGCAACAACTTGTCAA

Antibiogram of *A. hydrophila* BT14

Antimicrobials	Antimicrobial class	Concentration (µg)	Zone diameter (mm)	Zone diameter (mm) Interpretive criteria			Result	Standard protocol
				Resistant	Intermediate	Susceptible		
Ampicillin	Penicillins	10	0	≤ 13	14 - 16	≥ 17	Resistant	CLSI M100
Tetracycline	Tetracyclines	30	11	≤ 11	12 - 14	≥ 15	Resistant	CLSI M45-A3
Sulfamethoxazole -Trimethoprim	Folate pathway inhibitors	23.75 - 1.25	0	≤ 10	11 - 15	≥ 16	Resistant	CLSI M45-A3
Ciprofloxacin	Flouroquinolones	5	30	≤ 15	16 - 20	≥ 21	Susceptible	CLSI M45-A3
Chloramphenicol	Phenicols	30	15	≤ 12	13 - 17	≥ 18	Intermediate	CLSI M45-A3

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Table S2. Water parameters in Nile tilapia culture tank during 10 min NB-O_3 treatment in MRS

Treatment	Measurement time	Temperature (°C)		pH		DO (mg/L)		ORP (mV)	
		Control	NB-O_3 treatment	Control	NB-O_3 treatment	Control	NB-O_3 treatment	Control	NB-O_3 treatment
1st	Before treatment	28.1 ± 0.2	27.7 ± 0.0	7.76 ± 0.03	6.64 ± 0.42	6.58 ± 0.08	6.97 ± 2.21	470.4 ± 3.1	421.3 ± 1.0
	10 min treatment	28.1 ± 0.3	28.3 ± 0.0	8.06 ± 0.06	8.03 ± 0.05	6.57 ± 0.04	15.33 ± 1.76	412.8 ± 0.1	367.8 ± 7.5
	10 min post treatment	ND	28.6 ± 0.0	ND	7.98 ± 0.03	ND	12.69 ± 0.68	ND	328.4 ± 6.1
3rd	Before treatment	30.3 ± 0.0	28.6 ± 0.1	7.89 ± 0.03	7.63 ± 0.08	4.98 ± 0.42	5.82 ± 0.04	326.7 ± 3.8	465.5 ± 0.7
	10 min treatment	30.5 ± 0.1	29.2 ± 0.1	7.98 ± 0.08	7.92 ± 0.17	4.82 ± 0.40	12.29 ± 0.88	323.8 ± 1.8	387.2 ± 8.2
	10 min post treatment	ND	29.3 ± 0.1	ND	7.97 ± 0.16	ND	9.55 ± 1.24	ND	350.6 ± 11
5th	Before treatment	29.8 ± 0.1	29.8 ± 0.6	7.59 ± 0.28	7.82 ± 0.00	4.72 ± 1.25	4.89 ± 0.31	433.8 ± 1.2	432.8 ± 0.8
	10 min treatment	29.5 ± 0.1	30.0 ± 0.4	7.98 ± 0.21	8.08 ± 0.04	5.12 ± 0.40	12.7 ± 0.32	405 ± 10.3	400.3 ± 9.0
	10 min post treatment	ND	30.3 ± 0.2	ND	8.14 ± 0.02	ND	9.28 ± 0.80	ND	386.2 ± 1.1
7th	Before treatment	29.1 ± 0.1	29.4 ± 0.3	7.91 ± 0.03	7.95 ± 0.06	4.91 ± 0.12	5.39 ± 0.08	296.7 ± 9.3	309.9 ± 1.4
	10 min treatment	29.1 ± 0.1	29.7 ± 0.3	7.92 ± 0.06	8.01 ± 0.04	5.19 ± 0.30	12.26 ± 2.25	290.4 ± 4.6	310.3 ± 4.7
	10 min post treatment	ND	29.8 ± 0.4	ND	8.06 ± 0.04	ND	9.66 ± 1.70	ND	310.1 ± 4.9

DO: Dissolve Oxygen, ORP: Oxidation Reduction Potential, OB-O_3: ozone-nanobubbles, ND: Not done

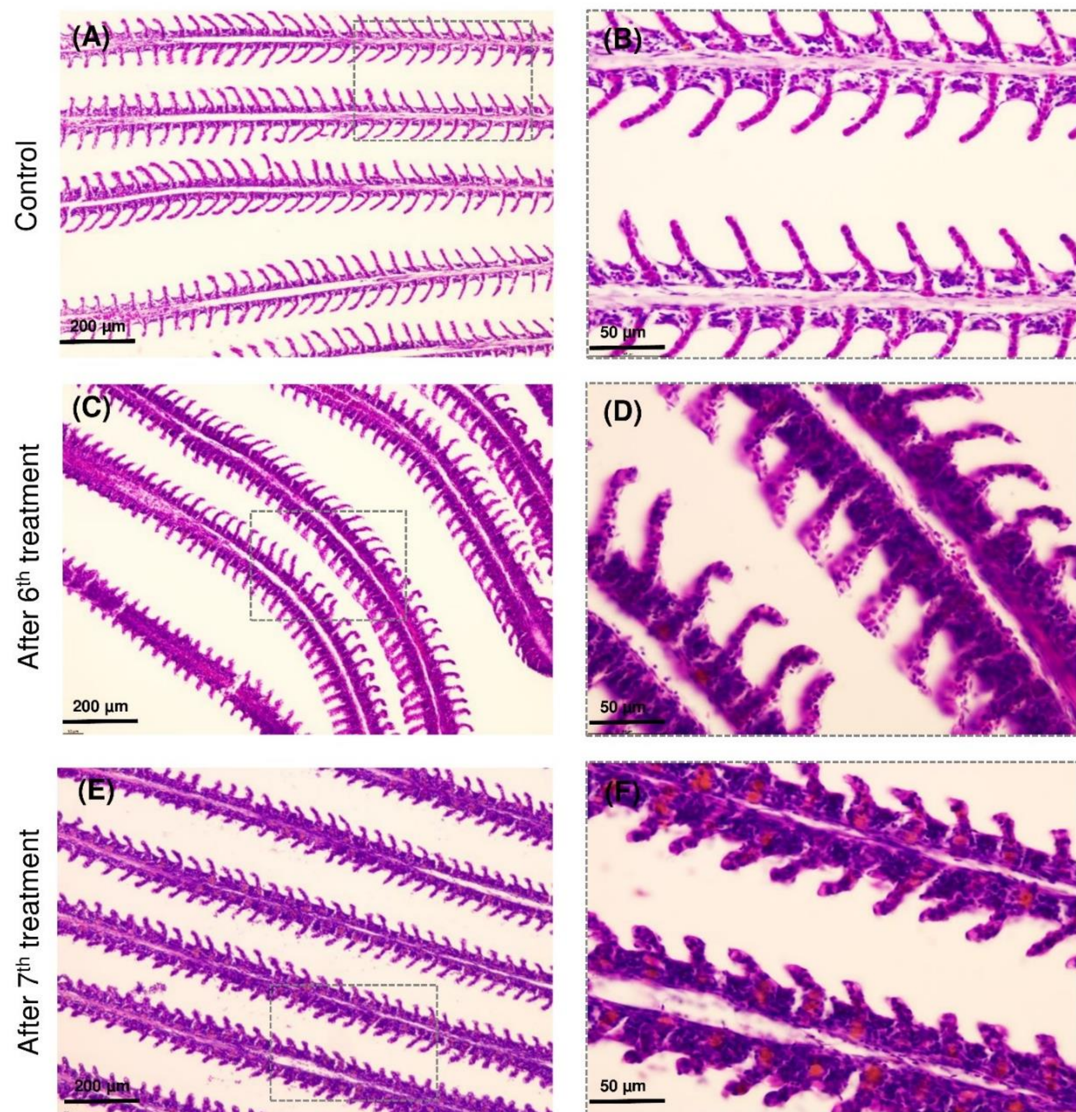


Figure S1. Representative photomicrographs of H&E stained sections of the gills taken at low and high magnifications. A, B, normal gill morphology from fish in control group. C, D, slight alterations in the gill lamella observed after 6th treatment. E, F, alteration and increasing melanin containing cells in the gill filaments after 7th treatment.

Supplementary data for Chapter 5

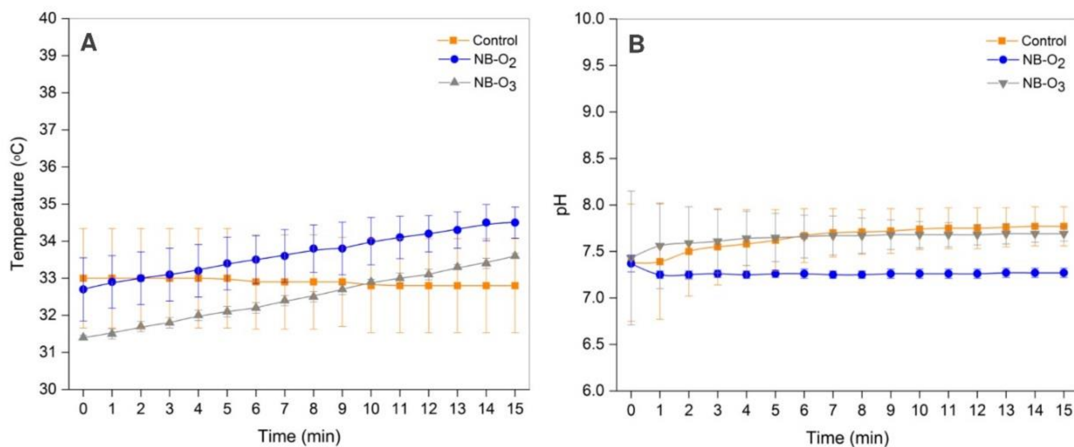
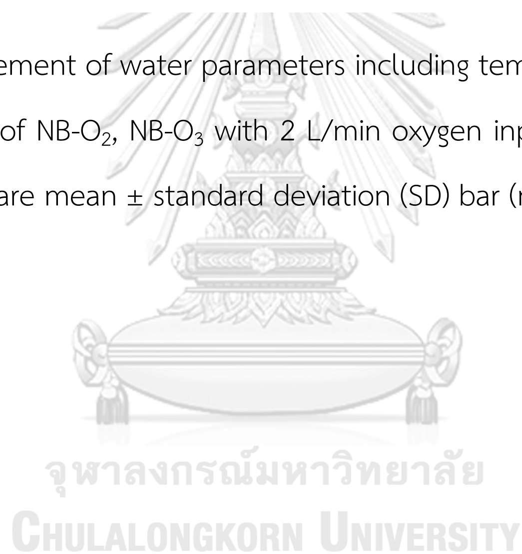


Figure S1. Measurement of water parameters including temperature (**A**), pH (**B**) during 15 min treatment of NB-O_2, NB-O_3 with 2 L/min oxygen input in tap water. Values of water parameters are mean ± standard deviation (SD) bar (n = 2).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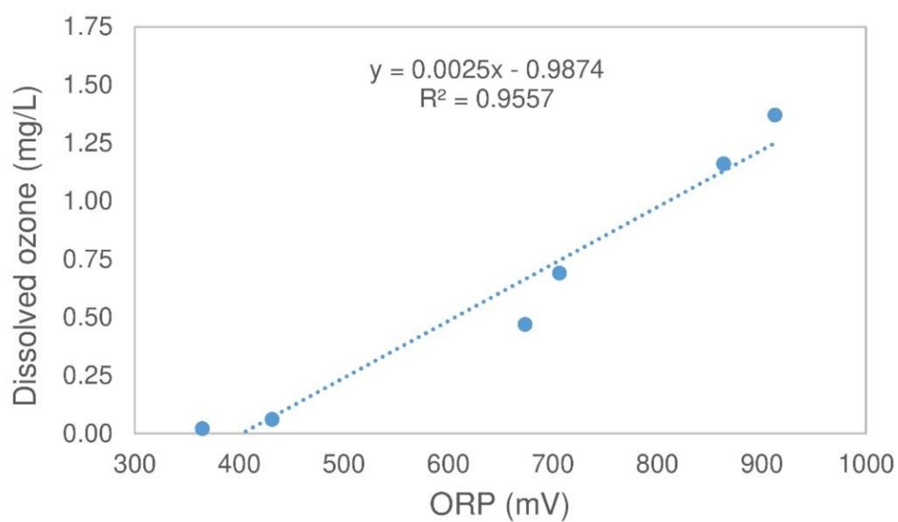

Figure S2. Plot of the dissolved ozone concentration vs. oxidative reduction potential (ORP) measurements. A linear regression shows the relationship between dissolved ozone and ORP. The ORP was measured by a multi-parameter meter (YSI Professional Plus, YSI Incorporated, USA), while dissolved ozone (ppm-mg/L) was measured by a K-7434 Ozone Vacu-vials Kit (Oxidation Technologies, USA).

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PUBLICATION	Dien LT, Linh NV, Sangpo P, Senapin S, St-Hilaire S, Rodkhum C, Dong HT (2021), Ozone nanobubble treatments improve survivability of Nile tilapia (Oreochromis niloticus) challenged with a pathogenic multidrug-resistant Aeromonas hydrophila, Journal of Fish Diseases, Vol 00: 1-13. Nguyen Vu Linh, Le Thanh Dien, Wattana Panphut, Anat Thapinta, Saengchan Senapin, Sophie St-Hilaire, Channarong Rodkhum, Ha Thanh Dong (2021), Ozone nanobubble modulates the innate defense system of Nile tilapia (Oreochromis niloticus) against Streptococcus agalactiae, Fish and Shellfish Immunology, Vol 112, 64-73. Le Thanh Dien, Nguyen Trong Tin, Nguyen Minh Tan, Nguyen Thi Thuy Hien, Nguyen Van Thanh Binh (2017), Antibiotic resistance profiling of Escherichia coli and Klebsiella pneumoniae in Nguyen Dinh Chieu Hospital, Vietnam Journal of Preventive Medicine, Vol 27, No. 11, 180-185.

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