MYCOBACTERIAL INFECTION IN SIAMESE FIGHTING FISH (*BETTA SPLENDENS*): PATHOGEN CHARACTERISTICS, PATHOGENICITY, PATHOGENESIS, AND CONTROL STRATEGIES

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การติดเชื้อมัยโคแบคทีเรียในปลากัดไทย (*เบทต้า สเปรนเดนส์*): ลักษณะเฉพาะของเชื้อก่อโรค ความรุนแรง พยาธิกำเนิด และวิธีการในการควบคุมโรค

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Thesis Title	MYCOBACTERIAL INFECTION IN SIAMESE FIGHTING FISH (*BETTA SPLENDENS*): PATHOGEN CHARACTERISTICS, PATHOGENICITY, PATHOGENESIS, AND CONTROL STRATEGIES
By	Mr. Nguyen Dinh Hung
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เหวียน ดินห์ ฮูง : การติดเชื้อมัยโคแบคทีเรียในปลากัดไทย (*เบทต้า สเปรนเดนส์*): ลักษณะเฉพาะของเชื้อก่อโรค ความรุนแรง พยาธิกำเนิด และวิธีการในการควบคุมโรค. (MYCOBACTERIAL INFECTION IN SIAMESE FIGHTING FISH (*BETTA SPLENDENS*): PATHOGEN CHARACTERISTICS, PATHOGENICITY, PATHOGENESIS, AND CONTROL STRATEGIES) อ.ที่ปรึกษาหลัก : ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาร่วม : สาทิต ฉัตรชัยพันธ์

การศึกษานี้ได้ตรวจสอบโรค mycobacteriosis ในปลากัดไทย (*Betta splendens*) ซึ่งเป็นโรคที่ส่งผลต่อความยั่งยืนของอุตสาหกรรมปลากัด ซึ่งการวิจัยแบ่งเป็น 3 ส่วน คือ การวิจัยส่วนที่ 1 เป็นการศึกษาโดยจำแนก *Mycobacterium* 5 สปีชีส์ ได้แก่ *M. chelonae, M. cosmeticum, M. farcinogenes, M. mucogenicum* และ *M. senegalense* เพื่อให้ได้ข้อมูลลักษณะปรากฏ, คุณสมบัติชีวเคมี รวมถึงข้อมูลความต้านทานต่อยาปฏิชีวนะของแบคทีเรีย ทั้งนี้มีค่าดัชนี MAR อยู่ในช่วง 0.22-0.61 แสดงถึงความเป็นไปได้ในการวางแผนการรักษา การทดสอบสารฆ่าเชื้อแสดงให้เห็นว่า ethanol, formalin, chlorine และ povidoneiodine มีประสิทธิภาพในการฆ่า Mycobacterium ได้เป็นอย่างดี ในขณะที่ด่างทับทิมมีประสิทธิภาพในการฆ่าเชื้อในระดับต่ำ การทดสอบโรคด้วยการฉีด *Mycobacterium* เข้าช่องท้องปลากัดไทย แสดงให้เห็นว่า *Mycobacterium* ทั้ง 5 สปีชีส์ เป็นสาเหตุของโรคในปลากัดไทย โดยที่ *M. chelonae* มีความรุนแรงสูงที่สุด การวิจัยส่วนที่ 2 เป็นการศึกษาการช่องทางการก่อโรคจาก *M. chelonae* ได้แก่ การฉีดเข้าช่องท้อง (IP), การฉีดเข้ากล้ามเนื้อ (IM), การรับเชื้อทางปาก และการรับเชื้อจากการจุ่มที่มีหรือไม่มีบาดแผลบนผิวหนัง ผลการวิจัยแสดงความเป็นไปได้ของการติดเชื้อว่าขึ้นอยู่กับเส้นทางการแพร่กระจาย โดยวิธี IP และ IM จะทำให้เกิดการติดเชื้อที่รุนแรง ทั้งนี้ทุกช่องทางการก่อโรคนำไปสู่สภาพเรื้อรัง ซึ่งแสดงให้เห็นว่าการควบคุม mycobacteriosis ต้องการการลดปัจจัยเสี่ยงที่เกี่ยวข้องกับเส้นทางเหล่านี้ การวิจัยส่วนที่ 3 เป็นการใช้นวัตกรรมโอโซนนาโนบับเบิล (NB-O_3) เพื่อกำจัด *Mycobacterium* ในน้ำเพื่อลดความเสี่ยงของ mycobacteriosis ในปลากัดไทย หลังจากการบำบัดน้ำด้วย NB-O_3 นาน 60 นาที พบว่าความเข้มข้นของ *M. chelonae* ลดลงอย่างมากถึง 99.92% ทั้งนี้การทดลองภาคสนามด้วยการบำบัดน้ำในฟาร์มด้วย NB-O_3 นาน 10 นาที ส่งผลให้มีการลดจำนวนแบคทีเรียทั้งหมดมากกว่า 90% แสดงให้เห็นว่าการใช้ NB-O_3 กับน้ำที่มี *M. chelonae* สามารถลดความเสี่ยงของการติดเชื้อและเพิ่มอัตราการรอดชีวิตของปลากัดไทยได้ ดังนั้น NB-O_3 เป็นวิธีที่มีศักยภาพสูงในการควบคุมโรค ซึ่งถือเป็นช่องทางใหม่ในการดูแลสุขภาพของปลากัดไทย กล่าวโดยสรุปคือ การศึกษาครั้งนี้แสดงถึงข้อมูลสำคัญเกี่ยวกับลักษณะเฉพาะของเชื้อ ความเป็นไปได้ในการทำให้เกิดโรค การก่อให้เกิดโรค และมาตรการป้องกันต่อโรค ซึ่งได้ข้อมูล mycobacteriosis ที่เป็นประโยชน์ และสามารถใช้ส่งเสริมการจัดการอุตสาหกรรมปลากัดไทยอย่างยั่งยืนต่อไป

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Nguyen Dinh Hung : MYCOBACTERIAL INFECTION IN SIAMESE FIGHTING FISH (*BETTA SPLENDENS*): PATHOGEN CHARACTERISTICS, PATHOGENICITY, PATHOGENESIS, AND CONTROL STRATEGIES. Advisor: Assoc. Prof. CHANNARONG RODKHUM Co-advisor: Asst. Prof. Satid Chatchaiphan

This study conducted a comprehensive investigation into mycobacteriosis in Siamese fighting fish (*Betta splendens*), a disease that poses major challenges to the sustainability of the sector. In the first phase, five different *Mycobacterium* species (*i.e.*, *M. chelonae, M. cosmeticum, M. farcinogenes, M. mucogenicum,* and *M. senegalense*) were meticulously characterized. Bacterial speciation was determined through phenotypic and biochemical characteristics. Concurrently, antibiotic resistance of the bacteria with MAR indices ranging from 0.22 to 0.61, indicated potential challenges in treatment strategies. Furthermore, disinfectant susceptibility revealed that ethanol, formalin, chlorine, and povidone-iodine were effective in killing the bacteria, while potassium permanganate was less effective. Intraperitoneal injection confirmed that all five isolates were pathogenic to betta fish, with *M. chelonae* exhibiting the highest virulence. In the second phase, the infection progression was explored by exposing *B. splendens* to the pathogenic *M. chelonae* isolate using various exposure methods, including injection (IP and IM), oral administration, and immersion with or without skin trauma. Results showed that pathogenicity was largely dependent on the transmission routes, with IP and IM methods causing particularly severe infections. It is also likely that infection occurs naturally primarily through an injured body surface and/or the digestive tract. All routes led to chronic conditions, suggesting that controlling mycobacteriosis requires a rigorous reduction of risk factors associated with these routes. In the third phase, an innovative approach using ozone nanobubbles (NB-O_3) to disinfect water was introduced to mitigate the risk of mycobacteriosis in betta fish. After a 60 min incubation in NB-O_3 treated water, *M. chelonae* concentration was significantly reduced by 99.92%. Direct treatment of farm water with NB-O_3 for 10 min resulted in over 90% reduction in total bacterial count. Application of NB-O_3 to water contaminated with *M. chelonae* effectively reduced the risk of infection and improved the fish's survivability. Thus, NB-O_3 represents a promising strategy for disease control, offering a new avenue for enhancing betta fish health. Taken together, these multifaceted studies provide essential insights into the pathogen characteristics, pathogenicity, pathogenesis, and preventive measures against the disease. This work offers a valuable perspective on mycobacteriosis and contributes significantly to the sustainable management of the betta fish industry.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2023	Advisor's Signature .....
		Co-advisor's Signature .....

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"Pursuing my PhD has been an arduous yet fulfilling journey"

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Nguyen Dinh Hung

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LIST OF ABBREVIATIONS

ADC	albumin dextrose catalase
DO	dissolved oxygen
H&E	hematoxylin and eosin
IP	intraperitoneal injection
IM	Intramuscular injection
LD_{50}	median lethal dose
MDR	multidrug-resistant MDR
MMCs	melano-macrophage centers
NB-O_3	ozone nanobubbles
NTM	non-tuberculous mycobacteria
OADC	oleic albumin dextrose catalase
ORP	oxidation-reduction potential
PBS	phosphate-buffered saline
RGM	rapidly-growing mycobacteria
SEM	scanning electron microscopy
SGM	slowly-growing mycobacteria

CHAPTER 1

Introduction

1.1. Importance and rationale

Siamese fighting fish (*Betta splendens*), colloquially known as betta, is a fascinating freshwater fish species native to the Southeast Asian region (U.S. Fish and Wildlife Service, 2019). Their vivid pigmentation combined with their elegantly curved fins make bettas not only an aesthetic sight in global aquarium trade, but also give them significant economic importance (Sermwatanakul, 2019). Nevertheless, bettas are particularly susceptible to mycobacteriosis, a problem that can significantly affect the sustainability of the industry (Puttinaowarat et al., 2002; Beran et al., 2006; Sirimalaisuwan et al., 2017; Weerakhun et al., 2019; Narendrakumar et al., 2022). Moreover, the potential for zoonotic transmission (Ziarati et al., 2022) makes these infections even more complex and poses not only a significant obstacle to culturing of betta fish, but also a serious public health problem. Therefore, it is imperative to develop a more comprehensive understanding of these infections and implement effective control strategies to ensure the long-term sustainability and expansion of the sector. Such strategies will not only benefit the industry, but also address the potential public health risks associated with zoonotic transmission.

Regrettably, current science offers neither vaccines nor effective cures for mycobacterial infections in any fish species, including *Betta splendens* (Chong, 2022). It is important to emphasize that mycobacteriosis is not only an important fish disease, but also a zoonotic infection caused by several species of non-tuberculous mycobacteria (NTM). These mycobacterial species, ubiquitous in nature and living freely in aquatic ecosystems, underscore the need for in-depth understanding and management of this disease (Gauthier and Rhodes, 2009). Consequently, the regulation of mycobacteriosis in fish, particularly betta fish, underscores the

paramount importance of disease control strategies that include strict quarantine protocols and methodical water disinfection procedures (Francis-Floyd, 2011). Currently, there are no non-lethal diagnostic methods for screening mycobacteriosis in betta fish. At the same time, the use of chemical disinfectants is often problematic due to their potential residual toxicity (Pandian et al., 2022), and the waxy coating within the cell wall of NTM could compromise the efficacy of conventional disinfectants (Francis-Floyd, 2011). This scenario urgently requires research and implementation of alternative, environmentally friendly strategies to improve the control of mycobacteriosis in betta fish farming.

Research on mycobacteriosis in *B. splendens* has yielded significant progress in pathogen isolation and detection, as well as in understanding clinical manifestations. Several critical research gaps, however, remain in this field and need to be explored. First, comprehensive epidemiological studies are essential to determine the prevalence and distribution of the disease within betta fish populations. Such studies would not only clarify the infection status but also reveal the profound impact of the disease. Second, the pathogenesis of mycobacteriosis in bettas remains poorly understood, and further investigations are required to elucidate the mechanisms underlying bacterial infection, host immune response, and disease progression. Additionally, the development of reliable and non-lethal diagnostic techniques specific to mycobacteriosis in *B. splendens* is essential for accurate and timely detection. Moreover, studies focusing on the development of effective vaccines or immunostimulants against mycobacteriosis in bettas are critically important for improving disease prevention and control measures. Finally, research exploring the potential zoonotic implications of mycobacteriosis in bettas is necessary to assess the risk to human health and provide appropriate guidelines for handling and husbandry practices. Addressing these research gaps will contribute

significantly to our understanding of mycobacteriosis in betta fish and facilitate the development of effective strategies to control the disease.

Thus, this study aimed to conduct a comprehensive investigation of the phenotypic and biochemical characteristics, as well as the antibiotic and disinfectant susceptibility of *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*). The virulence and pathogenicity of these isolates to their host species were also examined by experimentally infecting the fish through intraperitoneal injection. Furthermore, the pathogenesis of a selected pathogenic isolate was studied using various routes of exposure, including injection (both intraperitoneal and intramuscular), oral administration, and immersion with or without skin trauma. Additionally, the efficacy of ozone nanobubbles (NB-O_3) in treating the water used for culturing betta fish, in order to mitigate the risk of mycobacteriosis, was investigated.

1.2. Objectives of the study

1. To investigate the characteristics and pathogenicity of *Mycobacterium* spp. isolated from betta fish.
2. To examine the pathogenesis of mycobacterial infection in betta fish via various infection routes.
3. To evaluate the applicability of NB-O_3 technology for water disinfection to mitigate the risk of mycobacteriosis in cultured betta fish.

1.3. Hypothesis

The present study formulates the hypothesis that different isolates or species of NTM have distinct pathophysiological properties. It is also postulated that the route of infection significantly influences the manifestation of varied pathogenesis. Additionally, the potential efficacy of NB-O_3 technology is suggested as a promising approach to mitigate the risk of mycobacteriosis in betta fish farms.

1.4. Conceptual framwork

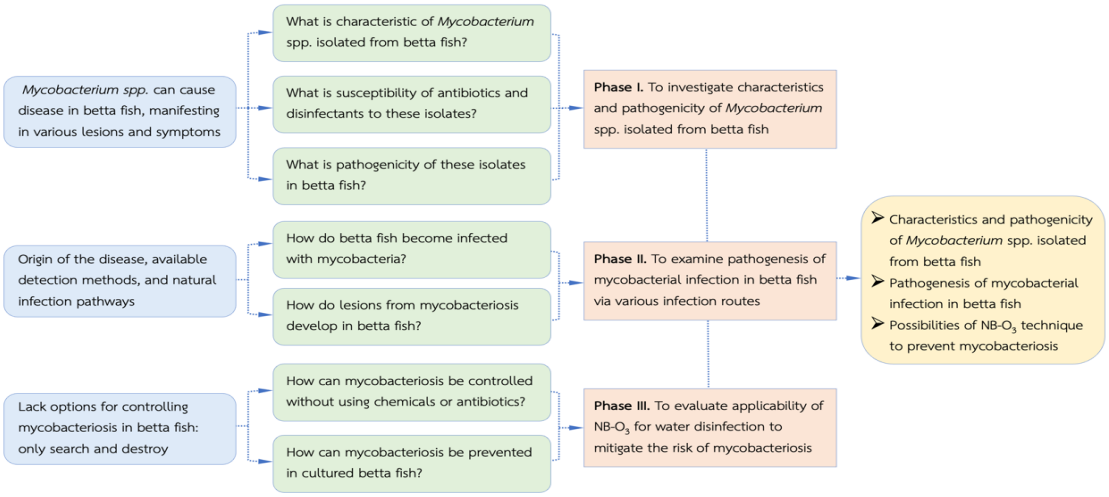

Figure 1.1. A conceptual framework illustrates the organization of currently available information (blue), research questions (green), the conduct of experiments to address these questions (orange), and the expected outcomes of research activities (yellow).

1.5. Reference

Beran V, Matlova L, Dvorska L, Svastova P and Pavlik I 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. J. Fish Dis. 29(7): 383-393.

Chong RS-M 2022. Mycobacteriosis. In: Aquaculture Pathophysiology. Elsevier. 407-415.

Francis-Floyd R 2011. Mycobacterial infections of fish. In: Southern Regional Aquaculture Center USA.

Gauthier DT and Rhodes MW 2009. Mycobacteriosis in fishes: A review. Vet J. 180(1): 33-47.

Narendrakumar L, Sudhagar A, Preena PG, Nithianantham SR, Mohandas SP and Swaminathan TR 2022. Detection of *Mycobacterium marinum* and multidrug-resistant bacteria in a chronic progressive disease outbreak among Siamese fighting fish (*Betta splendens*) in India. Biologia. 77(9): 2725-2733.

Pandian AMK, Rajamehala M, Singh MVP, Sarojini G and Rajamohan N 2022. Potential risks and approaches to reduce the toxicity of disinfection by-product–A review. Sci. Total Environ. 822: 153323.

Puttinaowarat S, Thompson K, Kolk A and Adams A 2002. Identification of *Mycobacterium* spp. isolated from snakehead, *Channa striata* (Fowler), and Siamese fighting fish, *Betta splendens* (Regan), using polymerase chain reaction–reverse cross blot hybridization (PCR–RCBH). J. Fish Dis. 25: 235-243.

Sermwatanakul A 2019. Capacitating the local farmers to enhance global marketing of Thailand's national aquatic animal, the Siamese fighting fish. Fish for the People. 17(2): 42-48.

Sirimalaisuwan A, Teeraruk P, Kanjanapitakchai P, Kaewsakhorn T, Potibut P and Pikulkaew S 2017. Detection of *Mycobacterium marinum* in clinically asymptomatic Siamese fighting fish (*Betta splendens*) from ornamental fish shops in Chiang Mai Province, Thailand. Asian Pac. J. Trop. Dis.. 7: 344-346.

U.S. Fish and Wildlife Service 2019. Subject: Siamese fighting fish (*Betta splendens*) ecological risk screening summary (online). Available: https://www.fws.gov/sites/default/files/documents/Ecological-Risk-Screening-Summary-Siamese-Fighting-Fish.pdf.

Weerakhun S, Sukon P and Hatai K 2019. *Mycobacterium marinum* and *Mycobacterium fortuitum* infections in Siamese fighting fish, *Betta splendens* (Regan), in Thailand. Thai J Vet Med. 49(2): 137-145.

Ziarati M, Zorriehzahra MJ, Hassantabar F, Mehrabi Z, Dhawan M, Sharun K, Emran TB, Dhama K, Chaicumpa W and Shamsi S 2022. Zoonotic diseases of fish and their prevention and control. Vet Q. 42(1): 95-118.

CHAPTER 2

Literature review

2.1. Overview of the betta fish industry and the challenges posed by mycobacteriosis

Betta splendens, known commercially as Siamese fighting fish or simply betta, are highly prized by enthusiasts around the world for their distinct color variations and unique fin morphologies. Historically, betta fish were originally bred to increase their aggressiveness for sparring competitions and served as a form of entertainment and gambling centuries ago (Sermwatanakul, 2019). However, this changed when local breeders in Thailand began to develop different varieties of the fish through selective breeding. The goal of these efforts was to achieve a range of colors and fin textures that would be attractive to customers. The unique and eye-catching appearance of betta fish has not only contributed to their popularity but has also inspired numerous artistic works and creative products (Monvises et al., 2009). These expressions can be found in various media such as photography, painting, sculpting, weaving, 3D graphics, furniture, cosmetics, and fashion, which has further increased the popularity of betta fish in the global market (Lichak et al., 2022). Economically, this species is of considerable value; the price of a single fish ranges from less than a dollar to over a thousand dollars, depending on coloration and fin shape (Sermwatanakul, 2019). The fish enjoyed great popularity in the aquarium trade with Thailand accounting for the largest share of breeders and exporters (Sermwatanakul, 2019). As one of the world's major sources of ornamental fish, the export of betta fish in Thailand reached about 22.82 million pieces with an estimated value of $5.55 million in 2018 (DOF, 2018). According to DOF (2018), the average annual export volume and value of betta fish in the country between 2014 and 2018 were $23.92 million and $4.29 million, respectively. It is worth noting that

in 2018, despite a sudden decrease in export volume, export value increased significantly to about $5.55 million, which is the highest turnover within the five-year period (2014-2018). According to the study of Sermwatanakul (Sermwatanakul, 2019), there is a correlation between the characteristics and features of betta fish and the corresponding prices in different markets. In the United States and Europe, these fish are most sought after as esthetically pleasing pets. In the Middle East, however, they are often exchanged as lively gifts on special occasions. In Asian countries, there is a widespread preference for acquiring high-end fish with which to participate in fishing competitions. There are also individuals who buy betta fish at wholesale prices and then resell them at a premium. For exports of betta fish from Thailand in 2018 (DOF, 2018), the United States, China, France, Iran, and Singapore were the top five destination countries in terms of trade value. Although France has the lowest import volume among these countries, the combination of exceptional fish quality and significant logistical effort has helped France achieve the highest average price for betta fish, about $0.5 per fish.

The exponential growth of the ornamental fish sector coupled with inconsistent culturing practices has led to an alarming increase in the prevalence of various diseases which can hinder the progress of betta farming (Purivirojkul and Sumontha, 2013; Senapin et al., 2014; Goldstein, 2015; Maceda-Veiga and Cable, 2019). Mycobacterial infections have been shown to be of particular concern and have potential economic impacts in the betta fish industry (Pleeging and Moons, 2017; Dong et al., 2018; Lichak et al., 2022). The infection is characterized by several recognizable symptoms, including lethargy, decreased appetite, weight loss, and the appearance of skin ulcers. These symptoms are typically observed in the context of chronic progressive infections and are often accompanied by external manifestations such as emaciation and fin accumulation (Narendrakumar et al., 2022), as well as the

occurrence of big belly syndrome and skin nodule syndrome (Dong et al., 2018). Histopathological analysis revealed widespread granulomatous inflammation in multiple organs, a finding consistent with other affected fish species (Dong et al., 2018). Due to the ability of the bacteria to persist in the aquatic environment, controlling the spread of the disease is a major challenge. Treatment of mycobacteriosis has proven to be an arduous endeavor that is often unsuccessful, as there are currently no vaccines or effective therapeutic measures for this particular infection (Chong, 2022). When clinical signs of infection occur, the only feasible method of containing the spread of the disease is to cull the affected animals and then rigorously disinfect the environment (Francis-Floyd, 2011). Consequently, these infections can result in significant economic losses to the betta industry, as they significantly affect both production and trade. The situation is further exacerbated by the possible transmission of infections from infected farmed animals to their offspring, a scenario that must be seriously considered. The reproduction of infected fish not only poses a significant economic burden due to the likelihood of stunted growth and chronic mortality but is also a constant threat to other fish in the ecosystem as well as to betta keepers, pet store employees, and aquarists. The complexity of the disease combined with the lack of effective preventative or curative measures makes it a critical issue for all stakeholders in the betta industry.

2.2. Overview of mycobacteriosis in fish

2.2.1. Pathogen characteristics

The genus *Mycobacterium* belongs to the family *Mycobacteriaceae*, class *Corynobacteriales*, phylum *Actinobacteria* and kingdom *Bacteria* (Shinnick and Good, 1994; Chapman, 2012; Tortoli, 2019). To date, more than 220 species and subspecies of the genus have been identified and described (Pereira et al., 2020). This large group includes mycobacteria that cause tuberculosis (*M. tuberculosis* complex and

M. bovis complex) and leprosy (*M. leprae*), as well as non-tuberculous mycobacteria (NTM, atypical mycobacteria or environmental mycobacteria) that are widely distributed in the environment (Runyon, 1959; Wolinsky, 1992; Shinnick and Good, 1994). *Mycobacterium* spp. are characterized, at phenotypic level, by unique characteristics. The main characteristic features of this genus include acid resistance and the presence of mycolic acids. Mycobacteria are slender, non-spore-forming, Gram-positive straight or slightly curved rods, aerobic bacteria that live freely in soil and water (Runyon, 1959; Shinnick and Good, 1994). These bacteria are acid-alcohol fast, meaning that they resist decolorization with acidified alcohol as well as strong mineral acids after staining. The property of acid resistance, which is due to waxy materials in the cell walls, is particularly important for the detection of mycobacteria. Staining procedures must be performed carefully because other Gram-positive bacteria (*e.g.*, *Nocardia*, *Corynebacterium* and *Rhodococcus*) are often partially acid fast (Kenneth J. Ryan, 1994). In addition, *Mycobacterium* spp. are characterized by a variety of features, including growth rate, colony morphology and pigmentation in the presence and absence of light (Velayati et al., 2019). According to Runyon (1959), mycobacteria can be classified into distinct groups based on specific phenotypic criteria, including pigmentation and growth rate on agar media. The classification includes Types I, II, and III, all of which are categorized as slowly growing mycobacteria (SGM), requiring 7 days or more to grow, and are differentiated by their coloration. Type I mycobacteria, known as photochromogens, produce pigment only when exposed to light; Type II, referred to as scotochromogens, exhibit pigmentation even when grown in the dark; and Type III, termed nonphotochromogens, are not strongly pigmented. Additionally, there is the category of rapidly growing mycobacteria (RGM-Type IV), characterized by almost no pigment production and growth in less than 7 days, albeit more slowly than most bacteria. Intriguingly, most pathogenic mycobacteria are found within the slowly growing

category, suggesting a direct correlation between growth rate and virulence, as documented in recent research (Tortoli et al., 2017; Johansen et al., 2020).

Genetically, mycobacteria are distinguished from the large majority of bacteria by their high G + C content (between 62% and 70%) (Goodfellow and Wayne, 1982; Tortoli, 2012). The number of copies of the ribosomal operon is low: two copies in rapid growers and only one in slow growers; there are very few exceptions (Böddinghaus et al., 1990). The 16S rRNA-based phylogeny endorses a division between rapid and slow growers, clustering most species in well-defined phylogenetic groups, however, the moderate variability of 16S rRNA among mycobacteria with high intraspecific identity (ranging from 94 to 100%) limits the distinction of several species (Tortoli, 2003). Rapid and slow-growing species appeared clearly separated in the phylogenetic trees (Tortoli, 2019). Arecent study by Gupta *et al.* (2018) has proposed a significant revision to the taxonomy of mycobacteria. In this revision, 150 species formerly classified within the genus *Mycobacterium* have been redistributed into five newly designated genera: *Mycobacterium, Mycobacteroides, Mycolicibacter, Mycolicibacterium,* and *Mycolicibacillus.* This updated nomenclature was subsequently validated in a publication in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM), , a journal that represents the official voice of the International Committee on Systematics of Prokaryotes, by Oren and Garrity (Oren and Garrity, 2018). It is worth noting that this revised taxonomy has generated some controversy, and there are concerns that the reference to NTM in recent publications may lead to confusion, particularly among those who are not specialists in the field of mycobacteriology. Despite the contentious nature of the changes, an editorial by Tortoli *et al.* (2019) has acknowledged that both the new and former nomenclature systems may coexist and be used appropriately. Consequently, it is plausible to

conclude that the nomenclature for NTM species (*i.e.*, *Mycobacterium* spp.) remains unaffected by these taxonomic adjustments.

2.2.2. Pathogenecity

Mycobacteriosis, a disease caused by members of the NTM family, is a critical disease in fish, especially in intensive aquaculture systems and aquarium settings (Gauthier and Rhodes, 2009). This disease occurs with remarkable frequency in tropical aquarium fish, *including B. splendens* (Puttinaowarat et al., 2002; Beran et al., 2006; Sirimalaisuwan et al., 2017; Weerakhun et al., 2019; Narendrakumar et al., 2022). Infections with NTM in fish usually present with a range of nonspecific clinical symptoms that often mirror other fish diseases (Francis-Floyd, 2011). Notably, a prominent histopathological feature of these infections is systemic granulomatosis, characterized by granulomatous lesions in multiple organs (Delghandi et al., 2020). In Betta fish, mycobacterial infections typically manifest as chronic progressive diseases characterized by external symptoms such as emaciation, grouped fins, big belly syndrome, and skin nodule syndrome. (Narendrakumar et al., 2022) (Dong et al., 2018). Although the identification of NTM species in the aquatic environment has increased, three primary species (*M. marinum, M. fortuitum,* and *M. chelonae*) are recognized as the most prominent pathogens affecting fish, with other species remaining inadequately characterized (Delghandi et al., 2020).

Previous artificial experiments have shown different mortality rates in different fish species after intraperitoneal injections with *Mycobacterium* species. For example, Arakawa and Frye (1984) reported mortality rates ranging from 20 to 52% in rainbow trout (*Oncorhynchus mykiss*) over an 80 day period and an astonishing 98% in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in only 10 days after infection with M. chelonae at a concentration of 1.0×10^{7} CFU/fish. Similarly, Talaat *et al.* (1999) recorded a mortality rate of 10.5-40% in goldfish (Carassius auratus)

infected with Mycobacterium smegmatis at doses of 10^7 to 10^8 CFU/fish over an 8 week period. Additionally, Fukano *et al.* (2015) observed a significant 95% mortality rate within three weeks in thread-sail filefish (*Stephanolepis cirrhifer*) infected with *Mycobacterium stephanolepidis* at 9.0×10^4 CFU/fish. In a study conducted by Weerakhun *et al.* (2019), the researchers identified significant mortality rates, ranging from 72 to 100%, in goldfish (*Carassius auratus*) that were infected with isolates of *M. marinum* and *M. fortuitum*. These results were found to be consistent across both low and high dosages, specifically 10^2 or 10^4 CFU/fish, over a period of 210 days. In a more recent investigation, Machida *et al.* (2021) observed a cumulative mortality rate between 30 and 50% in cultured koi carp (*Cyprinus carpio*) 100 days after being infected with *Mycobacterium paragordonae* and two other species of *Mycolicibacterium* spp. Overall, these studies highlight the variability of mycobacterial pathogenicity in fish. The virulence characteristics of mycobacteria in fish are influenced not only by the causative pathogens, but also by the infected fish host and other environmental factors.

2.2.3. Pathogenesis

The exact transmission routes of *Mycobacterium* spp. are currently unknown. It can be administered orally, through the skin, urogenital and by inhalation. However, it is likely that infection occurs after contact with environmental sources or an infected individials, either directly or indirectly. NTM can survive in the environment and contaminated water sources have been reported as a substantial risk factor for NTM infection (Le Dantec et al., 2002; Dowdell et al., 2019; Falkinham III, 2021). Biofilms are also a known reservoir for NTM species . Fish become infected through direct contact with biofilms, contaminated water, food, and infected individuals (Delghandi et al., 2020). The organisms typically colonize the hosts from cutaneous lesions and may form nodules or ulcers. They may also spread to the

viscera and form granulomas in organs with or without skin abnormalities. Despite the fact that NTM are widespread in the environment and exposure to these organisms is inevitable, the acquisition of mycobacteriosis involves complex interactions between host, pathogen and environmental determinants and risk factors (Delghandi et al., 2020). Although mycobacteriosis is considered to be precipitated by stress and occurs in immunodepressed host, specific factors leading to disease outbreaks are seldom defined and appear to vary among systems. The disease is typically a chronic, slow progressive disease in immunocompetent individuals, however in stressed or immunosuppressed, the course of infection is much more severe, leading to acute and systemic disease. In stressed and immunodepressed fish, such as those housed in crowded environments or with incorrect temperature or water parameters, the course of infection is much more severe, leading to systemic and acute disease (Chai, 2011). Based on the rare numbers of definitive reports of mycobacteria being isolated from free-ranging populations, this supports that animals debilitated in captivity are particularly susceptible (Martinho and Heatley, 2012). The initial dose also influences disease outcome, as evidenced by the study (Ramakrishnan et al., 1997) in the leopard frog (*Rana pipiens*), in which *M. marinum* produces chronic disease with a low initial innoculum and acute or subacute disease with higher infecting doses. Also emphasize that the simple isolation of mycobacteria alone does not necessarily indicate disease, because again, they are widely distributed in the environment (Pavlik et al., 2022). The pathogenesis of mycobacteria is therefore likely to be a complex interaction of numerous interrelated factors and variables that include host characteristics, environmental conditions, bacterial virulence, and other risk factors (Figure 2.1).

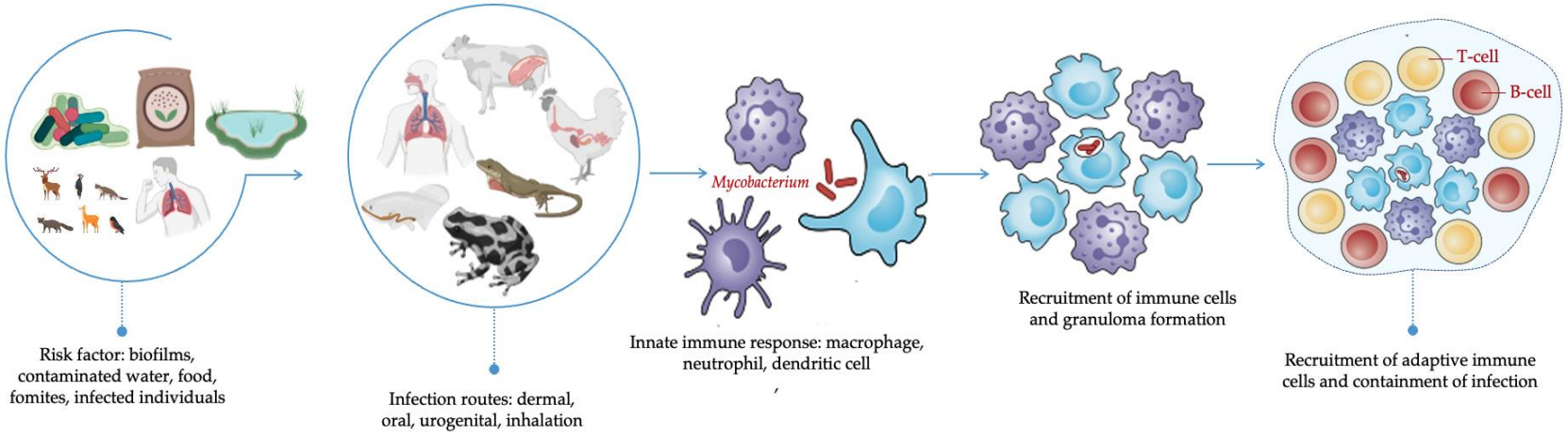

Figure 2.1. A conceptual pathogenesis of mycobacteriosis is complicated and the exact routes of transmission are unknown. These include oral, dermal, urogenital, and inhalational methods, typically through contact with the environment or infected individuals. Contaminated water, dust, food, and biofilms are risk factors and reservoirs for *Mycobacterium* spp. Although immune cells attempt to eliminate the bacteria, *Mycobacterium* spp. can escape destruction and cause infection, leading to granuloma formation, immune system activation, and recruitment of B and T lymphocytes as part of the adaptive immune response. (*The image was personally created by BioRender.com*).

2.2.4. Control strategies

Controlling fish mycobacteriosis necessitates a multifaceted approach. According to Francis-Floyd (Francis-Floyd, 2011), once fish populations are affected by mycobacteriosis, the recommended actions often include euthanizing the entire affected group and thoroughly cleaning and disinfecting all contact areas. This process involves removing organic biofilm and utilizing mycobacteriocidal agents such as 50 to 70% ethyl alcohol, sodium chlorite, or LysolR (containing 1% benzyl-4-chlorophenol-2-phenylphenol). The unique wax coating of the mycobacterial cell wall confers resistance to chlorine (up to 50 g/L is only moderately effective) and several commercial disinfectants, such as RoccalR and VirkonR.

To prevent the re-introduction of the pathogen, health testing of new fish stocks through histology, culture, and PCR methods, in conjunction with quarantine measures, is essential infection (Chong, 2022). The potential zoonotic risk associated with mycobacteria requires protective measures for individuals working with infected fish, water, or fish systems. These include the use of face shields, gloves, waterproof clothing and footwear, regular hand sanitization with alcohol-based disinfectants, and seeking medical attention if symptoms arise (Delghandi et al., 2020).

Treatment options are limited, and antibiotic therapy is generally not recommended for controlling mycobacteriosis in ornamental fish, given existing resistance to multiple antibiotics and the likelihood of reinfection through cannibalism or carrier fish infection (Chong, 2022). Experimental treatments, such as administering oral rifampicin, streptomycin, or erythromycin to infected yellowtail (*Seriola quinqueradiata*) (Kawakami and Kusuda, 1990), have proven ineffective as the mycobacteria persisted. Additionally, an experimental DNA vaccine targeting *M. marinum* has been found to provide only short-term protection for striped bass and lacks efficacy for an extended duration (Hashish et al., 2018).

In particular, disinfection of water contaminated with mycobacteria is therefore an important strategy to control mycobacteriosis and should be implemented in betta fish facilities. Various methods are available, including the use of chemicals, radiation, and physical methods like filtration and ozone treatment (Huyben et al., 2018). Chemical disinfectants like chlorine and hydrogen peroxide are effective in killing mycobacteria but can be toxic to fish and other aquatic organisms. Radiation methods, such as ultraviolet (UV) light, can also be effective, but they require specialized equipment and may not be appropriate for large-scale water treatment (Summerfelt, 2003). Physical methods such as filtration and ozone treatment are becoming increasingly popular in aquaculture and aquarium settings (Huyben et al., 2018).

2.3. NB-O_3 technology and its application in aquaculture.

Ozone (O_3), recognized as a potent oxidizing agent, has found increasing acceptance across various water treatment applications, with its demonstrated efficacy in disinfection, deodorization, and the elimination of both organic and inorganic contaminants (Langlais et al., 2019). Nonetheless, the widespread implementation of ozone has been curtailed by certain challenges, including its inherently poor stability, low solubility, and potential adverse impacts on aquatic organisms (Powell and Scolding, 2018). In recent years, the aquaculture industry has shown growing interest in an innovative approach that utilizes ozone in the form of nanobubbles (NB), a development that provides significant advantages over traditional ozone delivery systems. These benefits encompass enhanced solubility and stability, along with sustained ozone delivery (Gurung et al., 2016; Atkinson et al., 2019; Fan et al., 2020). Nanobubble technology, as it is understood today, entails the introduction of nanobubbles (<100 nm) containing a selected gas into water (Atkinson et al., 2019). These nanobubbles are characterized by neutral buoyancy

and elevated solubility, attributes that stem from their substantial surface-to-volume ratio (Gurung et al., 2016). During implosion, nanobubbles are generated that acquire a negative charge and this charge enables the nanobubbles to disperse superiorly in water due to the forces of mutual repulsion (Agarwal et al., 2011; Atkinson et al., 2019). When encapsulated within nanobubbles, ozone maintains its stability for extended periods and enables rapid reactions to degrade pollutants, simultaneously augmenting the concentration of dissolved oxygen in water (Seridou and Kalogerakis, 2021; Pal et al., 2022). Furthermore, the oxygen-free radicals produced within this context exhibit greater reactivity and oxidation potential in comparison to conventional disinfectants like chlorine (Takahashi et al., 2007). Consequently, ozone nanobubbles (NB-O_3) have the potential to significantly enhance water quality by achieving a multifaceted effect, such as reducing pathogen presence, optimizing dissolved oxygen levels, and mitigating the formation of harmful disinfection byproducts (Imaizumi et al., 2018; Jhunkeaw et al., 2021; Thanh Dien et al., 2021; Pal et al., 2022).

Application of NB-O_3 technology in aquaculture has received increasing attention in recent years due to its ability to eradicate aquatic pathogens. In a previous study by Imaizumi *et al.* (2018), disinfection experiments using NB-O_3 seawater with an ORP of 960 mV were conducted against *Vibrio parahaemolyticus*, a Gram-negative bacterium implicated in marine shrimp diseases. The results showed that NB-O_3 treatment has a high disinfection effect, as more than 99.99% of 1.8×10^5 CFU/mL of the tested bacteria were killed after a 1 min incubation time, and after an incubation time of 5 min or more, the sterilization effect reached 100%. Nghia *et al.* (2021) also found that a 6 min treatment with NB-O_3, which has an ORP of 830 ± 70 mV, completely inactivated *V. parahaemolyticus* at a concentration of 10^6 CFU/mL in laboratory experiments. In addition, Jhunkeaw *et al.* (2021) showed that treatment

with NB-O_3 with an ORP of 860 ± 42 mV had a comparable disinfection effect against pathogenic Gram-positive (*Streptococcus agalactiae*) and Gram-negative (*Aeromonas veronii*) bacteria in freshwater. The first 10 min treatment resulted in 26-fold and 48-fold reductions in bacterial loads at ~10^6 CFU/mL of *S. agalactiae* and *A. veronii*, corresponding to 96.11 and 97.92% reductions, respectively. Two subsequent 10 min NB-O_3 treatments further reduced the bacterial load in the water, reducing both pathogenic bacteria by over 99.99%. Dien *et al.* (2021) reported that despite the high bacterial concentration (~2×10^7 CFU/mL), several NB-O_3 treatments with an ORP of 870.1 ± 12.4 mV reduced the bacterial concentration of *A. hydrophila* by 15.9 to 35.6% of the total water load within the first 2 days. In contrast, bacterial concentrations in the untreated control group increased from 13.1 to 27.9%.

The study conducted by Jhunkeaw *et al.* (2021) also provides compelling evidence for the safety of NB-O_3 technology for fish. The study shows that a single exposure of 10 min to NB-O_3, resulting in an ORP of 860 ± 42 mV, is not harmful to fish. Consequently, treatment with NB-O_3 is a promising means of disease prevention because it effectively suppresses the proliferation of pathogenic bacteria in the water, thereby reducing the incidence of bacterial diseases. Moreover, this non-chemical disinfection technology has the potential to serve as an alternative to antibiotics in aquaculture, thereby reducing the use of antibiotics and indirectly mitigating the risk of antimicrobial resistance. Therefore, NB-O_3 may be a promising alternative solution to address mycobacteriosis in betta fish, although its potential has not yet been explored.

2.4. References

Agarwal A, Ng WJ and Liu Y 2011. Principle and applications of microbubble and nanobubble technology for water treatment. Chemosphere. 84(9): 1175-1180.

Arakawa CK and Fryer J 1984. Isolation and characterization of a new subspecies of *Mycobacterium chelonei* infectious for salmonid fish. Helgoländer Meeresuntersuchungen. 37: 329-342.

Atkinson AJ, Apul OG, Schneider O, Garcia-Segura S and Westerhoff P 2019. Nanobubble technologies offer opportunities to improve water treatment. Acc Chem Res. 52(5): 1196-1205.

Beran V, Matlova L, Dvorska L, Svastova P and Pavlik I 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. J. Fish Dis. 29(7): 383-393.

Böddinghaus B, Rogall T, Flohr T, Blöcker H and Böttger EC 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. 28(8): 1751-1759.

Chai N 2011. Mycobacteriosis in Amphibians. In: 224-230.

Chapman J 2012. The atypical mycobacteria and human mycobacteriosis. In: Springer Science & Business Media.

Chong RS-M 2022. Mycobacteriosis. In: Aquaculture Pathophysiology. Elsevier. 407-415.

Delghandi MR, El-Matbouli M and Menanteau-Ledouble S 2020. Mycobacteriosis and Infections with non-tuberculous mycobacteria in aquatic organisms: A Review. Microorganisms. 8(9).

DOF 2018. Statistics of freshwater aquaculture production 2016. Fisheries development policy and strategy division no. 8/2018, Department of Fisheries. Ministry of Agriculture and Cooperatives, Bangkok, Thailand., Bangkok: Department of Fisheries, Ministry of Agriculture and Cooperatives (2018).

Dong HT, Senapin S, Phiwsaiya K, Techatanakitarnan C, Dokladda K, Ruenwongsa P and Panijpan B 2018. Histopathology and culturable bacteria associated with “big belly” and “skin nodule” syndromes in ornamental Siamese fighting fish, *Betta splendens*. Microb Pathog. 122: 46-52.

Dowdell K, Haig SJ, Caverly LJ, Shen Y, LiPuma JJ and Raskin L 2019. Nontuberculous mycobacteria in drinking water systems - the challenges of characterization and risk mitigation. Curr Opin Biotechnol. 57: 127-136.

Falkinham III JO 2021. Ecology of nontuberculous mycobacteria. Microorganisms. 9(11): 2262.

Fan W, An WG Huo MX, Yang W, Zhu SY and Lin SS 2020. Solubilization and stabilization for prolonged reactivity of ozone using micro-nano bubbles and ozone-saturated solvent: A promising enhancement for ozonation. Sep. Purif. Technol. 238: 116484.

Francis-Floyd R 2011. Mycobacterial infections of fish. In: Southern Regional Aquaculture Center USA.

Fukano H, Wada S, Kurata O, Mizuno K, Nakanaga K and Hoshino Y 2015. Nontuberculous mycobacteriosis in farmed thread-sail filefish *Stephanolepis cirrhifer*. 魚病研究. 50(2): 68-74.

Gauthier DT and Rhodes MW 2009. Mycobacteriosis in fishes: A review. Vet J. 180(1): 33-47.

Goldstein RJ 2015. The betta handbook. In: Sourcebooks, Inc.

Goodfellow M and Wayne LG 1982. Taxonomy and nomenclature. The biology of the mycobacteria. 1: 471-521.

Gupta RS, Lo B and Son J 2018. Phylogenomics and comparative genomic studies robustly support division of the genus *Mycobacterium* into an emended genus *Mycobacterium* and four novel genera. Front Microbiol. 9: 67.

Gurung A, Dahl O and Jansson K 2016. The fundamental phenomena of nanobubbles and their behavior in wastewater treatment technologies. Geosystem Eng. 19(3): 133-142.

Hashish E, Merwad A, Elgaml S, Amer A, Kamal H, Elsadek A, Marei A and Sitohy M 2018. *Mycobacterium marinum* infection in fish and man: Epidemiology, pathophysiology and management; A review. Vet Q. 38(1): 35-46.

Huyben D, Bevan D, Stevenson R, Zhou H and Moccia R 2018. Evaluation of membrane filtration and UV irradiation to control bacterial loads in recirculation aquaculture systems. Aquac Int. 26(6): 1531-1540.

Imaizumi K, Tinwongger S, Kondo H and Hirono I 2018. Disinfection of an EMS/AHPND strain of *Vibrio parahaemolyticus* using ozone nanobubbles. J Fish Dis. 41(4): 725-727.

Jhunkeaw C, Khongcharoen N, Rungrueng N, Sangpo P, Panphut W, Thapinta A, Senapin S, St-Hilaire S and Dong HT 2021. Ozone nanobubble treatment in freshwater effectively reduced pathogenic fish bacteria and is safe for Nile tilapia (*Oreochromis niloticus*). Aquaculture. 534: 736286.

Johansen MD, Herrmann JL and Kremer L 2020. Non-tuberculous mycobacteria and the rise of *Mycobacterium abscessus*. Nat Rev Microbiol. 18(7): 392-407.

Kawakami K and Kusuda R 1990. Efficacy of rifampicin, streptomycin and erythromycin against experimental *Mycobacterium* infection in cultured yellowtail. Nippon Suisan Gakkaishi=Bulletin of the Japanese Society of Scientific Fisheries. 56(1): 51-53.

Kenneth J. Ryan e 1994. Sherris medical microbiology : An introduction to infectious diseases. In: Third edition. Norwalk, Conn: Appleton & Lange, 1994.

Langlais B, Reckhow DA and Brink DR 2019. Ozone in water treatment: Application and engineering. In: Routledge.

Le Dantec C, Duguet J-P, Montiel A, Dumoutier N, Dubrou S and Vincent V 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl. Environ. Microbiol. 68(11): 5318-5325.

Lichak MR, Barber JR, Kwon YM, Francis KX and Bendesky A 2022. Care and use of Siamese fighting fish (*Betta Splendens*) for research. Comp Med. 72(3): 169-180.

Maceda-Veiga A and Cable J 2019. Diseased fish in the freshwater trade: From retailers to private aquarists. Dis Aquat Organ. 132(2): 157-162.

Machida Y, Tang BCC, Yamada M, Sato S, Nakajima K, Matoyama H, Kishihara T, Endo M, Sano M and Kato G 2021. Mycobacteriosis in cultured Koi *carp Cyprinus carpio* caused by *Mycobacterium paragordonae* and two *Mycolicibacterium* spp. Aquaculture. 539: 736656.

Martinho F and Heatley J 2012. Amphibian Mycobacteriosis. The veterinary clinics of North America. Exotic animal practice. 15: 113-119, vii.

Monvises A, Nuangsaeng B, Sriwattanarothai N and Panijpan B 2009. The Siamese fighting fish: Well-known generally but little-known scientifically. ScienceAsia. 35: 8-16.

Narendrakumar L, Sudhagar A, Preena PG, Nithianantham SR, Mohandas SP and Swaminathan TR 2022. Detection of *Mycobacterium marinum* and multidrug-resistant bacteria in a chronic progressive disease outbreak among Siamese fighting fish (*Betta splendens*) in India. Biologia. 77(9): 2725-2733.

Nghia NH, Van PT, Giang PT, Hanh NT, St-Hilaire S and Domingos JA 2021. Control of *Vibrio parahaemolyticus* (AHPND strain) and improvement of water quality using nanobubble technology. Aquac. Res. 52(6): 2727-2739.

Oren A and Garrity GM 2018. List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol. 68(11): 3379-3393.

Pal P, Joshi A and Anantharaman H 2022. Nanobubble ozonation for waterbody rejuvenation at different locations in India: A holistic and sustainable approach. Results Eng. 16: 100725.

Pavlik I, Ulmann V, Hubelova D and Weston RT 2022. Nontuberculous mycobacteria as sapronoses: A review. Microorganisms. 10(7).

Pereira AC, Ramos B, Reis AC and Cunha MV 2020. Non-tuberculous mycobacteria: Molecular and physiological bases of virulence and adaptation to ecological niches. Microorganisms. 8(9).

Pleeging C and Moons CPH 2017. Potential welfare issues of the Siamese fighting fish (*Betta splendens*) at the retailer and in the hobbyist aquarium. Vlaams Diergeneeskundig Tijdschrift. 86: 213-223.

Powell A and Scolding JWS 2018. Direct application of ozone in aquaculture systems. Rev Aquac. 10(2): 424-438.

Purivirojkul W and Sumontha M 2013. *Euclinostomum heterostomum* (Rudolphi, 1809) metacercarial infection in three osphronemid fish specieshree osphronemid fish species. Walailak J. Sci. & Tech. 10(1): 97-102.

Puttinaowarat S, Thompson K, Kolk A and Adams A 2002. Identification of *Mycobacterium* spp. isolated from snakehead, *Channa striata* (Fowler), and Siamese fighting fish, *Betta splendens* (Regan), using polymerase chain reaction–reverse cross blot hybridization (PCR–RCBH). J. Fish Dis. 25: 235-243.

Ramakrishnan L, Valdivia RH, McKerrow JH and Falkow S 1997. *Mycobacterium marinum* causes both long-term subclinical infection and acute disease in the leopard frog (*Rana pipiens*). Infect Immun. 65(2): 767-773.

Runyon EH 1959. Anonymous mycobacteria in pulmonary disease. Med Clin North Am. 43(1): 273-290.

Senapin S, Phiwsaiya K, Laosinchai P, Kowasupat C, Ruenwongsa P and Panijpan B 2014. Phylogenetic analysis of parasitic trematodes of the genus

Euclinostomum found in Trichopsis and Betta fish. J. Parasitol. 100(3): 368-371.

Seridou P and Kalogerakis N 2021. Disinfection applications of ozone micro- and nanobubbles. Environ. Sci. Nano. 8(12): 3493-3510.

Sermwatanakul A 2019. Capacitating the local farmers to enhance global marketing of Thailand's national aquatic animal, the Siamese fighting fish. Fish for the People. 17(2): 42-48.

Shinnick TM and Good RC 1994. Mycobacterial taxonomy. Eur J Clin Microbiol Infect Dis. 13(11): 884-901.

Sirimalaisuwan A, Teeraruk P, Kanjanapitakchai P, Kaewsakhorn T, Potibut P and Pikulkaew S 2017. Detection of *Mycobacterium marinum* in clinically asymptomatic Siamese fighting fish (*Betta splendens*) from ornamental fsih shops in Chiang Mai Province, Thailand. Asian Pac. J. Trop. Dis.7: 344-346.

Summerfelt ST 2003. Ozonation and UV irradiation - An introduction and examples of current applications. Aquac Eng. 28(1): 21-36.

Takahashi M, Chiba K and Li P 2007. Free-radical generation from collapsing microbubbles in the absence of a dynamic stimulus. J. Phys. Chem. B. 111(6): 1343-1347.

Talaat AM, Trucksis M, Kane AS and Reimschuessel R 1999. Pathogenicity of *Mycobacterium fortuitum* and *Mycobacterium smegmatis* to goldfish, *Carassius auratus.* Vet Microbiol. 66(2): 151-164.

Thanh Dien L, Linh NV, Sangpo P, Senapin S, St-Hilaire S, Rodkhum C and Dong HT 2021. Ozone nanobubble treatments improve survivability of Nile tilapia (*Oreochromis niloticus*) challenged with a pathogenic multi-drug-resistant *Aeromonas hydrophila.* J Fish Dis. 44(9): 1435-1447.

Tortoli E 2003. Impact of genotypic studies on mycobacterial taxonomy: The new mycobacteria of the 1990s. Clin. Microbiol. Rev. 16(2): 319-354.

Tortoli E 2012. Phylogeny of the genus *Mycobacterium*: Many doubts, few certainties. Infect. Genet. Evol. 12(4): 827-831.

Tortoli E 2019. Chapter 1 - The taxonomy of the genus *Mycobacterium*. In: Nontuberculous mycobacteria (NTM). Ali Akbar Velayati and Parissa Farnia (eds). Academic Press. 1-10.

Tortoli E, Brown-Elliott BA, Chalmers JD, Cirillo DM, Daley CL, Emler S, Floto RA, Garcia MJ, Hoefsloot W, Koh WJ, Lange C, Loebinger M, Maurer FP, Morimoto K, Niemann S, Richter E, Turenne CY, Vasireddy R, Vasireddy S, Wagner D, Wallace RJ, Jr., Wengenack N and van Ingen J 2019. Same meat, different gravy: Ignore the new names of mycobacteria. Eur Respir J. 54(1).

Velayati AA, Farnia P and Saif S 2019. Chapter 2 - Identification of nontuberculous *Mycobacterium*: Conventional versus rapid molecular tests. In: Nontuberculous mycobacteria (NTM). Ali Akbar Velayati and Parissa Farnia (eds). Academic Press. 11-59.

Weerakhun S, Sukon P and Hatai K 2019. *Mycobacterium marinum* and *Mycobacterium fortuitum* infections in Siamese fighting fish, *Betta splendens* (Regan), in Thailand. Thai J Vet Med. 49(2): 137-145.

Wolinsky E 1992. Mycobacterial diseases other than tuberculosis. Clin Infect Dis. 15(1): 1-10.

CHAPTER 3

Insight into characteristics and pathogenicity of five rapidly growing non-tuberculous *Mycobacterium* species isolated from the Siamese fighting fish, *Betta splendens*

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3.1. Highlights

- Characteristics and pathogenicity of rapidly growing non-tuberculous mycobacteria isolated from betta fish, were investigated for the first time.
- Preliminary data for the bacterial speciation was provided through phenotypic and biochemical characteristics.
- Antibiotic resistance profiles of the bacteria with multiple antibiotic resistance indexes ranging from 0.22 to 0.61 indicated potential challenges for treatment strategies.
- Disinfectant susceptibility tests showed that ethanol, formalin, chlorine, and povidone-iodine were effective in killing the bacteria, whereas potassium permanganate was less effective.
- Experimental infection revealed that all five non-tuberculous *Mycobacterium* spp., namely *Mycobacterium chelonae, M. cosmeticum, M. farcinogenes, M. mucogenicum,* and *M. senegalense*, are pathogenic to betta fish.

Keywords: Non-tuberculous mycobacteria; Antimicrobial susceptibility; Disinfectants; Pathogenicity; Histopathology; Betta fish.

3.2. Abstract

Siamese fighting fish (*Betta splendens*) play an important role in the global aquarium trade, but their susceptibility to mycobacteriosis could challenge the long-term sustainability of the industry. Thus, this study aimed to characterize rapidly growing non-tuberculous mycobacteria (RGM) isolated from the fish and to investigate their pathogenicity. Five RGM species were investigated, namely *Mycobacterium chelonae, M. cosmeticum, M. farcinogenes, M. mucogenicum,* and *M. senegalense*. The isolates were phenotypically and biochemically characterized and assessed for susceptibility to 18 antibiotics and five disinfectants. The pathogenicity of the isolated bacteria was evaluated through experimental infection of the fish by intraperitoneal injection. The results of phenotypic and biochemical typing allowed

the identification of some of the isolates and provided preliminary information to distinguish between the bacteria. All isolates were resistant to at least four antibiotics, with multiple antibiotic resistance indexes ranging from 0.22 to 0.61, with *M. chelonae* having the highest value. Four disinfectants (*i.e.*, ethanol, formalin, chlorine, and povidone-iodine) showed strong antibacterial activity, whereas potassium permanganate proved less effective. An LD_{50} value within 7 days showed that *M. chelonae* had the highest virulence at 7.12×10^5 CFU/fish, followed by *M. mucogenicum* at 4.25×10^6 CFU/fish and a range of $1.01\text{-}1.65\times10^7$ CFU/fish for the other isolates. Depending on the dose and the isolate administered, some fish displayed acute disease symptoms, while others developed a chronic condition. The acute disease was characterized by short median survival, severe peritonitis, and tissue necrosis. Fish with a chronic infection survived the 42-day trial but were emaciated and had systemic granulomas within their viscera. The findings of this study have not only improved our understanding of the nature of these RGM species but also promoted the development of control strategies to mitigate the negative impact of mycobacteriosis on the Siamese fighting fish industry.

3.3. Introduction

Betta splendens, commonly known as Siamese fighting fish or betta, are known for their diverse colours and fin types, making them a favourite amongst hobbyists worldwide. The species is of considerable economic value, with prices for individual fish varying from under a dollar to more than a thousand dollars, depending on their colour and fin shape (Sermwatanakul, 2019). The highest price recorded for a betta fish that resembled the colours of the Thai national flag was $1,530 (Bangkok Post, 2016). Trade in betta is a growing industry, with Thailand accounting for the largest share of breeding and exports (Sermwatanakul, 2019), and the country has also declared the betta as a National Aquatic Animal in recognition of its cultural and economic importance (Bangkok Post, 2019). The rapid expansion of

the ornamental fish industry, together with variable culture conditions, has contributed to a remarkable increase in the prevalence of various diseases (Purivirojkul & Sumontha, 2013; Senapin et al., 2014; Goldstein, 2015; Maceda-Veiga & Cable, 2019), which could also have negative impacts on the development of betta fish farming. Among the numerous diseases affecting betta fish, mycobacterial infections have emerged as a particular problem leading to potential economic impacts (Pleeging & Moons, 2017; Dong et al., 2018; Lichak et al., 2022). Furthermore, the possibility of zoonotic transmission (see Ziarati et al., 2022) makes these infections not only a major challenge for the farming of betta fish but also a public health concern. A better understanding of these infections is therefore critical to ensure the long-term sustainability and growth of the betta fish industry.

Mycobacteria are aerobic, Gram-positive, acid-fast, and non-motile bacilli that cause disease in both humans and animals (Wolinsky, 1992). The genus *Mycobacterium* consists of over 200 known species and sub-species (Tortoli, 2019), which are classified into the *M. tuberculosis* complex and non-tuberculous mycobacteria (NTM). The NTM are characterized by their diverse phenotypes that differ in growth rate, colony pigmentation, environmental distribution, and pathogenic potential (Shinnick & Good, 1994). Classification of NTM, however, is commonly divided into slowly growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM) based on their ability to form visible colonies under optimal nutrient and temperature conditions (Runyon, 1959). The RGM are characterized as species that can form visible colonies in less than 7 days on selective media, while the SGM are defined as species that require more than 7 days to grow. In addition, a recent study by Gupta *et al.* (2018) proposed a revision of the taxonomy of mycobacteria in which 150 species of the genus *Mycobacterium* were redistributed into five new genera, namely *Mycobacterium, Mycobacteroides, Mycolicibacter, Mycolicibacterium,* and *Mycolicibacillus*. Validation of the updated nomenclature

was published in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) (Oren & Garrity, 2018), which serves as the official publication of the International Committee on Systematics of Prokaryotes. Regarding the updated nomenclature, the reference to NTM in recent publications may therefore cause confusion among non-mycobacteriologists. This taxonomy remains controversial; an editorial by Tortoli *et al.* (2019), however, acknowledged that both the new and former nomenclature systems can coexist and be used appropriately. It is therefore plausible that the nomenclature for NTM species (*i.e.*, *Mycobacterium* spp.) remains unaffected.

Mycobacteriosis is an important disease in fish caused by NTM members and is particularly common in intensive aquaculture facilities and aquaria settings (Gauthier & Rhodes, 2009). The disease has been observed with high frequency in tropical aquarium fish (Zanoni et al., 2008; Francis-Floyd, 2011; Puk et al., 2018; Phillips Savage et al., 2022), including *B. splendens* (Puttinaowarat et al., 2002; Beran et al., 2006; Sirimalaisuwan et al., 2017; Weerakhun et al., 2019; Narendrakumar et al., 2022). Generally, NTM infections in fish show variable, nonspecific clinical signs that often resemble other fish diseases (Francis-Floyd, 2011), with systemic granulomatosis emerging as a striking histopathological feature characterized by granulomatous lesions in multiple organs (Delghandi et al., 2020). Specifically in betta fish, mycobacterial infections often occur as chronic progressive infections typically characterized by external symptoms such as emaciation and grouped fins (Narendrakumar et al., 2022), big belly syndrome (Dong et al., 2018), and skin nodule syndrome (Dong et al., 2018). Despite the growing number of NTM species identified in aquatic ecosystems, three primary species (*i.e.*, *M. marinum, M. fortuitum,* and *M. chelonae*) remain the best-known pathogenic species affecting fish (Delghandi et al., 2020), while other documented species are still poorly characterized and understood. Thus, this study aimed to conduct a comprehensive investigation of the

phenotypic and biochemical characteristics as well as antibiotic and disinfectant susceptibility of RGM isolated from Siamese fighting fish (*Betta splendens*). Specifically, the study included six isolates of five RGM species: *Mycobacterium chelonae, M. cosmeticum, M. farcinogenes, M. mucogenicum,* and *M. senegalense*, also known as *Mycobacteroides chelonae, Mycolicibacterium cosmeticum, Mycolicibacterium farcinogenes, Mycolicibacterium mucogenicum,* and *Mycolicibacterium senegalense* in the new taxonomic schema of Gupta *et al.* (2018). The virulence and pathogenicity of these isolates towards their host species were also evaluated by experimentally infecting the fish by intraperitoneal injection. Insights obtained from this study are expected to enhance our understanding of these infections and contribute to the formulation of effective disease prevention and control strategies, to ultimately mitigate the negative impact of mycobacteriosis on the betta fish industry.

3.4. Materials and methods

3.4.1. Bacteria isolation and identification

A total of six RGM isolates were included in this study, obtained from naturally occurring betta fish displaying signs of either big belly syndrome (BBS) or skin nodule syndrome (SNS), as previously described by Dong *et al.* (2018). Of these, four isolates were collected in 2016 and the remaining two were collected in 2021 as part of the present study (see Table 3.1). Briefly, the bacteria were isolated from the nodules of SNS-affected fish by inserting a sterile 2-mm-diameter loop into the nodular incision, or from the fluid obtained from the abdominal cavity of BBS-affected fish, both of which were streaked onto Middlebrook 7H11 agar (HiMedia, India) containing 10% OADC growth supplement (*i.e.*, Oleic Albumin Dextrose Catalase), and incubated at 30°C. The plates were inspected daily for 7 days until colonies were observed. These colonies were examined for the presence of acid-fast

bacteria using an acid-fast stain (Ziehl-Neelsen stain). The acid-fast colonies were then selected for purification and species identification by 16S rRNA analysis.

Bacterial genomic DNA was extracted from individual colonies using the boiling method described by Dinh-Hung *et al.* (2023). A 16S rRNA PCR reaction of 25 µL was prepared consisting of 12.5 µL 2× Terra PCR Direct Polymerase Mix (Takara Bio, Japan), 0.5 µL each of 10 µM forward and reverse primers (Uni-Bact F: 5'-AGAGTTTGATCMTGGCTCAG-3', Uni-Bact-R: 5'-ACGGHTACCTTGTTACGACTT-3') (see Weisburg et al., 1991), 0.5 µL 1.25 U/µL Terra Taq enzyme (Takara Bio, Japan), 2 µL bacterial DNA, and distilled water. The PCR cycling conditions included a denaturation step at 98°C for 5 min, followed by 28 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 80 s, with a 5 min extension at 68°C. The expected size of the amplified product was approximately 1.5 kb. Following agarose gel electrophoresis, the amplified products were visualized using a gel documentation system (Aplegen Omega Fluor™, USA) and subsequently purified using the NucleoSpin Gel and PCR Clean-up Kit (Takara Bio, Japan). The purified DNA amplicons were then sequenced *via* a barcode-tagged sequencing technique (Celemics, Inc., South Korea). The obtained DNA sequences were subjected to BLAST-n query against the available nucleotide sequences in the GenBank database (www.ncbi.nlm.nih.gov) to determine taxonomic identification. The closest known relatives and several sequences from related *Mycobacterium* species were included in the phylogenetic analysis. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstraps based on multiple alignments generated using ClustalW in MEGA 11 version 11.0.11 (Tamura et al., 2021). The tree was rooted using the 16S rRNA sequences of *Mycobacterium tuberculosis* (GenBank accession no: MG995516).

3.4.2. Phenotypic and biochemical characteristics analysis

The isolated mycobacteria were phenotypically characterized using conventional phenotypic and biochemical tests. Characterization included colony morphology, growth at various temperatures, and pigment production on Middlebrook 7H11 agar plates containing 10% OADC, as well as some selected standard biochemical tests (*e.g.*, nitrate reduction, tellurite reduction, urease, citrate, and utilization of carbohydrate) according to Bhalla *et al.* (2018). The ultrastructure of the bacteria was examined using scanning electron microscopy (SEM). For this, a culture of the bacteria was incubated at 30°C with shaking at 250 rpm for 24-28 h in Middlebrook 7H9 broth (HiMedia, India) supplemented with 10% ADC (*i.e.*, Albumin Dextrose Catalase). Following this, the culture fluids (5 mL) were then concentrated by centrifugation at 5000 g for 5 min. The bacterial pellet was then resuspended in 0.5 mL of 1× phosphate-buffered saline (PBS), spread on 0.01% poly-L-lysine-coated coverslips (Sigma-Aldrich, USA), and allowed air-dry for 3 h. Afterward, the samples were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide followed by dehydration with a series of ethanol at various concentrations. The samples were then dried under CO_2 using a critical point dryer (Hitachi, Japan) and coated with a gold layer by sputtering. The ultrastructure of the bacteria was then examined and photographed under a scanning electron microscope (Hitachi, Japan).

3.4.3. Antibiotic and disinfectant susceptibility test

Antibiotic susceptibility of the isolates was determined using a disc diffusion method on Mueller-Hinton (MH) agar with commercially available discs (HiMedia, India) according to Clinical and Laboratory Standards Institute guidelines (CLSI) (see CLSI, 2020). A total of 18 antibiotics were utilized in this study, with their respective concentration (µg/disc) given in parentheses: amoxicillin (10), imipenem (10), azithromycin (15), clarithromycin (15), doxycycline (30), oxytetracycline (30),

minocycline (30), sulphamethoxazole/trimethoprim (25), trimethoprim (5), ofloxacin (5), enrofloxacin (5), levofloxacin (5), ciprofloxacin (5), moxifloxacin (5), sparfloxacin (5), amikacin (30), rifampicin (5), and isoniazid (10). Briefly, the isolates were incubated in Middlebrook 7H9 broth supplemented with 10% ADC at 30°C for 24-48 h with shaking at 250 rpm. The bacterial suspension was diluted to a turbidity equivalent to a 0.5 McFarland standard (*i.e.*, approximately 10^8 CFU/mL). The bacterial suspension was then aseptically applied to the entire surface of an MH agar plate using sterile swabs. Antibiotic discs were placed on the agar surface using sterile forceps, and the agar plates were then incubated at 30°C for 4 nights. The test was repeated twice to ensure its reliability. The diameter of the zone of inhibition (mm) was measured to establish the degree of antibiotic susceptibility. The results were interpreted as sensitive (≥18 mm), intermediate (13-17 mm), and resistant (<13 mm) following the guidelines of CLSI (2020). The multiple antibiotic resistance (MAR) index was determined for the isolates based on the antibiotic susceptibility test results according to the methodology proposed by Krumperman (1983). The index is calculated using the formula MAR = a/b, where “a” indicates the number of antibiotics to which the isolate was resistant and “b” represents the total number of antibiotics used for susceptibility testing of the isolates.

The antibacterial profile of a disinfectant was tested using the agar spreading method (see Sanders, 2012) to determine bacterial growth on the surface of agar plates. Five commercially available disinfectants (*i.e.*, ethanol, formalin, chlorine, povidone-iodine, and potassium permanganate) were used at various selected concentrations. The bacterial suspensions were prepared in the same way as in the antibiotic susceptibility test. The disinfectants were diluted with sterile distilled water to obtain the solution with the desired concentration. Exposure was performed by diluting the bacterial suspensions 100-fold with the selected disinfectant solution, ultimately resulting in a final concentration of approximately 10^6 CFU/mL. The

disinfectant was allowed to act for a specified time, *i.e.,* 1 min, 5 min, 1 h, and 24 h. The exposed bacterial suspensions were then spread on Middlebrook 7H11 agar plates containing 10% OADC, which were incubated at 30°C for 4 nights to allow the growth of surviving bacteria. The number of bacterial colonies growing on the agar was counted and compared with the number of colonies on the control plates streaked with a bacterial suspension of approximately 10^6 CFU/mL without disinfectant exposure. To ensure reliability, the test was performed on two separate occasions. The results were reported as total inhibition if no colonies grew on the agar plate.

3.4.4. Pathogenicity challenge

To assess the virulence and pathogenicity of the tested isolates, a cohort of 220 adult betta fish (4-5 months old) was obtained from a local commercial fish farm. Each fish was individually housed in a 5 × 5 × 15 cm aquaria and acclimated for one week prior to the experiment. Opaque white plastic dividers were placed between the aquaria to prevent aggressive behavior among the fish. The fish were fed a commercial betta pellet diet containing 28% protein (Optimum, Thailand) three times per week. The water in each aquaria was exchanged by 50% every week. A representative subsample of the fish population was subjected to thorough clinical, bacteriological, and molecular analysis according to Weerakhun *et al.* (2019) to confirm the absence of mycobacterial infections.

The isolates were cultured in Middlebrook 7H9 broth supplemented with 10% ADC and incubated for 24-48 h at 30°C with constant shaking at 250 rpm. The bacterial suspension was pelleted by centrifugation at 5000 g for 5 min. The pellet was washed twice and resuspended with 1× PBS to achieve an OD_{600} of 1.0, which corresponds to a concentration of approximately 10^8 CFU/mL, as determined by conventional plate counting. The experimental design comprised 18 groups, each

consisting of 10 fish. The fish in each group were injected intraperitoneally with 0.1 mL of a bacterial suspension of one of the six isolates to achieve a final concentration of approximately 10^7, 10^6, or 10^5 CFU/fish. Two control groups were also performed, one of which received an intraperitoneal injection of 0.1 mL of 1× PBS per fish, while the other was not injected. The lethal dose (LD_{50}) of all experimental groups was calculated on the 7^{th} day of the challenge according to Reede & Muench (1938). The clinical signs and mortalities of the fish in each group were recorded daily until 6 weeks post-injection (wpi). Bacterial re-isolation was carried out on representative fish from all groups. The experimental procedures and use of animals were approved by the Institutional Animal Care and Use Committee of Kasetsart University (approval ID: ACKU65-FIS-001).

3.4.5. Histopathological examination

At least two control fish (pre- and post-challenge) and three challenged fish were collected from each group. The infected fish were divided into two categories: moribund, those that became moribund within one-week post-infection (1 wpi); and survivor, those that were alive at the end of the study (*i.e.*, 6 wpi). Whole fish were fixed in 10% neutral buffered formalin and routinely processed for histology using standard procedures. The collected specimens were embedded in paraffin, sectioned into 5 µm thickness, and stained with hematoxylin and eosin (H&E). The histopathological scoring system was adapted from the previous study (Reimschuessel et al., 1992) and graded the severity of tissue damage as normal (0), mild (1), moderate (2), or severe (3). Lesions were scored according to both the number and extent of affected areas. Mild lesions were defined as those present in small numbers and have minimal effect on the organ, whereas severe lesions were characterized by extensive tissue damage that barely recognized normal tissue. Moderate lesions were classified as falling between these two extremes.

Histological sections were also subjected to acid-fast staining according to the established protocols of Dong *et al.* (2018), with slight modifications. This procedure allowed visualization and characterization of the acid-fast bacteria in the histological sections. In brief, the deparaffinized sections were immersed in a 60°C carbon-fuchsin solution for 1 h. The slides were then rinsed under running tap water and decolorized with 1% acidic alcohol for 30 s to 1 min. To facilitate contrast and differentiation of the stained bacteria, the slides were counterstained with a methylene blue solution for 30 s. The slides were then rinsed again with tap water, dried, coverslipped, examined, and photographed under a digital light microscope (Olympus, Japan).

3.4.6. Statistic test

Data on cumulative mortality and histopathological scores of fish infected with different isolates within the dose were statistically compared using the SPSS program (SPSS Inc., USA). Significant differences were determined using a one-way ANOVA followed by Duncan's new multiple-range test. A *p*-value of less than 0.05 was considered significant.

3.5. Results

3.5.1. Data of the bacterial isolates

Bacteria, positive for acid-fast staining, were successfully recovered from the nodular incision of the external skin nodules and fluid from the distended abdomen of naturally infected betta fish (see Figure 3.1). Six isolates were molecularly identified by 16S rRNA sequencing and used for further *in vitro* studies. Based on BLAST-n analysis in the GenBank database, the isolates were assigned to five different *Mycobacterium* species, including *Mycobacteroides chelonae, Mycolicibacterium cosmeticum, Mycolicibacterium farcinogenes, Mycolicibacterium mucogenicum,* and *Mycolicibacterium senegalense.* Further confirmation was provided by the

relationship of the isolates from this study to other recognized *Mycobacterium* species by phylogenetic tree analysis (Figure 3.2). Details of the isolates used in this study are given in Table 3.1.

3.5.2. Phenotypic and biochemical characteristics

Colony morphology. Colonies cultured on Middlebrook 7H11 agar plates containing 10% OADC and incubated for 4 nights at 30°C showed different morphologies depending on *Mycobacterium* species (see Figure 3.3 and Table 3.2). Colonies of *M. chelonae* were rough, white, irregular, dry, and averaged 2-4 mm in diameter. In contrast, the isolates of *M. cosmeticum* formed smooth, round colonies less than 2 mm in diameter. These colonies resembled those of *M. farcinogenes* and *M. senegalense*; however, they differed in that they produced a distinct yellow pigment while the other isolates remained white. The isolate of *M. mucogenicum* was easily recognized by its elongated, smooth, opaque white, and moist colonies.

Ultrastructural morphology. SEM results confirmed that all isolates had rod-shaped morphology with distinguishable cell structures among *Mycobacterium* species (see Figure 3.4). The length of the individual bacterial cells could serve as a distinguishing feature between these species. *Mycobacteroides chelonae* was the longest with a length of approximately 4-6 µm, while *M. cosmeticum* was the shortest with a length of approximately 2-4 µm; both species had a diameter of approximately 1.0-1.2 µm. *Mycolicibacterium farcinogenes*, *M. mucogenicum*, and *M. senegalense* had similar lengths of approximately 3-5 µm and smaller diameters (*i.e.*, <0.8 µm) compared with *M. chelonae* and *M. cosmeticum.*

Biochemical characteristics. Biochemical tests performed on the isolates show some similarities, including the ability to grow at both 30°C and 37°C, positive nitrate reduction, and utilization of some carbohydrates. Differences in biochemical

profiles, however, were observed in nitrate reduction and urease activity, as shown in Table 3.2.

3.5.3. Antibiotic and disinfectant susceptibility patterns

Antibiotic resistance profiles. The results of the susceptibility tests for the 18 antibiotics performed on the isolates are shown in Table 3.3. Among the isolates, uniform susceptibility to clarithromycin, levofloxacin, and ciprofloxacin was observed, while they all showed resistance to rifampicin. Resistance rates for each antibiotic were as follows: Rifampicin (100%), isoniazid and sulfamethoxazole/trimethoprim (83.3%), trimethoprim (67.7%), amoxicillin (50%), and 16.7% for the other remaining antimicrobials except clarithromycin, levofloxacin, and ciprofloxacin. Of note, all of the isolates exhibited multiple antibiotic resistance (*i.e.*, they were all resistant to at least 4 antibiotics). The MAR indexes of the studied isolates had values ranging from 0.22 to 0.61, as indicated in Table 3.4. Of these, *M. chelonae* had the highest MAR index and was resistant to 11 of the 18 antibiotics examined (*i.e.,* to seven of the nine tested antibiotic classes).

Disinfectant susceptibility. The inhibitory effect of the disinfectants on the isolates was evaluated by observing bacterial colony growth on selective agar plates after treatment (see Table S3.1). The results show that ethanol (either 70 or 95%), chlorine (both 1 and 2%), povidone-iodine (0.06 and 0.6%), and formalin (5%) exhibited strong inhibition against all the bacteria investigated in this study. No colonies were observed on plates spreading the bacteria treated with these disinfectants for periods ranging from 1 min to 24 h, whereas control plates had innumerable colonies. In contrast, formalin (1%) and potassium permanganate (at concentrations of 10 and 30 ppm) showed limited antibacterial activity against the isolates, as evidenced by the presence of more than 300 colonies of certain isolates on the agar plates even after an exposure period of 24 h (Table S3.1). The number of

isolates that were completely inhibited by the disinfectants tested (*i.e.*, no bacterial growth) is shown in Table 3.5.

3.5.4. Virulence properties

Clinical signs and virulence. The challenge resulted in either acute or chronic disease, depending on the dose of bacteria administered and the isolates involved. Within 24 hours of infection, fish showed ulceration and fin rot at the injection site (Figure 3.5A), which were frequently observed (*i.e.*, more than 50%) in all the experimental fish groups regardless of isolate or dose used. During the acute phase of the disease, mortality was observed between 2 and 5 days after infection. Affected fish showed characteristic symptoms, such as abdominal distension (Figure 3.5B) with severe necrosis of visceral organs at necropsy (Figure 3.5C). All the tested isolates, at both injected low or high doses, resulted in a significant proportion (*i.e.*, over 60%) of fish were affected by chronic diseases leading to emaciation, reduced vitality, and aesthetic impairment (Figure 3.5D). Only a small number of fish exhibited clinical signs of big belly syndrome (Figure 3.5E) with the presence of ascites fluid during necropsy. The cumulative mortality data over the experimental period (*i.e.*, 42 days) are shown graphically in Figure 3.6, and the calculated LD_{50} values are provided in Table 3.6. The LD_{50} values determined within 7 days indicated that *M. chelonae* exhibited the highest virulence at 7.12×10^5 CFU/fish, followed by *M. mucogenicum* at 4.25×10^6 CFU/fish, and a range of $1.01\text{-}1.65\times10^7$ CFU/fish for the remaining isolates. No mortality was observed in the control groups, which included fish injected with PBS and those that did not receive an injection.

Histopathological findings. As the histopathological lesions caused by the different isolates appear to follow a similar pattern, a scoring system was used to evaluate the histopathology caused by each isolate, as shown in Figure S3.1 and Table 3.6. Histopathological examination revealed that the fish exhibited severe

peritonitis and tissue necrosis due to the acute disease (Figure 3.7B). Upon completion of the experiment (*i.e.,* 6 wpi), the surviving fish exhibited a remarkable histopathological feature of progressive systemic granuloma formation within their visceral organs. Early-stage granuloma formation was observed in the liver, as shown in Figure 3.7C. A considerable portion of the spleen and kidney tissues were occupied by granulomas that developed in numerous fish (Figure 3.7D, E). Multiple granulomas were frequently found in the parenchyma of the peritoneum (Figure 3.7F). At high magnification, the granulomas exhibited diverse features, such as onion-ring-shaped granuloma with epithelioid macrophages surrounding a necrotic center (Figure 3.7G), granuloma formation with foamy macrophages (Figure 3.7H), and caseous granuloma (Figure 3.7I). The acid-fast stained section confirmed the presence of numerous rod-shaped bacterial cells within the granuloma (Figure 3.7K).

3.6. Discussion

3.6.1. Phenotypic characteristics remain useful to distinguish NTM

NTM infection is a common chronic disease in aquatic animals, including aquarium fish, and is manifested by non-specific clinical signs such as emaciation, scale loss, reproductive failure, abdominal dropsy, and skin lesions (Francis-Floyd, 2011). These signs are similar due to causation by different NTM species and may overlap with other fish diseases, complicating diagnosis and treatment. Identification of the causative NTM species is therefore critical for selecting effective treatment options and control strategies (*e.g.*, appropriate antibiotics selection) (Brown-Elliott et al., 2012; Brown-Elliott & Philley, 2017). The results of this study show that phenotypic and biochemical characteristics can be a helpful approach for preliminary detection and identification of NTM species. For example, *Mycobacterium shottsii* and *Mycobacterium pseudoshottsii* prefer a lower temperature of 25°C (Gupta et al., 2013), whereas the five species studied here thrive at 30°C or even 37°C. In addition,

M. cosmeticum produces pigments and forms smooth colonies, while *M. chelonae* forms rough and unpigmented colonies. On the other hand, *M. farcinogenes* and *M. senegalense* have similar colonial morphology, they differ in ultrastructural morphology and urease activity, which is also consistent with a previous study (Hamid, 2014). Although molecular techniques allow accurate and rapid detection and identification of bacterial isolates, they are challenging due to their complexity and associated sequencing costs. For many years, taxonomic and phylogenetic studies of mycobacterial species have been based mainly on targeted genes such as 16S rRNA, *hsp65*, and *rpoB* (Tortoli, 2012). The use of a single or a limited number of target genes for diagnosis and taxonomy within the genus *Mycobacterium*, however, has its limitations. These lie primarily in the lack of sequence diversity, which can lead to potential misidentification risks to discriminate mycobacteria species (Nolte et al., 2005; Kim et al., 2019). To overcome these challenges, whole-genome sequencing and next-generation sequencing have emerged as the gold standard for precise identification within the genus *Mycobacterium* (Dohál et al., 2021; Khieu et al., 2021). In this study, the 16S rRNA gene was used for identification because it is widely used and its sequence is currently available in public databases for all known mycobacterial species (Tortoli, 2019). Genomic analysis also may not always be feasible in resource-limited laboratories due to inadequate infrastructure, trained personnel, and access to molecular methods. Many laboratories, therefore, use a combination of conventional and genomic methods to improve mycobacterial identification whenever possible. This study demonstrates that phenotypic methods are important and continue to be useful and can serve as a reliable guide for the preliminary identification of specific mycobacteria.

3.6.2. Concern of multiple antibiotic resistance of NTM from betta

There is currently no widely available treatment for mycobacteriosis in fish, although antibiotic therapy has been considered as a possible option (Delghandi et

al., 2020). Previous studies have shown that treatment of mycobacterial infections in fish usually involves the administration of antibiotics such as rifampicin, streptomycin, erythromycin, ethambutol, isoniazid, doxycycline, kanamycin, ethionamide, minocycline, and tetracycline, which have varying degrees of efficacy (Kawakami & Kusuda, 1990; Lewis & Chinabut, 2011). Kawakami and Kusuda (1990) found that rifampicin, streptomycin, and erythromycin were relatively effective against unidentified *Mycobacterium* spp. in yellowtail (*Seriola quinqueradiata*). In addition, ethambutol, isoniazid, and/or rifampicin have occasionally been used to treat mycobacteriosis in valuable aquarium animals (Lewis & Chinabut, 2011). The results of the present study show that clarithromycin, levofloxacin, and ciprofloxacin were effective against all isolates, which is consistent with a previous study on the same species isolated from human clinical specimens (Brown-Elliott et al., 2012). Antibiotic susceptibility testing of isolates of piscine mycobacteria, however, is rarely performed, and resistance appears to be highly dependent on the infecting species and strains. The present study shows for the first time the alarming extent of antibiotic resistance in NTM species isolated from betta fish, with *M. chelonae* being of particular concern. It is also essential to underscore that each isolate showed resistance to multiple antibiotics, indicating the increase in MAR strains of NTM species. This finding is particularly concerning, as MAR mycobacteria are often associated with unsuccessful treatments and could pose a significant public health risk (Brown-Elliott et al., 2012). Bacteria with a MAR index value of 0.2 or higher are thought to originate from high-risk sources of bacterial contamination where antimicrobials are frequently used (Krumperman, 1983). The higher than average MAR index values (*i.e.*, ≥ 0.22) were observed for *Mycobacterium* spp. isolates from betta fish may imply misuse of antimicrobials in the betta fish industry. All of these findings underscore the need for increased monitoring and regulation of antimicrobial use in

the betta fish industry and other aquaculture sectors to curb the emergence and spread of antimicrobial resistance in bacterial pathogens.

3.6.3. Several common disinfectants may reduce the risk of mycobacterial infection in fish and the zoonotic potential

The alarming results on antibiotic resistance patterns of NTM isolated in this study also highlight the importance of proactive prophylaxis of disease spread, which would be greater beneficial and effective than therapy after disease onset. Once fish populations are affected by mycobacteriosis, the recommended actions are often to euthanize the entire affected group, followed by thoroughly cleaning and disinfecting all contact areas (Francis-Floyd, 2011). The use of disinfectants in betta fish facilities, therefore, is an important strategy to control mycobacteriosis that should be taken. This study has demonstrated the antibacterial efficacy of various disinfectants and provided valuable data for formulating potentially optimal disinfection procedures in betta fish farming and other areas where mycobacterial infections may pose a threat. Despite these promising *in vitro* results, further studies need to be conducted to evaluate the safety and efficacy of disinfectants in fish farming, taking into account aspects such as active ingredients, concentration, exposure duration, and application methods. Choosing an appropriate disinfectant in fish farms also requires careful consideration of its benefits and drawbacks. For example, ethanol may be recommended because of its potential environmental friendliness and lower harmfulness. Exposure to povidone-iodine, however, could alter the immune response and microbiota of the gills and skin of koi carp, *Cyprinus carpio* (see Zhang et al., 2023). Formalin, a disinfectant commonly used in aquaculture, could negatively affect fish and water quality (Leal et al., 2018) and possibly pose a carcinogenic risk to humans (Duong et al., 2011). In addition, chlorine compounds are known to be highly corrosive and toxic (Jørgensen et al., 2009).

Moreover, it is important to emphasize that all NTM species isolated in this study have zoonotic potential. While *M. chelonae* (along with *M. marinum* and *M. fortuitum*) is commonly found in fish (Delghandi et al., 2020), isolates of *M. cosmeticum, M. farcinogenes, M. mucogenicum,* and *M. senegalense* were detected in betta fish in our study. All of these isolates are associated with infections in humans and have been detected in various water sources, including natural water, tap water, and wastewater (Le Dantec et al., 2002; Dowdell et al., 2019; Falkinham III, 2021). For instance, these isolates have been detected in various opportunistic infections in humans, such as *M. chelonae* in soft tissue infections (Hay, 2009; Gonzalez-Diaz et al., 2018), *M. cosmeticum* in infections associated with beauty and nail salons (Cooksey et al., 2004), *M. farcinogenes* in skin infections (Cheng & Lee, 2022), *M. mucogenicum* as a possible source of nosocomial infections (Fernandez-Rendon et al., 2012), and *M. senegalense* in osteomyelitis of the distal tibia (Maupin et al., 2019). Thus, betta keepers and aquarists are at increased risk of mycobacterial infection through direct contact with infected fish or contaminated water. Activities such as handling infected specimens or cleaning contaminated tanks may increase the risk of mycobacterial exposure. Hence, implementing strict disinfection procedures is of utmost importance to reduce the risk of mycobacterial infection. Our findings are therefore critical not only for developing effective proactive strategies to control mycobacteriosis in the interest of a sustainable betta industry but also for advancing the One Health approach.

3.6.4. Five NTM species are pathogenic to betta fish

Despite previous studies that have investigated the pathogenicity of mycobacteria in various fish species, this study is, to our knowledge, the first time that Koch's postulates have been fulfilled for *Mycobacterium* spp. isolated from betta fish. Over 7 days after intraperitoneal injection, the LD_{50} values showed that *M. chelonae* had the highest virulence, followed by *M. mucogenicum* with respective

values of 7.12×10^5 and 4.26×10^6 CFU/fish. The remaining isolates (*i.e.*, *M. cosmeticum, M. farcinogenes,* and *M. senegalense*) exhibited lower virulence, requiring $1.02\text{-}1.63\times10^7$ CFU/fish to kill 50% of the experimental fish population. Artificial challenges have previously documented differential mortality rates in different fish species following intraperitoneal injection with *Mycobacterium* species. For example, Arakawa and Frye (1984) found that rainbow trout (*Oncorhynchus mykiss*) had a mortality rate of 20-52% in 80 days and juvenile Chinook salmon (*Oncorhynchus tshawytscha*) had a mortality rate of 98% in only 10 days when infected with *M. chelonae* at a dose of 1.0×10^7 CFU/fish. In another study, Talaat et al (1999) reported a mortality rate of 10.5-40% in goldfish (*Carassius auratus*) infected with *Mycobacterium smegmatis* at a dose of $10^7\text{-}10^8$ CFU/fish over 8 weeks. In addition, Fukano *et al.* (2015) observed a significant 95% mortality rate within three weeks in thread-sail filefish (*Stephanolepis cirrhifer*) infected with *Mycobacterium stephanolepidis* at 9.0×10^4 CFU/fish. Weerakhun *et al.* (2019) found high mortality rates (*i.e.*, 72-100%) in goldfish (*Carassius auratus*) infected with *M. marinum* and *M. fortuitum* isolates, even at low or high doses (*i.e.*, 10^2 or 10^4 CFU/fish) over 210 days. Recently, Machida *et al.* (2021) documented a cumulative mortality rate of 30-50% after 100 days post-infection in cultured koi carp (*Cyprinus carpio*) infected with *Mycobacterium paragordonae* and two other *Mycolicibacterium* spp. at doses of 2.0×10^6 to 3.0×10^8 CFU/fish. All this indicates that the pathogenicity of mycobacteria in fish is variable. Not only the causative pathogens but also the infected fish host and further environmental factors may influence the virulence properties of piscine mycobacteria. It should also be emphasized that the termination of our experiment on day 42 may not have provided sufficient time to observe additional deaths or symptoms (*e.g.*, skin nodules) that might be associated with chronic progressive disease.

The histological findings confirmed that all the isolates caused abnormal changes in fish tissues characterized by granulomatous lesions. Granulomatous lesions were observed even in acute disease (*i.e.*, within 7 days of infection), indicating a relatively high level of pathogen-host interaction. Chronic disease is more clearly characterized by progressive systemic granuloma formation in the visceral organs. Granulomas with various histopathological features (*e.g.*, necrotizing, non-necrotizing, and caseous) were observed, consistent with those found in other fish species affected by mycobacteriosis (Talaat et al., 1998; Talaat et al., 1999; Dong et al., 2018). Further evidence of the etiologic involvement of mycobacteria in these lesions was the detection of acid-fast bacilli and the successful re-isolation of these same bacteria from the affected tissues. In addition to the high mortality rate during acute infection, chronically infected fish frequently showed symptoms of emaciation, decreased vitality, and esthetic impairment. Taken together, these findings demonstrated that these five NTM species are pathogenic to betta fish, potentially posing a challenge to the long-term sustainability of the betta fish industry.

3.6.5. Potential economic impact on the betta fish industry

Unfortunately, there are currently no vaccines or effective treatments for mycobacterial infections in betta fish. Once clinical signs of infection become apparent, destruction of the affected stock and subsequent disinfection of the environment is the only way to control the spread of the disease. Thus, the infections can cause significant economic losses in the betta industry, as the disease can significantly affect production and trade. It was estimated that in a commercial betta farm with up to 100,000 fish per harvest, about 1% suffer from these clinical conditions, resulting in reduced saleability. For instance, for a high-quality individual fish priced at $10-20, the total loss per harvest per farm would be $10,000-20,000 (Dong et al., 2018). Considering that many farms export 3-4 harvests of fish per year, the total loss would be substantial, especially for high-priced fish. The situation

becomes even more critical when it must be assumed that infected brood-stock can transmit infections to their offspring. Production of infected fish is economically detrimental because fish from infected parents are likely to exhibit stunted growth, die chronically, and pose a further threat to other fish, betta keepers, pet store employees, and ultimately aquarists.

3.7. Tables and figures

Table 3.1. Details of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*) used in this study.

Isolates	Year isolation	Sources	Identification	GenBank accession number (16S rRNA gene)	% best match identity (GenBank database)
M. chelonae BN 1983	2016	(Dong et al., 2018)	*Mycobacteroides chelonae*	MG438529	99.53
M. cosmeticum BN 1984	2016	(Dong et al., 2018)	*Mycolicibacterium cosmeticum*	MG438530	99.46
M. cosmeticum N041	2021	This study	*Mycolicibacterium cosmeticum*	ON684603	99.71
M. farcinogenes SNSK5	2021	This study	*Mycolicibacterium farcinogenes*	ON684376	100.0
M. mucogenicum BN1956	2016	(Dong et al., 2018)	*Mycolicibacterium mucogenicum*	MG438542	100.0
M. senegalense BN1985	2016	(Dong et al., 2018)	*Mycolicibacterium senegalense*	MG438531	99.59

Table 3.2. Phenotypic and biochemical characteristics of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*).

Characteristics		Isolates					
		M. chelonae BN 1983	*M. cosmeticum* BN 1984	*M. cosmeticum* N041	*M. farcinogenes* SNSK5	*M. mucogenicum* BN 1956	*M. senegalense* BN 1985
Colony	Surface	Rough, irregular, dry	Smooth, round, moist	Smooth, round, moist	Smooth, round, moist	Smooth, elongated, moist	Smooth, round, moist
	Pigmentation	- White	+ Yellow	+ Yellow	- White	- Opaque white	- White
Bacilli	Length (µm)	4 - 6	2 - 4	2 - 4	3 - 5	3 - 5	3 - 5
	Diameter (µm)	1.0 - 1.2	1.0 - 1.2	< 0.8	< 0.8	< 0.8	< 0.8
Growth at 30°C (after 4-5 days)		+	+	+	+	+	+
Growth at 37°C (after 6-7 days)		+	+	+	+	+	+
Nitrate reduction		-	-	-	-	+	-
Tellurite reduction		+	+	+	+	+	+
Urease		+	+	+	-	-	+
Utilization of:	Citrate	-	-	-	-	-	-
	Glucose	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+

Table 3.3. Antibiotic susceptibility patterns of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*).

Antimicrobial classes	Antimicrobial agents	Concentration (µg)	Number of isolates resistant to antibiotics (%)			Resistance profile					
			S	I	R	*M. chelonae* BN 1983	*M. cosmeticum* BN 1984	*M. cosmeticum* N041	*M. farcinogenes* SNSK5	*M. mucogenicum* BN 1956	*M. senegalense* BN 1985
1. Penicillins (PNs)	Amoxicillin (AML)	10	3 (50)	0 (0)	3 (50)	R	S	S	R	S	R
2. B-Lactam/ß-Lactamase inhibitor combination (BL/BLI)	Imipenem (IPM)	10	5 (83.3)	0 (0)	1 (16.7)	R	S	S	S	S	S
3. Macrolides (MCs)	Azithromycin (AZM)	15	5 (83.3)	0 (0)	1 (16.7)	R	S	S	S	S	S
	Clarithromycin (CLR)	15	6 (100)	0 (0)	0 (0)	S	S	S	S	S	S
4. Tetracyclines (TCs)	Doxycycline (DO)	30	5 (83.3)	0 (0)	1 (16.7)	S	S	S	S	R	S
	Oxytetracycline (OT)	30	4 (66.7)	1 (16.7)	1 (16.7)	I	S	S	S	R	S
	Minocycline (MI)	30	5 (83.3)	0 (0)	1 (16.7)	S	S	S	S	R	S

5. Sulfonamides (SULs) (Folate pathway inhibitors)	Sulphamethoxazole /Trimethoprim (SXT)	25	1 (16.7)	0 (0)	5 (83.3)	R	R	R	R	S	R
	Trimethoprim (TR)	5	2 (33.3)	0 (0)	4 (66.7)	R	R	R	R	S	S
6. Fluoroquinolones (FQNs)	Ofloxacin (OF)	5	5 (83.3)	0 (0)	1 (16.7)	R	S	S	S	S	S
	Enrofloxacin (ENR)	5	5 (83.3)	0 (0)	1 (16.7)	R	S	S	S	S	S
	Levofloxacin (LE)	5	6 (100)	0 (0)	0 (0)	S	S	S	S	S	S
	Ciprofloxacin (CIP)	5	6 (100)	0 (0)	0 (0)	S	S	S	S	S	S
	Moxifloxacin (MO)	5	5 (83.3)	0 (0)	1 (16.7)	R	S	S	S	S	S
	Sparfloxacin (SPX)	5	5 (83.3)	0 (0)	1 (16.7)	R	S	S	S	S	S
7. Aminoglycosides (AMGs)	Amikacin (AK)	30	5 (83.3)	1 (16.7)	0 (0)	I	S	S	S	S	S
8. Rifamycin	Rifampicin (RIF)	5	0 (0)	0 (0)	6 (100)	R	R	R	R	R	R
9. Hydrazide derivatives	Isoniazid (INH)	10	1 (16.7)	0 (0)	5 (83.3)	R	R	R	S	R	R
Summary						R (11) I (2) S (5)	R (4) I (0) S (14)	R (4) I (0) S (14)	R (4) I (0) S (14)	R (5) I (0) S (13)	R (4) I (0) S (14)

R, resistant; S, sensitive; I, intermediate.

Table 3.4. Multiple antibiotic resistance index of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*).

Number of antibiotic resistant (resistance pattern)*	Isolates	MAR index
4 (SXT+RIF+TR+INH)	*M. cosmeticum* BN 1984 and *M. cosmeticum* N041	0.22
4 (SXT+RIF+ TR +AML)	*M. farcinogenes* SNSK5	0.22
4 (SXT+RIF+AML+INH)	*M. senegalense* BN 1985	0.22
5 (OT+RIF+MI+DO+INH)	*M. mucogenicum* BN 1956	0.28
11 (ENR+SXT+AML+RIF+OF+IPM+AZM+MO+SPX+TR+INH)	*M. chelonae* BN 1983	0.61

*The abbreviations used are listed in Table 3.3.

Table 3.5. Number of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*) were completely inhibited (*i.e.*, no colonies grew on the agar plate) by the tested disinfectants.

Disinfectant agents		Exposure time			
		1 min	5 min	1 hour	24 hour
Ethanol	0%	6/6	6/6	6/6	6/6
	95%	6/6	6/6	6/6	6/6
Formalin	1%	0/6	0/6	5/6 (except *M. mucogenicum* BN 1956)	6/6
	5%	6/6	6/6	6/6	6/6
Chlorine	1%	6/6	6/6	6/6	6/6
	2%	6/6	6/6	6/6	6/6
Povidone-iodine	0.6%	6/6	6/6	6/6	6/6
	0.06%	6/6	6/6	6/6	6/6
Potassium permanganate	10 ppm	0/6	0/6	0/6	5/6 (except *M. farcinogenes* SNSK5)
	30 ppm	0/6	0/6	2/6 (only M. *cosmeticum* BN 1984 and N041)	5/6 (except M. *farcinogenes* SNSK5)

Table 3.6. Results of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*) induced pathogenicity in experimentally challenged by intraperitoneal injection evaluated by LD_{50} and histopathological score.

Isolates	Bacterial suspension stock* (CFU/mL)	LD_{50} (CFU/fish)	Histology score** (number positive***)			
			Moribund (1 wpi)	Survivor (6 wpi)		
			10^7	10^7	10^6	10^5
M. chelonae BN 1983	2.25×10^8	7.12×10^5	2.33^{a} (3/3)	n/a	3^{a} (3/3)	3^{a} (3/3)
M. cosmeticum BN 1984	2.55×10^8	1.01×10^7	1^{bc} (0/3)	1.33^{c} (3/3)	1.33^{c} (3/3)	1.33^{cd} (1/3)
M. cosmeticum N041	2.71×10^8	1.43×10^7	1^{bc} (0/3)	2.33^{b} (3/3)	2^{b} (3/3)	1.33^{cd} (3/3)
M. farcinogenes SNSK5	2.52×10^8	1.65×10^7	2^{a} (0/3)	3^{a} (3/3)	3^{a} (3/3)	2.67^{ab} (3/3)
M. mucogenicum BN 1956	2.45×10^8	4.25×10^6	1.67^{ab} (0/3)	3^{a} (3/3)	2.67^{a} (3/3)	2^{bc} (3/3)
M. senegalense BN 1985	2.65×10^8	1.05×10^7	0.33^{c} (0/3)	1^{c} (1/3)	1^{c} (1/3)	1^{d} (1/3)

*The bacterial suspension stock indicates the concentration of bacteria in the stock suspension at a turbidity equivalent of OD_{600} = 1.0; however, fish were injected intraperitoneally with either 0.1 mL of the bacterial suspension stock or with 10-fold dilutions to achieve a final concentration of approximately 10^7, 10^6, or 10^5 CFU/fish.

**Histopathological scores were classified as (0) normal, mild (1), moderate (2), and severe (3), as shown in an example from Figure S3.1. Scores were calculated as the mean of three fish examined and statistically analyzed using a one-way ANOVA followed by Duncan's new multiple-range test. Significant differences between isolates within the dose are indicated by superscript alphabets (p-value <0.05). See text for additional details.

***Number of fish with granulomas in the histological specimen per the total number of fish examined. n/a, not applicable; wpi, weeks post-infection.

Figure 3.1. *Mycobacterium* spp. were recovered from the nodular incision of the external skin nodules (A) and fluid present in the distended abdomen (B) of naturally infected Siamese fighting fish (*Betta splendens*) (see inset boxes).

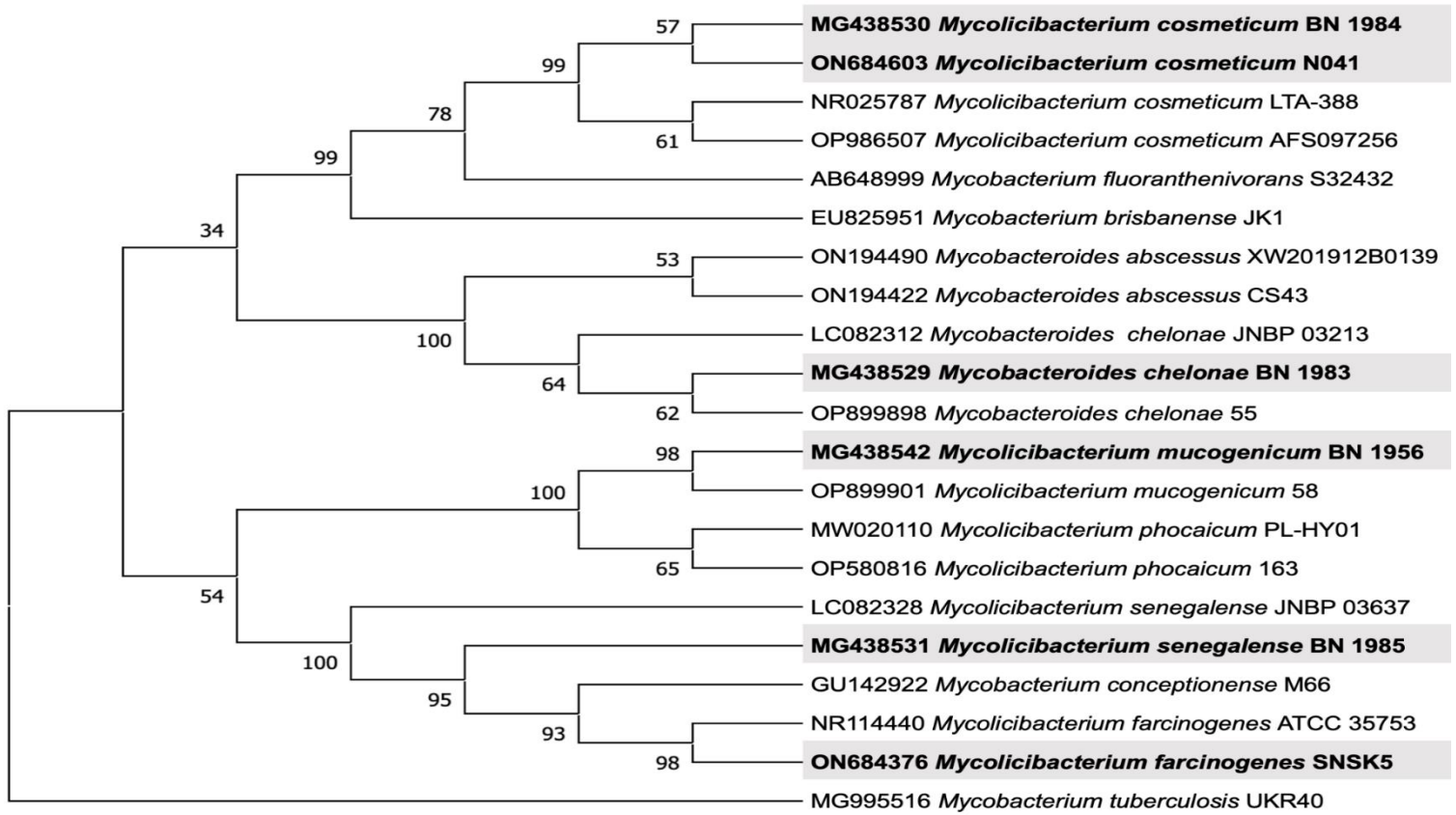

Figure 3.2. A phylogenetic tree was constructed based on the 16S rRNA sequence (~1.5 kb) of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*) (see highlights) and other closely related *Mycobacterium* species. The tree was constructed using the neighbor-joining method, with bootstrap values calculated from 1000 replications. The number at the node of the tree indicates the bootstrap value in percent. The sequences of *M. tuberculosis* were used as an outgroup.

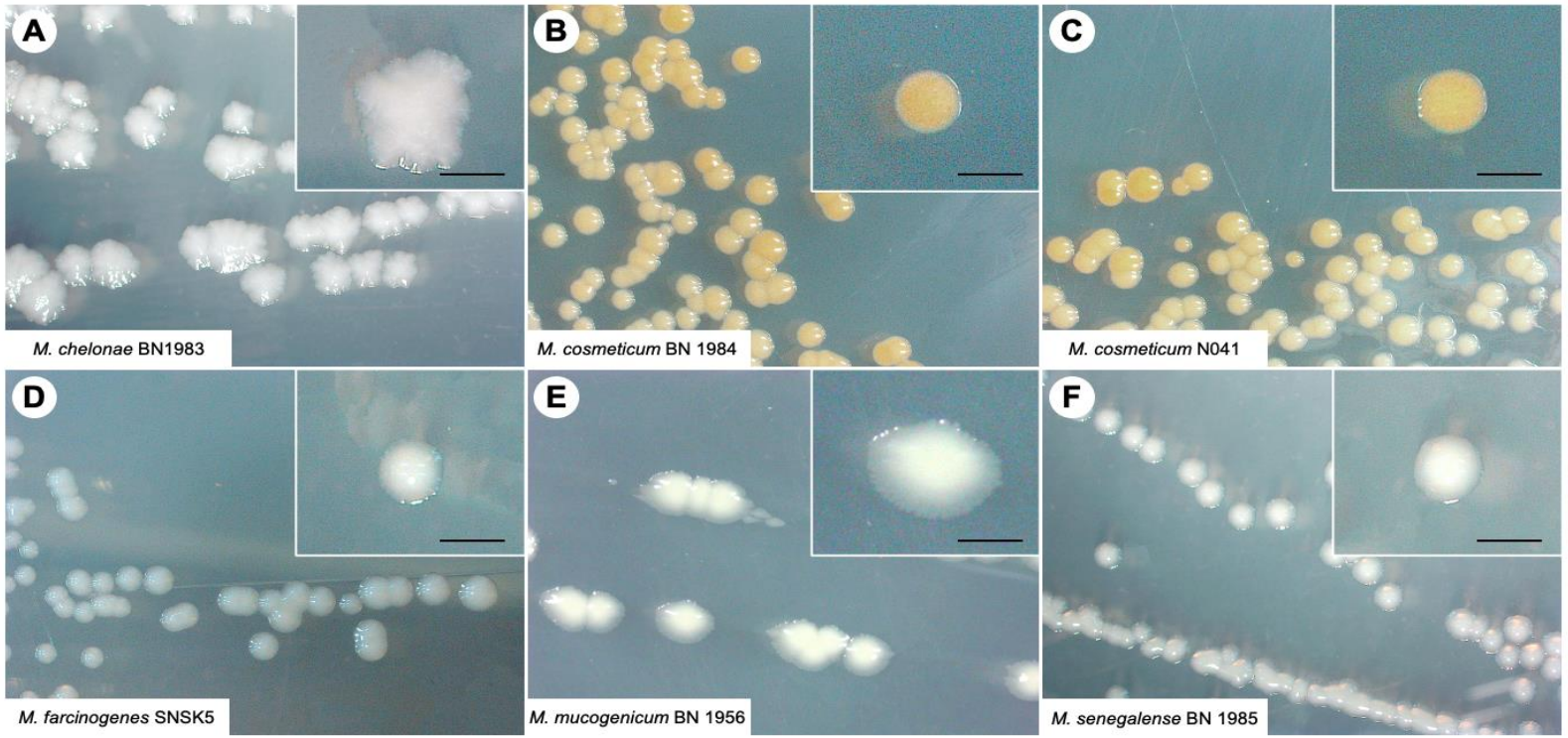

Figure 3.3. Colony characteristics of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*) were grown on Middlebrook 7H11 agar with 10% OADC enrichment after incubation at 30°C for 4 nights. *Mycobacteroides chelonae* with rough, white, irregular, and dry colonies (A). Two isolates of *M. cosmeticum* (B and C) had the same smooth-round morphology as the isolate of *M. farcinogenes* (D) and *M. senegalense* (F), but differed in yellow pigmentation, while the others remained white. *Mycolicibacterium mucogenicum* isolates were easily recognized by elongated, smooth, opaque-white, and moist colonies (E). Scale bars = 2 mm.

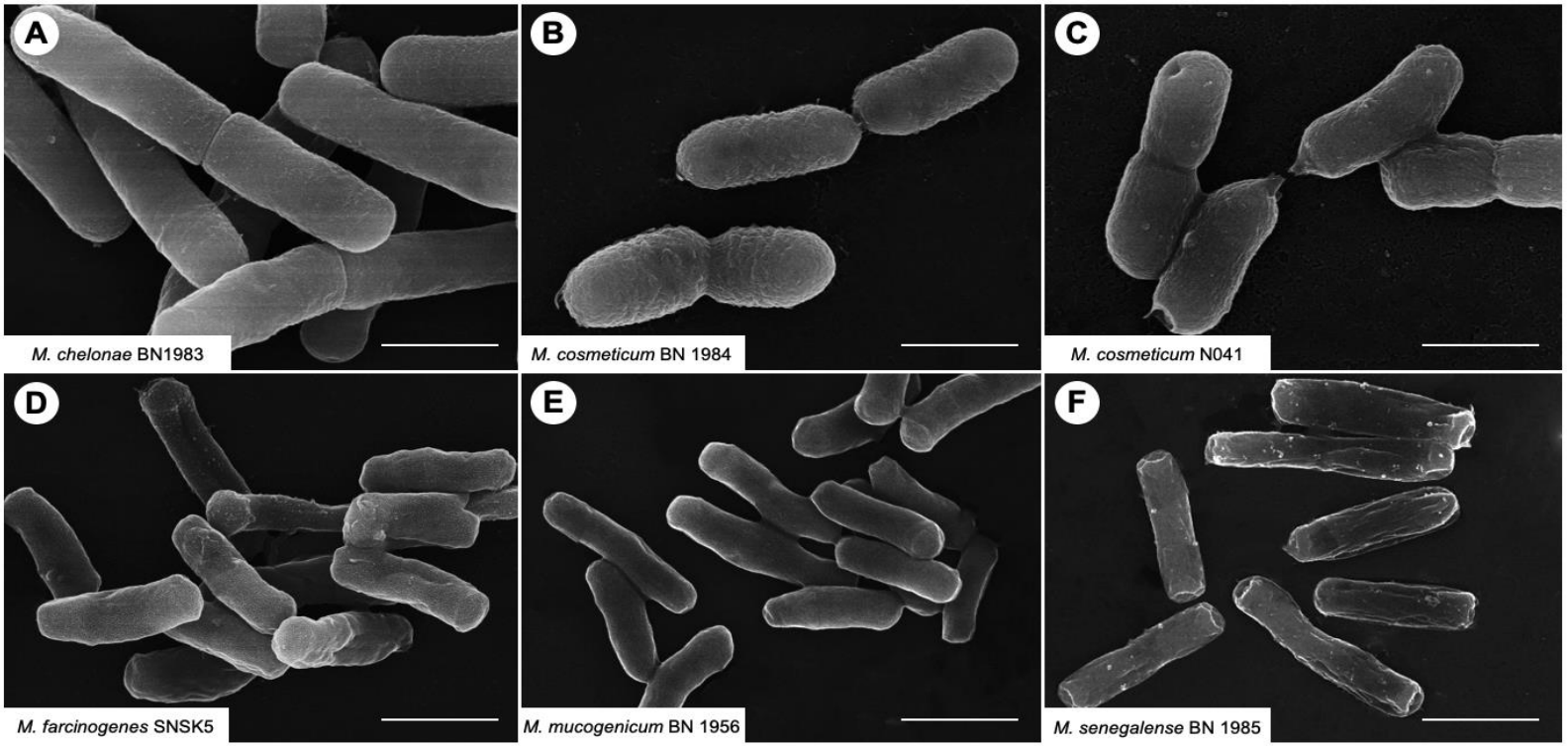

Figure 3.4. Scanning electron micrographs of the *Mycobacterium* spp. isolated from Siamese fighting fish (*Betta splendens*). Results from SEM reconfirm that all isolates had rod-shaped morphology but distinguishable cell structures. *Mycobacteriodes chelonae* was the longest with a length of about 4-6 µm (A), while *M. cosmeticum* was the shortest with a length of about 2-4 µm (B and C) and both species had a diameter of 1.0-1.2 µm. *Mycolicibacterium farcinogenes* (D), *M. mucogenicum* (E), and *M. senegalense* (F) were quite similar with a length of 3-5 µm and had a relatively smaller diameter than *M. chelone* and *M. cosmeticum*. Scale bars = 2 µm.

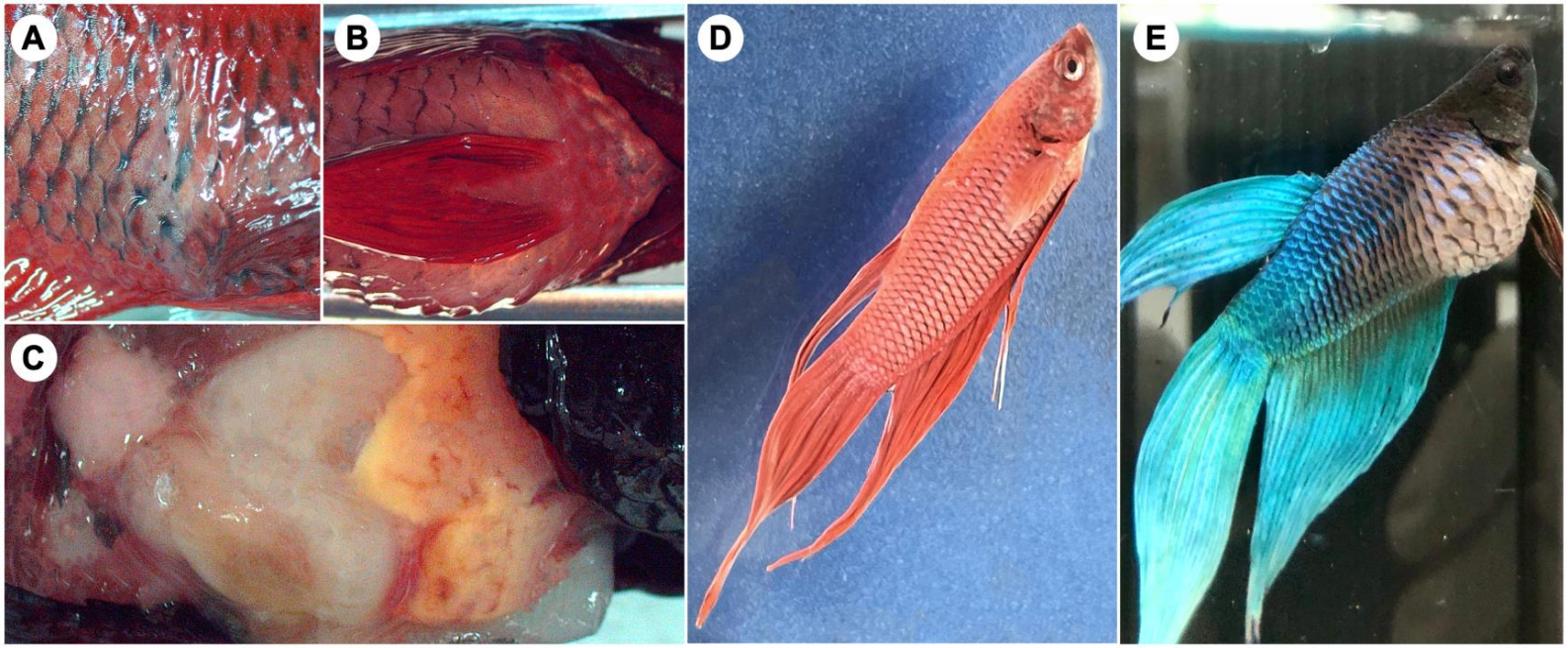

Figure 3.5. Representative clinical signs of experimentally infected Siamese fighting fish (*Betta splendens*). Acute or chronic disease occurred depending on the dose and isolate administered. Fish with ulceration and fin rot at the injection site after 24 h of infection (A). Acutely dead fish showed abdominal distension (B) with severe necrosis of visceral organs (C). Chronically diseased fish with emaciation, lack of vitality, and deterioration of esthetic condition (D) and some fish with big belly syndrome (E).

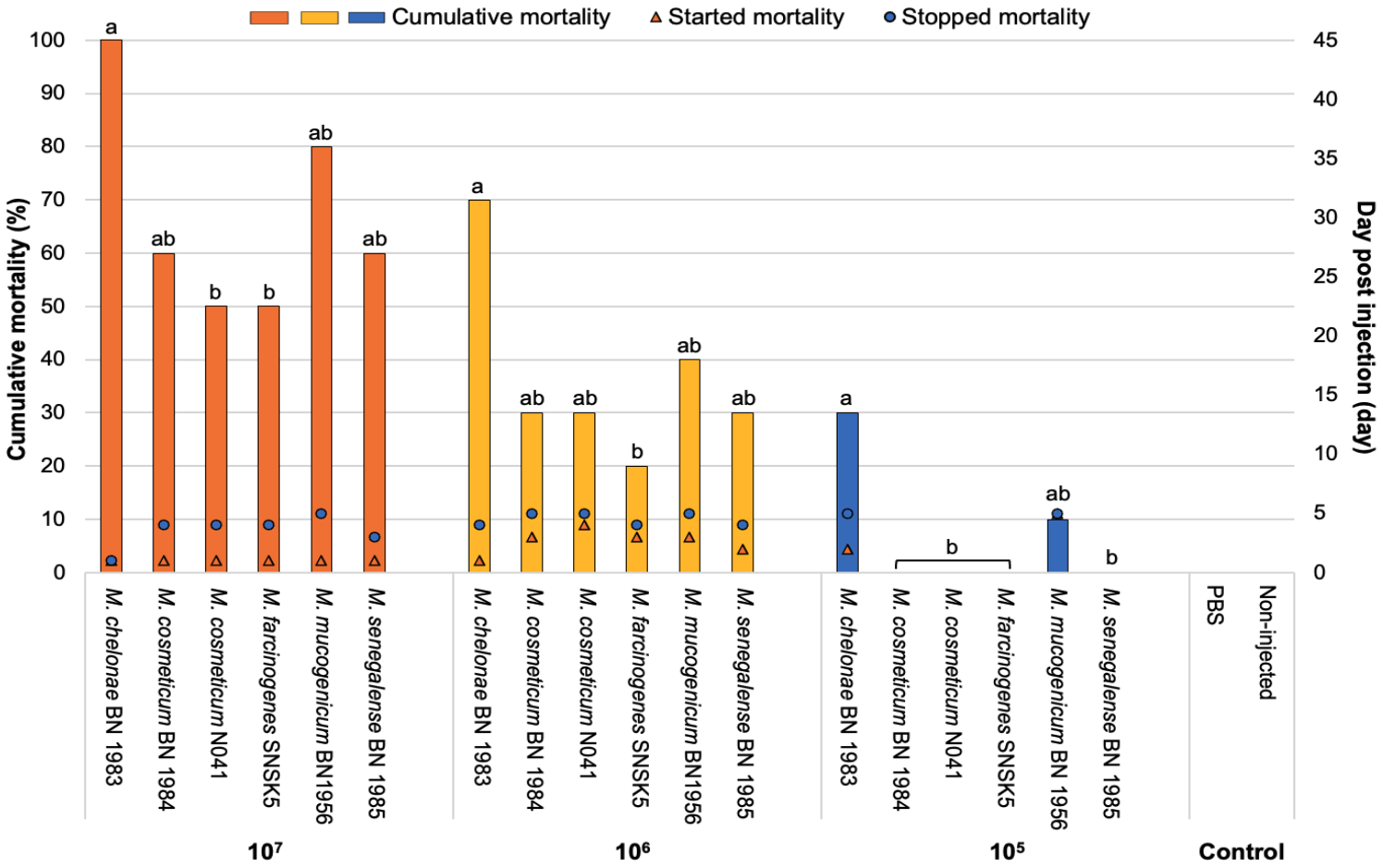

Figure 3.6. Mortality data for the experimentally infected Siamese fighting fish (*Betta splendens*). Comparison of significant differences between the isolates within each dose is indicated by superscript alphabets (*p*-value <0.05).

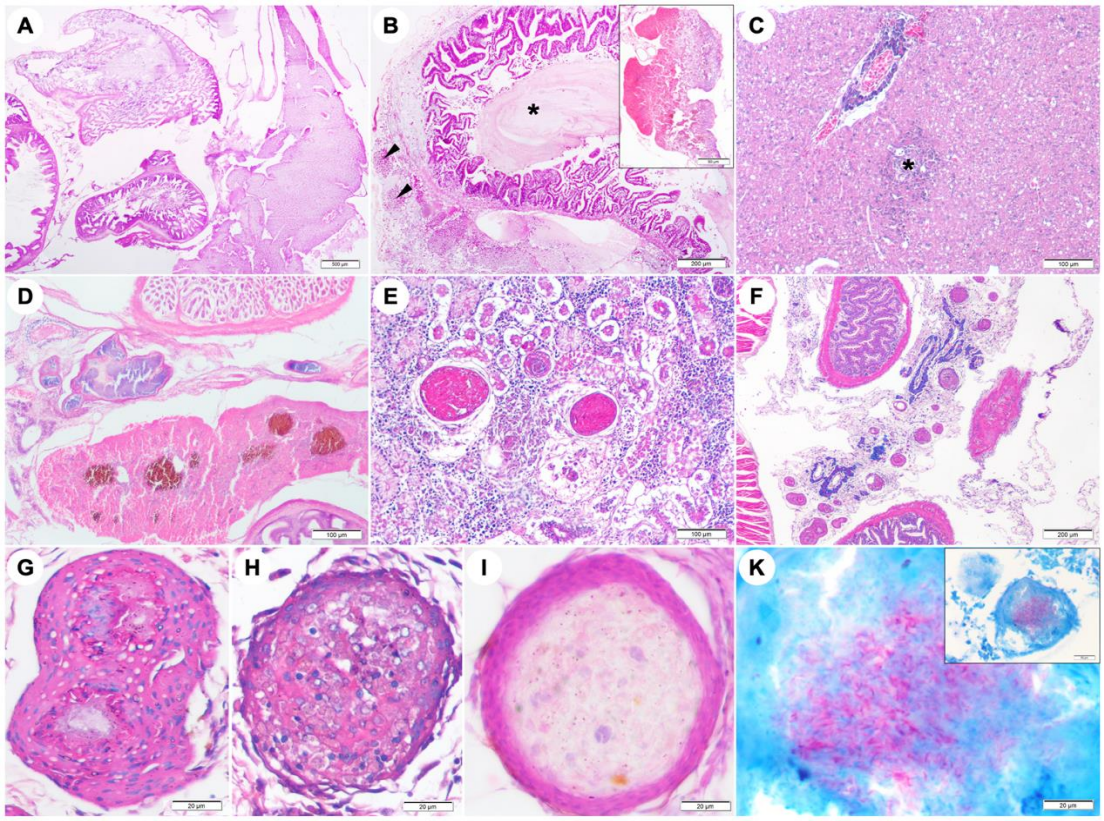

Figure 3.7. Representative histopathology of experimentally infected Siamese fighting fish (*Betta splendens*). Light micrographs of normal peritoneum, liver, and intestine of control group fish (A). Peritoneum, liver, and intestine of an acutely dead fish infected 3 days earlier (B). There is a region of extensive necrotic tissue (inset), bacterial cells (arrowheads), and exudate (asterisk). Histopathological sections of fish chronically infected for 6 weeks (C to I). Early granuloma formation in liver (asterisk in Fig. C). Granuloma formation occupied a large area of spleen and parenchyma of peritoneum (D). Multiple granulomas affect a large portion of kidney (E). Dense granuloma formation in parenchyma of peritoneum (F). At high magnification, granulomas show various features such as onion-ring-shaped granuloma with epithelioid macrophages surrounding a necrotic center (G), granuloma formation with foamy macrophages (H), and caseous granuloma (I). The acid-fast stained section shows numerous rod-shaped bacterial cells in a granuloma (K). Scale bars are shown in the pictures.

3.8. Supplementary data

Table S3.1. The number of colonies of the isolates that survive exposure to different disinfectants

Disinfectant agents		*M. chelonae* BN 1983				*M. cosmeticum* BN 1984 *M. cosmeticum* N041				*M. farcinogenes* SNSK5				*M. mucogenicum* BN 1956				*M. senegalense* BN 1985			
		1 min	5 min	1h	24h	1 min	5 min	1h	24h	1 min	5 min	1h	24h	1 min	5 min	1h	24h	1 min	5 min	1h	24h
EtOH	70%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	95%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Formalin	1%	>300	>300	0	0	>300	>300	0	0	>300	>300	0	0	>300	>300	40±8	0	>300	>300	0	0
	5%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlorine	1%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Povidone iodine	0.6%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.06%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Potassium permanganate	10 ppm	>300	>300	>300	0	>300	>300	>300	0	>300	>300	>300	>300	>300	>300	>300	0	>300	>300	>300	0
	30 ppm	>300	>300	>300	0	>300	>300	0	0	>300	>300	>300	>300	>300	>300	>300	0	>300	>300	>300	0
Control		tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc	tmtc

tmtc: too much to count

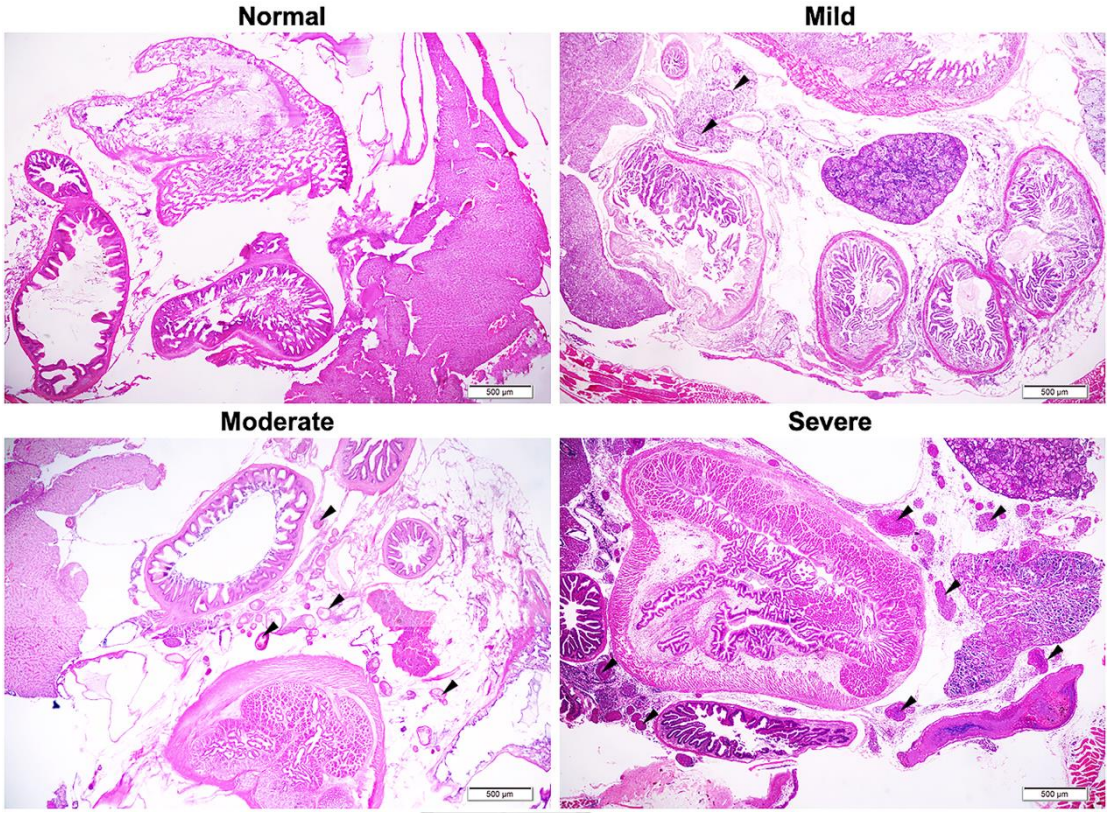

Figure S3.1. Representative histopathologic scores were classified as normal (0), mild (1), moderate (2), and severe (3). Lesions were scored according to both the number and extent of affected areas. Mild lesions were defined as those present in small numbers and have minimal effect on the organ, whereas severe lesions were characterized by extensive tissue damage that barely recognized normal tissue. Moderate lesions were classified as falling between these two extremes. Progressive systemic granuloma formation within the visceral organs (arrowheads). Scale bars are shown in the pictures.

3.9. References

Arakawa CK and Fryer J 1984. Isolation and characterization of a new subspecies of *Mycobacterium chelonei* infectious for salmonid fish. Helgoländer Meeresuntersuchungen. 37: 329-342.

Beran V, Matlova L, Dvorska L, Svastova P and Pavlik I 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. J. Fish Dis. 29(7): 383-393.

Bhalla GS, Sarao MS, Kalra D, Bandyopadhyay K and John AR 2018. Methods of phenotypic identification of non-tuberculous mycobacteria. Pract Lab Med. 12: e00107.

Brown-Elliott BA, Nash KA and Wallace RJ 2012. Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. Clin. Microbiol. Rev. 25(3): 545-582.

Brown-Elliott BA and Philley JV 2017. Rapidly growing mycobacteria. Microbiol Spectr. 5(1).

Cheng AY and Lee CH 2022. Skin infection by *Mycobacterium farcinogenes-senegalense* group in an immunocompetent patient: A case report. BMC Infect Dis. 22(1): 445.

Cooksey RC, de Waard JH, Yakrus MA, Rivera I, Chopite M, Toney SR, Morlock GP and Butler WR 2004. *Mycobacterium cosmeticum* sp. nov., a novel rapidly growing species isolated from a cosmetic infection and from a nail salon. Int J Syst Evol Microbiol. 54(Pt 6): 2385-2391.

Delghandi MR, El-Matbouli M and Menanteau-Ledouble S 2020. Mycobacteriosis and I\infections with non-tuberculous mycobacteria in aquatic organisms: A review. Microorganisms. 8(9).

Dinh-Hung N, Dong HT, Taengphu S, Soontara C, Rodkhum C, Senapin S and Chatchaiphan S 2023. *Streptococcus suis* is a lethal pathogen in snakeskin gourami, *Trichopodus pectoralis*. Aquaculture. 739173.

Dohál M, Porvazník I, Solovič I and Mokrý J 2021. Whole genome sequencing in the management of non-tuberculous mycobacterial infections. Microorganisms. 9(11): 2237.

Dong HT, Senapin S, Phiwsaiya K, Techatanakitarnan C, Dokladda K, Ruenwongsa P and Panijpan B 2018. Histopathology and culturable bacteria associated with “big belly” and “skin nodule” syndromes in ornamental Siamese fighting fish, *Betta splendens*. Microb Pathog. 122: 46-52.

Dowdell K, Haig SJ, Caverly LJ, Shen Y, LiPuma JJ and Raskin L 2019. Nontuberculous mycobacteria in drinking water systems - the challenges of characterization and risk mitigation. Curr Opin Biotechnol. 57: 127-136.

Duong A, Steinmaus C, McHale CM, Vaughan CP and Zhang L 2011. Reproductive and developmental toxicity of formaldehyde: A systematic review. Mutat Res. 728(3): 118-138.

Falkinham III JO 2021. Ecology of nontuberculous mycobacteria. Microorganisms. 9(11): 2262.

Fernandez-Rendon E, Cerna-Cortes JF, Ramirez-Medina MA, Helguera-Repetto AC, Rivera-Gutierrez S, Estrada-Garcia T and Gonzalez YMJA 2012. *Mycobacterium mucogenicum* and other non-tuberculous mycobacteria in potable water of a trauma hospital: A potential source for human infection. J Hosp Infect. 80(1): 74-76.

Francis-Floyd R 2011. Mycobacterial infections of fish. In: Southern Regional Aquaculture Center USA.

Fukano H, Wada S, Kurata O, Mizuno K, Nakanaga K and Hoshino Y 2015. Nontuberculous mycobacteriosis in farmed thread-sail filefish *Stephanolepis cirrhifer*. 魚病研究. 50(2): 68-74.

Gauthier DT and Rhodes MW 2009. Mycobacteriosis in fishes: A review. Vet J. 180(1): 33-47.

Goldstein RJ 2015. The betta handbook. In: Sourcebooks, Inc.

Gonzalez-Diaz E, Morfin-Otero R, Perez-Gomez HR, Esparza-Ahumada S and Rodriguez-Noriega E 2018. Rapidly growing mycobacterial infections of skin and soft tissues caused by *M. fortuitum* and *M. chelonae.* Curr. Trop. Med. Rep.5(3): 162-169.

Gupta RS, Lo B and Son J 2018. Phylogenomics and comparative genomic studies robustly support division of the genus *Mycobacterium* into an emended genus *Mycobacterium* and four novel genera. Front Microbiol. 9: 67.

Gupta T, Fine-Coulson K, Karls R, Gauthier D and Quinn F 2013. Internalization of *Mycobacterium shottsii* and *Mycobacterium pseudoshottsii* by *Acanthamoeba polyphaga.* Can J Microbiol. 59(8): 570-576.

Hamid M 2014. Current perspectives on *Mycobacterium farcinogenes* and *Mycobacterium senegalense*, the causal agents of bovine farcy. Vet. Med. Int. 2014: 247906.

Hay RJ 2009. M*ycobacterium chelonae* - A growing problem in soft tissue infection. Curr Opin Infect Dis. 22(2): 99-101.

CLSI 2020. Methods for antimicrobial broth dilution and disk diffusion susceptibility testing of bacteria isolated from aquatic animals, 2nd Edition. VET03. In: Clinical and Laboratory Standards Institute.

Jørgensen TR, Larsen TB and Buchmann K 2009. Parasite infections in recirculated rainbow trout (*Oncorhynchus mykiss*) farms. Aquaculture. 289(1): 91-94.

Kawakami K and Kusuda R 1990. Efficacy of rifampicin, streptomycin and erythromycin against experimental *Mycobacterium* infection in cultured yellowtail. Nippon Suisan Gakkaishi=Bulletin of the Japanese Society of Scientific Fisheries. 56(1): 51-53.

Khieu V, Ananta P, Kaewprasert O, Laohaviroj M, Namwat W and Faksri K 2021. Whole-genome sequencing analysis to identify infection with multiple species of nontuberculous mycobacteria. Pathogens. 10(7): 879.

Kim B-J, Kim G-N, Kim B-R, Shim T-S, Kook Y-H and Kim B-J 2019. New *Mycobacteroides abscessus* subsp. *massiliense* strains with recombinant hsp65 gene laterally transferred from *Mycobacteroides abscessus* subsp. *abscessus*: Potential for misidentification of *M. abscessus* strains with the hsp65-based method. PLOS ONE. 14(9): e0220312.

Krumperman PH 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl Environ Microbiol. 46(1): 165-170.

Le Dantec C, Duguet JP, Montiel A, Dumoutier N, Dubrou S and Vincent V 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl. Environ. Microbiol. 68(11): 5318-5325.

Leal JF, Neves MGPMS, Santos EBH and Esteves VI 2018. Use of formalin in intensive aquaculture: Poperties, application and effects on fish and water quality. Rev Aquac. 10(2): 281-295.

Lewis S and Chinabut S 2011. Mycobacteriosis and nocardiosis. In: Fish diseases and disorders. Volume 3: Viral, bacterial and fungal infections. CABI Wallingford UK. 397-423.

Lichak MR, Barber JR, Kwon YM, Francis KX and Bendesky A 2022. Care and use of Siamese fighting fish (*Betta Splendens*) for research. Comp Med. 72(3): 169-180.

Maceda-Veiga A and Cable J 2019. Diseased fish in the freshwater trade: From retailers to private aquarists. Dis Aquat Organ. 132(2): 157-162.

Machida Y, Tang BCC, Yamada M, Sato S, Nakajima K, Matoyama H, Kishihara T, Endo M, Sano M and Kato G 2021. Mycobacteriosis in cultured koi carp *Cyprinus carpio* caused by *Mycobacterium paragordonae* and two *Mycolicibacterium* spp. Aquaculture. 539: 736656.

Maupin J, Cantrell A, Kupiec K, Melendez DP and Haleem AM 2019. *Mycobacterium senegalense* osteomyelitis of the distal Tibia: A case report. J Bone Jt Infect. 4(3): 140-145.

Narendrakumar L, Sudhagar A, Preena PG, Nithianantham SR, Mohandas SP and Swaminathan TR 2022. Detection of *Mycobacterium marinum* and multidrug-resistant bacteria in a chronic progressive disease outbreak among Siamese fighting fish (*Betta splendens*) in India. Biologia. 77(9): 2725-2733.

Nolte O, Haag H and Häfner B 2005. A mutation in the 65,000Dalton heat shock protein gene, commonly used for molecular identification of non-tuberculous mycobacteria, leads to the misidentification of *Mycobacterium malmoense* as *Mycobacterium marinum.* Mol. Cell. Probes. 19(4): 275-277.

Oren A and Garrity GM 2018. List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol. 68(11): 3379-3393.

Phillips Savage ACN, Blake L, Suepaul R, McHugh OS, Rodgers R, Thomas C, Oura C and Soto E 2022. Piscine mycobacteriosis in the ornamental fish trade in Trinidad and Tobago. J. Fish Dis. 45(4): 547-560.

Pleeging C and Moons CPH 2017. Potential welfare issues of the Siamese fighting fish (*Betta splendens*) at the retailer and in the hobbyist aquarium. Vlaams Diergeneeskundig Tijdschrift. 86: 213-223.

Post B 2016. "Subject: Flag-coloured fighting fish for B53,500" (online). Available: https://www.bangkokpost.com/thailand/general/1138365/flag-coloured-fighting-fish-sold-for-b53-500.

Post B 2019. "Subject: Siamese fighting fish confirmed as national aquatic animal" (online). Available: https://www.bangkokpost.com/thailand/general/1624026/siamese-fighting-fish-confirmed-as-national-aquatic-animal.

Puk K, Banach T, Wawrzyniak A, Adaszek Ł, Zietek J, Winiarczyk S and Guz L 2018. Detection of *Mycobacterium marinum, M. peregrinum, M. fortuitum* and *M. abscessus* in aquarium fish. J Fish Dis. 41(1): 153-156.

Purivirojkul W and Sumontha M 2013. Euclinostomum heterostomum (Rudolphi, 1809) metacercarial infection in three osphronemid fish specieshree osphronemid fish species. Walailak J. Sci. & Tech. 10(1): 97-102.

Puttinaowarat S, Thompson K, Kolk A and Adams A 2002. Identification of *Mycobacterium* spp. isolated from snakehead, *Channa striata* (Fowler), and Siamese fighting fish, *Betta splendens* (Regan), using polymerase chain reaction–reverse cross blot hybridization (PCR–RCBH). J. Fish Dis. 25: 235-243.

Reed LJ and Muench H 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27(3): 493-497.

Reimschuessel R, Bennett RO and Lipsky MM 1992. Communications: A classification system for histological lesions. J. Aquat. Anim. Health. 4(2): 135-143.

Runyon EH 1959. Anonymous mycobacteria in pulmonary disease. Med Clin North Am. 43(1): 273-290.

Sanders ER 2012. Aseptic laboratory techniques: plating methods. J. Vis. Exp: JoVE. (63): e3064-e3064.

Senapin S, Phiwsaiya K, Laosinchai P, Kowasupat C, Ruenwongsa P and Panijpan B 2014. Phylogenetic analysis of parasitic trematodes of the genus *Euclinostomum* found in Trichopsis and Betta fish. J. Parasitol. 100(3): 368-371.

Sermwatanakul A 2019. Capacitating the local farmers to enhance global marketing of Thailand's national aquatic animal, the Siamese fighting fish. Fish for the People. 17(2): 42-48.

Shinnick TM and Good RC 1994. Mycobacterial taxonomy. Eur J Clin Microbiol Infect Dis. 13(11): 884-901.

Sirimalaisuwan A, Teeraruk P, Kanjanapitakchai P, Kaewsakhorn T, Potibut P and Pikulkaew S 2017. Detection of *Mycobacterium marinum* in clinically asymptomatic Siamese fighting fish (*Betta splendens*) from ornamental fish shops in Chiang Mai Province, Thailand. Asian Pac. J. Trop. Dis. 7: 344-346.

Talaat AM, Reimschuessel R, Wasserman SS and Trucksis M 1998. Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. Infect Immun. 66(6): 2938-2942.

Talaat AM, Trucksis M, Kane AS and Reimschuessel R 1999. Pathogenicity of *Mycobacterium fortuitum* and *Mycobacterium smegmatis* to goldfish, *Carassius auratus*. Vet Microbiol. 66(2): 151-164.

Tamura K, Stecher G and Kumar S 2021. MEGA11: Molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38(7): 3022-3027.

Tortoli E 2012. Phylogeny of the genus Mycobacterium: Many doubts, few certainties. Infect. Genet. Evol. 12(4): 827-831.

Tortoli E 2019. Chapter 1 - The Taxonomy of the genus *Mycobacterium*. In: Nontuberculous Mycobacteria (NTM). Ali Akbar Velayati and Parissa Farnia (eds). Academic Press. 1-10.

Tortoli E, Brown-Elliott BA, Chalmers JD, Cirillo DM, Daley CL, Emler S, Floto RA, Garcia MJ, Hoefsloot W, Koh WJ, Lange C, Loebinger M, Maurer FP, Morimoto K, Niemann S, Richter E, Turenne CY, Vasireddy R, Vasireddy S, Wagner D, Wallace RJ, Jr., Wengenack N and van Ingen J 2019. Same meat, different gravy: Ignore the new names of mycobacteria. Eur Respir J. 54(1).

Weerakhun S, Sukon P and Hatai K 2019. *Mycobacterium marinum* and *Mycobacterium fortuitum* infections in Siamese fighting fish, *Betta splendens* (Regan), in Thailand. Thai J Vet Med. 49(2): 137-145.

Weisburg WG, Barns SM, Pelletier DA and Lane DJ 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 173(2): 697-703.

Wolinsky E 1992. Mycobacterial diseases other than tuberculosis. Clin Infect Dis. 15(1): 1-10.

Zanoni RG, Florio D, Fioravanti M, Rossi M and Prearo M 2008. Occurrence of *Mycobacterium* spp. in ornamental fish in Italy. J. Fish Dis. 31: 433-441.

Zhang R, Wu GT, Zhu JY, Wang XW, Liu LL, Li HJ and Zhu H 2023. Povidone iodine exposure alters the immune response and microbiota of the gill and skin in Koi carp, *Cyprinus carpio*. Aquaculture. 563: 738926.

Ziarati M, Zorriehzahra MJ, Hassantabar F, Mehrabi Z, Dhawan M, Sharun K, Emran TB, Dhama K, Chaicumpa W and Shamsi S 2022. Zoonotic diseases of fish and their prevention and control. Vet Q. 42(1): 95-118

CHAPTER 4

Infection and histopathological consequences in Siamese fighting fish (*Betta splendens*) due to exposure to a pathogenic *Mycobacterium chelonae* via different routes

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4.1. Highlights

- Various routes of exposing *B. splendens* to *M. chelonae* were studied for the first time, including injection (IP and IM), oral administration, and immersion with/without skin trauma.
- Pathogenicity depends largely on transmission modes, with IP and IM leading to particularly severe infections.
- It is likely that infection occurs naturally primarily through an injured body surface and/or the digestive tract.
- All exposure routes led to chronic conditions, suggesting that control of mycobacteriosis requires rigorous reduction of associated risk factors.

Keywords: *Mycobacterium chelonae*; Betta fish; Infection routes; Pathogenicity; Histopathology; Granulomas; Mycobacteriosis

4.2. Abstract

Mycobacteriosis, a chronic progressive disease in fish caused by *Mycobacterium* spp., is typically characterized by granulomatous lesions that illustrate its harmful pathogenesis. This study was initiated to understand the progression of mycobacteriosis in Siamese fighting fish (*Betta splendens*) through various transmission modes. Experimental infections were conducted using a pathogenic isolate of *Mycobacterium chelonae* originally derived from a naturally infected betta fish. The bacteria were administered by five different routes at three doses (approximately 10^5, 10^6, 10^7 CFU/fish or CFU/mL), including intraperitoneal (IP) and intramuscular (IM) injections, oral administration, immersion, and immersion with skin trauma. Acute infections, typified by short survival, non-specific gross signs, and severe organ necrosis, were frequently observed in the IP and IM methods. The median lethal dose (LD_{50}) for these methods was 4.16×10^5 CFU/fish and 1.66×10^6 CFU/fish, respectively, within 7 days following infection. Conversely, oral

administration and immersion with or without skin trauma resulted in negligible fish mortality but triggered progressive chronic disease. Chronically infected fish survived the 28-day trial but exhibited macroscopic pathology such as emaciation, aesthetic deterioration, along with an increased presence of melanomacrophage centers (MMCs) in lymphoid tissues and/or systemic granulomas, as noted in histopathology. The results indicate that the course of *M. chelonae* infection is strongly correlated with the exposure routes, with injuries on the fish body and/or via the digestive tract being the most probable natural pathways. This study expands the understanding of the pathogenesis of *Mycobacterium* species in betta fish and emphasizes the need to mitigate risk factors associated with exposure routes to effectively control the disease.

4.3. Introduction

Betta splendens, commonly known as Siamese fighting fish or simply as betta, is a species highly prized in the worldwide aquarium trade for its vibrant colours and aggressive behavior (U.S. Fish and Wildlife Service, 2019). The species has considerable economic value, with the price of a single fish ranging from a few to a thousand dollars, depending on its colour and fin shape (Monvises et al., 2009; Sermwatanakul, 2019). The fish are subjected to intensive trade and rearing practices, which greatly heighten their susceptibility to various diseases (Senapin et al., 2014; Goldstein, 2015; Maceda-Veiga & Cable, 2019). Among the numerous diseases that affect betta fish, mycobacteriosis has emerged as a particularly threatening problem with potentially substantial economic impact (Pleeging & Moons, 2017; Dong et al., 2018; Lichak et al., 2022). Unfortunately, there are as yet no vaccines or effective therapeutic measures against this disease in fish (see Francis-Floyd, 2011), including *B. splendens*.

Mycobacterial infections in betta fish are frequently documented; however, most of the existing literature focuses narrowly on pathogen detection and isolation. Several *Mycobacterium* species have been identified as causative agents of mycobacteriosis in betta fish, including *M. chelonae, M. cosmeticum, M. farcinogenes, M. fortuitum, M. marinum, M. mucogenicum*, and *M. senegalense* (Beran et al., 2006; Sirimalaisuwan et al., 2017; Weerakhun et al., 2019; Dong et al., 2018; Narendrakumar et al., 2022; Dinh-Hung et al., 2023a). Our previous study comprehensively characterized six isolates, including five species (*i.e., M. chelonae, M. cosmeticum, M. farcinogenes, M. mucogenicum,* and *M. senegalense*), and found that *M. chelonae* was highly pathogenic to betta fish and exhibited multiple drug resistance (Dinh-Hung et al., 2023a). Beyond these findings, the modes of transmission and pathological effects of mycobacterial infections in many fish species, including *B. splendens*, remain largely unexplored. Addressing these knowledge gaps is critical to fully understand the complexity of the disease. Since experimental infections are a cornerstone in the study of infectious diseases in fish, this approach is essential to describe the role of host-microorganism interactions in pathological processes, to elucidate the mechanisms of disease development, and to evaluate the efficacy of future therapeutic or preventive interventions (Gudding et al., 1999). Hence, this study aims to investigate the pathogenesis of *Mycobacterium* spp. in betta fish using controlled infection models in the laboratory. Experimental infections were performed with a pathogenic, multidrug-resistant isolate of *Mycobacterium chelonae* originated from a naturally infected betta fish (Dong et al., 2018; Dinh-Hung et al., 2023a). The investigation involves a comparative analysis of various infection routes to assess their potential to induce mycobacteriosis and subsequent histopathological consequences. This study is expected to provide valuable insights into host-pathogen interactions (*i.e.*, infection progression, tissue tropism) that will enhance our understanding of mycobacterial pathogenesis. These

insights will enable us to mitigate risk factors and develop strategies to control the disease, thereby promoting the long-term sustainability of the betta fish industry.

4.4. Materials and methods

4.4.1. Bacteria preparation

The pathogenic *Mycobacterium chelonae* isolate BN1983 (GenBank accession no. MG438529) used in this study was originally sourced from a naturally infected betta fish (Dong et al., 2018; Dinh-Hung et al., 2023a). The isolate was retrieved from a frozen glycerol stock (-80°C) and routinely cultured on Middlebrook 7H11 agar (HiMedia, India) containing 10% OADC growth supplement (*i.e.,* Oleic Albumin Dextrose Catalase) for 4 nights at 30°C. Subsequently, a single pure colony was inoculated into a small volume of 5 mL Middlebrook 7H9 broth (HiMedia, India) supplemented with 10% ADC (*i.e.,* Albumin Dextrose Catalase) and incubated for 2 h at 30°C. The bacterial suspension (5 mL) was then transferred to 200 mL of the same medium broth and incubated at the same temperature for 48 h with constant shaking at 250 rpm. The harvested bacterial suspension was pelleted by centrifugation at 5000 g for 5 min. The pellet was then washed twice before resuspension with 1× phosphate-buffered saline (PBS) to achieve an OD_{600} of 1.0, which corresponds to 2.3×10^8 CFU/mL by conventional plate count. The bacterial stock solution (*i.e.,* 2.3×10^8 CFU/mL) was subjected to a serial dilution with 1× PBS to achieve the desired dosages for use.

4.4.2. Fish and ethics statement

A cohort of apparently healthy adult fish aged 4-5 months (n=320) was obtained from a reputable local commercial betta fish farm. The fish were housed individually in a 5×5×15 cm aquarium and allowed to acclimate for one week before the experiment. Opaque white plastic partitions were placed between the aquaria to prevent possible aggressive interactions between the fish. The fish were fed a

commercial betta pellet diet with 28% protein content (Optimum, Thailand) three times per week. Prior to the experiment, a representative subset of the fish (n=10) underwent comprehensive clinical, bacteriological, and molecular analysis (see Section 4.4.4) to confirm that no mycobacterial infections were present. To ensure the safe handling of *M. chelonae*, all water and equipment contaminated with the bacteria were disinfected using a 2% (*v/v*) chlorine solution for at least 60 minutes before disposal, as per the guidelines of a previous study (Dinh-Hung et al., 2023a). The experimental procedures and the purposes for which the fish were used for this trial were approved following ethical review by the Institutional Animal Care and Use Committee of Kasetsart University (approval ID: ACKU65-FIS-001).

4.4.3. Experimental infection

A total of 20 groups of 15 fish each were included in the experimental design. Of these, 15 experimental groups were infected via five different routes, with three different concentrations of bacteria administered for each route. In parallel, five control groups were set up corresponding to each infection route. Fish in these control groups underwent the same procedures as those in the experimental groups but were exposed to 1× PBS instead of the bacteria. Details of the experimental infection are described below.

Intraperitoneal (IP) and intramuscular (IM) injections

Prior to injection, fish were lightly anesthetized using a 50 ppm clove oil solution, with a recovery time of 30 to 45 s. Each fish received an injection either intraperitoneally just below the pectoral fin or intramuscularly into the muscle mass below the dorsal fin. The injections were administered using 1cc 29G×1/2-inch insulin syringes, with each syringe delivering 50 µL of the bacterial suspension. This procedure aimed to achieve final bacterial concentrations of approximately 1.15×10^7, 1.15×10^6, and 1.15×10^5 CFU/fish.

Oral exposure

Each fish was orally given the bacterial suspension by inserting 25 µL directly into its buccal cavity using a plastic dispensing needle. During the procedure, the operculum was gently held closed for approximately 5 s. The procedure was then repeated, ensuring a total delivery of 50 µL of the bacterial suspension per fish. This resulted in final bacterial concentrations of roundly 1.15×10^7, 1.15×10^6, and 1.15×10^5 CFU/fish.

Immersion challenge and immersion with skin trauma

In the immersion experiment, individual fish were immersed in a 50 mL Falcon tube containing 30 mL of prepared bacterial suspensions with concentrations of 2.3×10^7, 2.3×10^6, or 2.3×10^5 CFU/mL. After 1 h of immersion, the fish were returned to their respective aquaria. For the "immersion with skin trauma" approach, a superficial incision (~2×2×2 mm) was made near the dorsal fin using a surgical blade tip. Following this, the fish underwent the same procedures as those in the standard immersion experiment.

4.4.4. Data collection and analysis

Mortality, morbidity, and lethal dose

After each exposure, mortality and morbidity were monitored daily for 28 days. In this study, no individual fish were categorized outside the two defined categories: moribund, *i.e.,* those acutely infected within the first week post-infection (1 wpi), and survivors, *i.e.,* those still alive by the end of the study (4 wpi). Subsequently, these fish were subjected to comprehensive clinical, bacteriological, molecular, and histopathological examinations. The median lethal dose (LD_{50}) for all challenge groups was determined on the 7^{th} day according to the method of Reed and Muench (1938).

Confirmation of infection by bacteriology and PCR

Bacteria from the visceral organs of representative experimental fish were re-isolated on Middlebrook 7H11 agar plates supplemented with 10% OADC and then incubated at 30°C for up to 4 days. For detecting *M. chelonae*, several bacterial colonies were inspected based on colony characteristics previously described in the study (Dinh-Hung et al., 2023a). Selected colonies were subjected to acid-fast staining for further confirmation. Colonies were confirmed as positive for the bacteriology if they tested positive in the acid-fast staining, indicated by the bright red staining of the acid-fast bacilli.

The PCR was also performed with DNA extracted from pooled tissues (*i.e.,* liver, spleen, and intestine) of the experimental fish. Genomic DNA was extracted using the Tissue Genomic DNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's protocol. The presence of *M. chelonae* DNA was examined using primers Tb11 (5'-ACCAACGATGGTGTCCAT-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3') specific to the 65 kDa heat shock protein (hsp65) gene of the genus *Mycobacterium*, as described by Telenti *et al.* (1993). A 25 µL PCR reaction was performed using the following components: 12.5 µL 2× Terra PCR Direct Polymerase Mix (Takara Bio, Japan), 2 µL DNA template, 0.5 µL of each primer (10 µM), 0.5 µL 1.25 U/µL Terra Taq enzyme (Takara Bio, Japan), and 9.0 µL distilled water. The amplification protocol included one cycle at 95°C for 1 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The expected amplicons of the PCR method were 439 bp.

Histopathological examination

The entire fish body was fixed in 10% neutral buffered formalin for 24 h before being transferred to 70% ethanol for histological processing. The tissues were then dehydrated with increasing concentrations of alcohol, cleaned with xylene, and

embedded in paraffin by the standard method. These paraffin-embedded samples were sectioned into 5 µm slices with a microtome and then stained with hematoxylin and eosin (H&E). From each group, at least two control fish (both pre- and post-challenge) and three challenged fish were analyzed.

Histological sections were also subjected to acid-fast staining following established protocols (Dinh-Hung et al., 2023a). This procedure facilitated the observation and identification of acid-fast bacteria within histopathological specimens. Briefly, the deparaffinized sections were immersed in a hot 60°C carbol-fuchsin solution for 1 h and subsequently rinsed with running tap water. The slides were then decolorized with 1% acid alcohol and counterstained with a methylene blue solution for 30 s. After a water rinse and air drying, the slides were coverslipped and analyzed under a digital light microscope (Olympus, Japan). The presence of *M. chelonae* was confirmed by the bright red staining of the acid-fast bacilli after the staining process.

4.5. Results

4.5.1. Gross pathology and mortality

The challenges induced either acute or chronic infections, depending on both the bacterial dose and the infection route. During the acute disease phase, IP and IM infections led to rapid mortality within 24-48 h post-infection. The IP-infected fish displayed abdominal distension (Figure 4.1A), whereas IM infections typically caused skin ulcers at the injection site (Figure 4.1B). Necropsy examination these fish revealed pronounced necrosis in visceral organs and the presence of ascitic fluid (Figure 4.1C). Across all test routes, fish exhibited a loss of appetite and lethargy within a week of infection, with more severe effects noted in groups exposed to higher doses. Both oral and immersion infections resulted in non-specific clinical symptoms. Immersion associated with skin trauma frequently caused skin ulcers at

previously injured epidermal regions (Figure 4.1D). At the end of the experiment, some fish in this group had developed skin nodules at the incision sites (Figure 4.1E). A few fish showed clinical signs of “big belly syndrome” (associated with ascites at necropsy) in the injection and oral groups (see Figure 4.1F). Regardless of the routes of infection, most fish exhibited chronic conditions at the end of the study. Although the fish survived, they were emaciated, displayed reduced vitality and esthetic impairment (Figure 4.1G), with severe infections noted primarily in the IP and IM groups (Table 4.1).

The cumulative mortality data caused by the different infection routes during the 28-day experimental period are presented graphically in Figure. 4.2. The LD_{50} values determined within the first 7 days are provided in Table 4.1. For infection via the IP and IM routes, the LD_{50} values were 4.16×10^{5} and 1.66×10^{6} CFU/fish, respectively The LD_{50} could not be estimated for fish exposed orally or by immersion (both with and without skin trauma) because mortality was negligible even at the highest doses. No mortality was observed in the control groups.

4.5.2. Bacteriology and PCR

Mycobacterium chelonae was successfully isolated from the internal organs of fish that died acutely within a week of infection, regardless of the dose of bacteria administered or the route of infection (see Table 4.1 and Figure S4.1). Bacteriological examinations, however, failed to detect *M. chelonae* in fish that survived 28 days post-infection, except for some fish from the IP and IM groups that had received high doses (10^{7} or 10^{6} CFU/fish). In contrast, PCR analysis consistently yielded positive results from organs of fish in all challenged groups (see Table 4.1 and Figure S4.2). Both the bacteriological and PCR examinations of all control fish groups were negative when performed directly on the internal organs (Figure S4.2).

4.5.3. Histopathological consequences

Histopathology evaluations and localization of acid-fast bacteria in fish infected through various routes tested are summarized in Table 4.1.

Intraperitoneal injection. Upon histopathological examination, severe damage was evident in visceral organs, manifesting as pronounced peritonitis and tissue necrosis due to acute disease (Figure 4.3A). An increased presence of melano-macrophage centers (MMCs) was noted in the lymphoid tissues, particularly in the spleen (Figure 4.3A). Early granuloma formation was observed in several organs, including the peritoneum, kidney, and liver (Figure 4.3B, C, D). Acid- fast staining revealed numerous rod-shaped bacilli distributed throughout the peritoneum (Figure 4.3E). At the end of the 28-day infection period, the surviving fish exhibited progressive systemic granulomas in their visceral organs, a striking histopathological finding, with particularly dense formations in the peritoneum (Figure 4.3F, G). Additionally, multibacillary formations were detected in the core of these granulomas (see Figure 4.3G).

Intramuscular injection. Fish that died within a week of infection displayed moderate necrosis in several visceral organs, notably in the liver and kidney (Figure 4.4A, B). There was also a notable increase in the number of MMCs in lymphoid tissues, particularly in the kidney (see Figure 4.4B). Acid-fast multibacilli were predominantly found near the spine and abdominal muscles (Figure 4.4C), whereas paucibacilli were dispersed throughout the muscles, liver, and kidney (Figure 4.4D, E, F). Fish that survived showed chronic disease lessions, which were characterized by high numbers of granulomas in visceral organs, particularly concentrated in the peritoneum (Figure. 4.4G). Additionally, these granulomas were occasionally seen in the body muscles, with their cores containing numerous acid-fast bacteria (Figure 4.4H).

Oral exposure. Acutely deceased fish exhibited pronounced intestinal damage, as shown in Figure 4.5A. The bacteria were identified within the intestinal submucosa using acid-fast staining (Figure 4.5B). Occasionally, acid-fast rod-shaped bacilli were evident in the gill filaments of these fish (Figure 4.5C). Fish that survived infection beyond 28 days exhibited an increase in MMCs numbers in lymphoid tissues, notably in the liver and kidney (Figure 4.5D, E). Of note, some of these MMCs resembled early granuloma formations and sometimes had acid-fast rod-shaped bacteria present in them (Figure 4.5F).

Immersion and immersion with skin trauma. Fish that died acutely presented with mild to moderate intestinal damage (Figure 4.6A). Acid-fast rod-shaped bacilli were found in the intestinal submucosa of the affected region (Figure 4.6B). In cases of chronic disease, fish infected by immersion generally showed no specific lesions indicative of infection. Fish from the immersion group that had previously suffered skin trauma, however, showed local granuloma formation at the injury site (Figure 4.6C, E). These granulomas contained acid-fast multibacilli in their core, as shown in Figure 4.6F.

4.6. Discussion

Most previous studies of *Mycobacterium* spp. in fish have used IP injection to fulfill Koch's postulates and reproduce the disease in laboratory experiments (Arakawa & Fryer, 1984; Talaat et al., 1998; Fukano et al., 2015; Weerakhun et al., 2019; Machida et al., 2021; Dinh-Hung et al., 2023a); however, other exposure methods have not been performed and/or compared simultaneously. The only example of such studies in the literature is by Harriff *et al.* (2007), which found that the gastrointestinal tract was the primary route of infection in zebrafish (*Danio rerio*) exposed to *Mycobacterium marinum* and *Mycobacterium peregrinum* compared to immersion. The present study is the first attempt to compare five different infection

routes of *Mycobacterium* spp. in fish, specifically the infection of *B. splendens* with *M. chelonae*, leading to a better understanding of its pathogenesis.

Among the tested routes, IP and IM injections proved to be the most effective in rapidly achieving high-intensity and systemic infections. These protocols are routinely used because they provide a simple and rapid method for the precise delivery of suspended bacteria. On the other hand, ensuring uniform bacterial loads in all fish is challenging with oral and immersion methods. After the fish were returned to their aquaria following oral exposure, some bacteria were probably excreted, as evidenced by the presence of bacteria in the gill filaments of some acutely dead fish. Our results showed that both IP and IM methods successfully reproduced the characteristic features of mycobacteriosis at both macroscopic and microscopic levels. These findings manifested as either acute or chronic, in agreement with previous descriptions of mycobacterial infections in *B. splendens* (Dong et al., 2018; Weerakhun et al., 2019; Narendrakumar et al., 2022; Dinh-Hung et al., 2023a) and other fish species (Gauthier & Rhodes, 2009; Francis-Floyd, 2011; Delghandi et al., 2020; Machida et al., 2021). However, it is important to note that injectable infection methods could not fully replicate natural disease progression. This is because the bacteria can bypass important immune defense mechanisms in the skin, gills, and gastrointestinal mucosa through direct injection (Klesius et al., 2000). Despite this limitation, these injection techniques remain advantageous for future mycobacteriosis studies, particularly to rapidly and reliably obtain severely infected fish, fulfill Koch's postulates, perform systemic pathology studies, and evaluate the efficacy of therapeutics.

It has been suggested that exposure via the oral route probably mimics natural mycobacteriosis in fish, since fish can become infected with mycobacteria by consuming infected conspecifics or aquatic detritus (Belas et al., 1995). Mycobacteria are also known to infect numerous aquatic organisms other than fish and can survive

and multiply in various protozoan hosts (Skriwan et al., 2002; Pavlik et al., 2022), which could contaminate a variety of live food sources for betta fish (*e.g.,* tubificid worms, mosquito larvae, *Artemia*, rotifers). Of note, in our study, although oral administration resulted in negligible mortality, chronic gross signs and pathological tissue changes were observed at the end of the experiment, indicating an active disease process. Histological examinations revealed that the intestine was significantly damaged during the acute infection phase and the bacteria were detected, suggesting that the bacteria could compromise the intestine defense mechanisms. This aligns with the behavior of other environmental mycobacteria, such as *M. avium*, which can infect mice via the intestine due to their ability to invade intestinal mucosal cells (Sangari et al., 2001). Our study suggests that the intestine could serve as a potential entry port for the bacteria, allowing them to introduce, survive, and even replicate in macrophages. Subsequently, the bacteria could disseminate through the bloodstream to other parts of the fish, particularly the lymphoid tissue, where they stimulate the proliferation of MMCs - an important indicator of infection. The MMCs perform an important role in response to foreign bodies, including infectious agents (Gómez Manrique et al., 2014; Dinh-Hung et al., 2023b). The MMCs are primarily composed of melanoma macrophages; therefore, activation of these cells during mycobacterial infection may be associated with the formation and proliferation of MMCs in lymphoid tissues (Agius & Roberts, 2003). The proliferation of MMCs suggests a possible defense mechanism in which the fish may have attempted to eliminate the bacteria by shedding infected cells (*i.e.,* bacteria-laden tissue macrophages). Interestingly, the bacteria were occasionally detected in MMCs, along with fish that appeared healthy but tested positive in PCR, suggesting subclinical transmission of infection rather than complete elimination of the pathogen. Of note, no systemic granulomas were detected in this group, possibly due to the relatively short observation period (*i.e.,* 28 days). This duration could be

insufficient for the manifestation of granulomas, particularly in the context of low-circulating bacterial populations. Moreover, this paucibacillary condition could explain the low sensitivity observed in the bacteriological tests performed on the surviving fish.

Immersion led to a lower severity of infection compared to oral exposure, suggesting that the mucus and/or skin tissue effectively blocked bacterial invasion. Our results also showed that infection through immersion was effective only following skin trauma, supporting the assumption that natural infection also occurs via lesions. Given their aggressive nature, *B. splendens* are susceptible to skin lesions that could serve as ports of entry for bacteria. Once *Mycobacterium* spp. have overcome the physical and humoral defenses of fish, the innate immune cells (*i.e.,* macrophages, monocytes, and neutrophils) typically phagocytose and neutralize these invaders (Uribe et al., 2011). Ordinarily, this innate response would be sufficient to eliminate the bacteria; however, *Mycobacterium* spp., partly due to their cell wall properties, can resist these intracellular defense mechanisms (Ramakrishnan, 2012). These bacteria can trigger the release of inflammatory cytokines, such as tumor necrosis factor, which attract more immune cells to the infection site and can eventually lead to granuloma formation (Awuh & Flo, 2017). The adaptive immune response subsequently plays an important role in granuloma maturation by recruiting lymphocytes to the periphery of granulomas, where B and T cells, together with fibroblasts, form a peripheral fibrotic capsule that restricts the spread of bacterial cell populations (Martínez-Lara et al., 2021; Rajme-Manzur et al., 2021). In this context, evidence suggests that certain *Mycobacterium* species can leverage the host's macrophages and granulomas for its expansion and dissemination by recruiting new, uninfected macrophages into the granuloma to phagocytose infected dead cells, thereby promoting the spread of the bacteria (Ramakrishnan, 2012). We hypothesized that granulomas that form in the skin manifest externally as

recognizable skin nodules. This theory aligns with the observations that fish developed visible skin nodules at the incision sites following immersion in the bacterial suspension, which is further supported by histological evidence of granuloma formation. Such manifestations, particularly the clinically visible “skin nodule syndrome”, have also been documented in betta fish naturally infected with mycobacteria (Dong et al., 2018). The skin nodules observed in B. splendens, however, could be attributed to either mycobacteriosis or iridophoroma (Dong et al., 2018; Rahmati-Holasoo et al., 2019; Orós et al., 2022). Additionally, the presence of “big belly syndrome” in this species could also complicate diagnosis, as these manifestations could be due to either mycobacterial infection or polycystic kidney disease (Dong et al., 2018; Rahmati-Holasoo et al., 2020). Molecular techniques and a wide range of diagnostic methods are therefore recommended to accurately distinguish these diseases in B. splendens. Acid-fast staining is recommended to identify suspected mycobacterial infections (Dong et al., 2018; Dinh-Hung et al., 2023a), observation of cysts and nephrocalcinosis in the renal tubules is indicative of polycystic kidney disease (Rahmati-Holasoo et al., 2020), and the use of polarized light is essential for differential diagnosis of iridophores (Rahmati-Holasoo et al., 2019; Orós et al., 2022).

The transmission of mycobacteria in *B. splendens* is not well understood to date. Water serves as a natural habitat for *Mycobacterium* spp. including *M. chelonae* (Le Dantec et al., 2002; Dowdell et al., 2019; Falkinham III, 2021), suggesting that waterborne transmission is likely. Additionally, the ability of mycobacteria to form biofilms allows their persistence in water or fish containers (Falkinham, 2015; Esteban & García-Coca, 2018). In the natural environment, fish are unlikely to be exposed to high doses of bacteria. Instead, they could be exposed naturally through consumption of aquatic detritus, which could serve as a reservoir for mycobacteria, or through intermediate hosts (*e.g., Artemia*, amoebae, rotifers). Such consumption

could allow the bacteria to invade the intestinal mucosa and survive until a local or systemic immunosuppressive event triggers disease progression. Furthermore, infected fish are thought to excrete the bacteria into the water via feces, potentially serving as a reservoir for recurrent infections over extended periods. The disease, therefore, may not manifest significantly until the fish has surface lesions or has a weakened immune response. Several risk factors such as suboptimal husbandry practices, stress from handling, overcrowding, and concurrent infections with other pathogens, could weaken fish immunity. This weakened state creates an opportunity for opportunistic bacteria to invade the fish. If injuries to the fish body or infections via the digestive tract are indeed the primary natural routes of infection, effective measures could include water disinfection to reduce bacterial loads, reducing stocking densities in subadult fish, and refining husbandry practices. Such measures could greatly improve management and control of the disease.

4.7. Tables and figures

Table 4.1. Results of gross pathologicaly, histopathology, bacteria distribution, bacteriology, and PCR analysis.

Exposure	LD_{50} (CFU/fish)	Gross pathology		Histopathology**		Bacteria distribution		Bacteriology		PCR	
		Moribund	Survivor*	Moribund	Survivor	Moribund	Survivor	Moribund	Survivor	Moribund	Survivor
Intraperitoneal injection	4.16×10^5	Abdominal distension	> 60	Severe peritonitis and visceral organ necrosis (+)	Systemic granulomas (+)	Systemic	Core of granulomas	+	+/-	+	+
Intramuscular injection	1.66×10^6	Ulcers at injection site	> 50	Moderate visceral organ necrosis (+)	Systemic granulomas (+)	Systemic	Core of granulomas	+	+/-	+	+
Oral administration	ND	Non-specific signs	30 - 40	Severe intestinal damage (-)	Increasing MMCs in lymphoid tissues (+/-)	Intestinal submucosa	Within MMCs (occasionally)	+	-	+	+
Immersion challenge	ND	Non-specific signs	10 - 20	Mild to moderate intestinal damage (-)	No detectable lesions (-)	Intestinal submucosa	ND	+	-	+	+/-
Immersion with skin trauma	ND	Ulcers at incision site	30 - 40	Mild to moderate intestinal damage (-)	Granuloma formation at incision site (+/-)	Intestinal submucosa	Core of granulomas	+	-	+	+/-

*The percentage of fish showed macroscopic symptoms of chronic disease such as emaciation and deterioration of aesthetics.

**Within the parentheses indicates the presence of granulomas

+, positive: one or more of the tested samples were positive. -, negative: none of the tested samples was positive. +/-, indicates that positive results are not present in all exposure groups, *e.g.,* some groups infected with low dose of bacteria (*i.e.,* 10^5 or 10^6) have no positive results.

ND, not determined. Moribund, fish that were acutely infected within a week (1 wpi). Survivors, fish that were alive at the end of the study period (*i.e.,* 4 wpi)

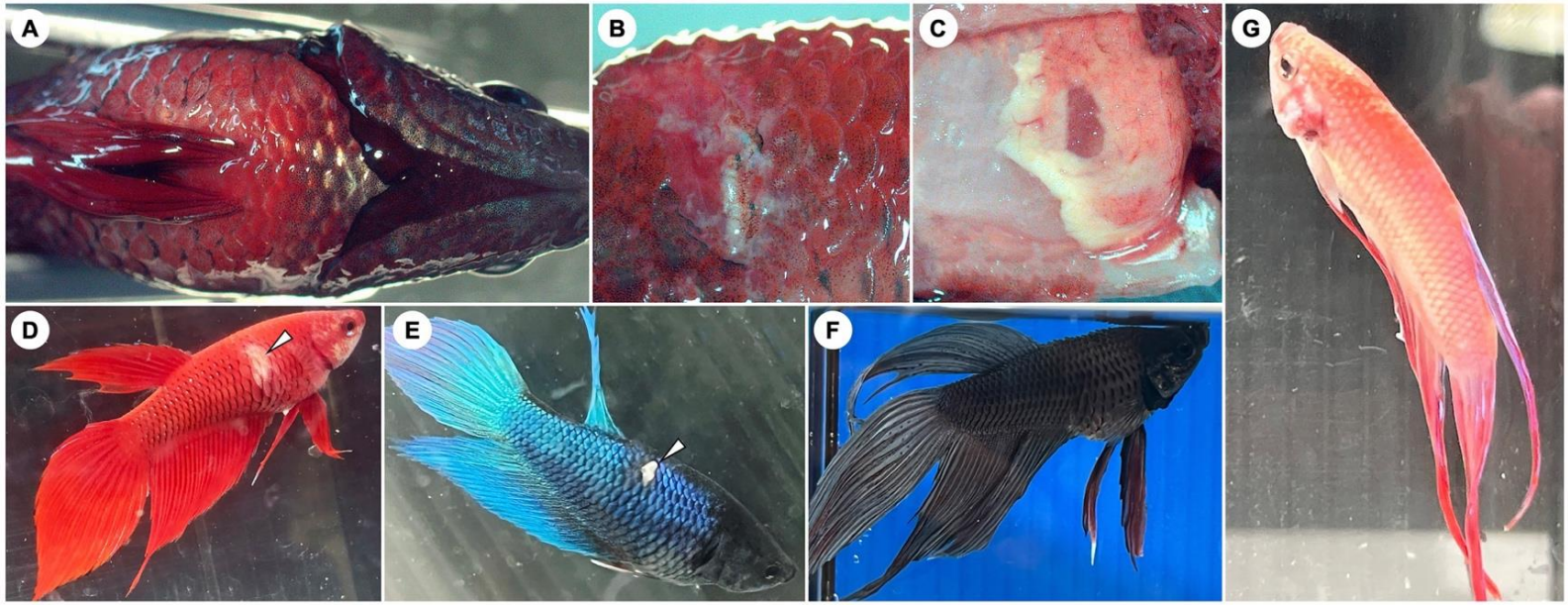

Figure 4.1. Representative gross pathology observed in *Betta splendens* exposed to *Mycobacterium chelonae* by various routes. Mortality occurred 24-48 h after infection and was typified by abdominal distention following IP injection (A) and skin ulceration at the site IM injection (B). Acutely deceased fish of all infection modes exhibited severe necrosis of visceral organs with ascitic fluid at necropsy (C). Skin ulcers appeared on the injured epidermis areas following immersion (arrowhead in Fig. D). Skin nodules formed at the incision sites at the conclusion of the experiment (arrowhead in Fig. E). Chronic disease, some fish in the injection and oral groups showed clinical signs of “big belly” (Fig. F), while a significant proportion of fish became emaciation, lecked vitality, and aesthetic deterioration, regardless of infection routes (G).

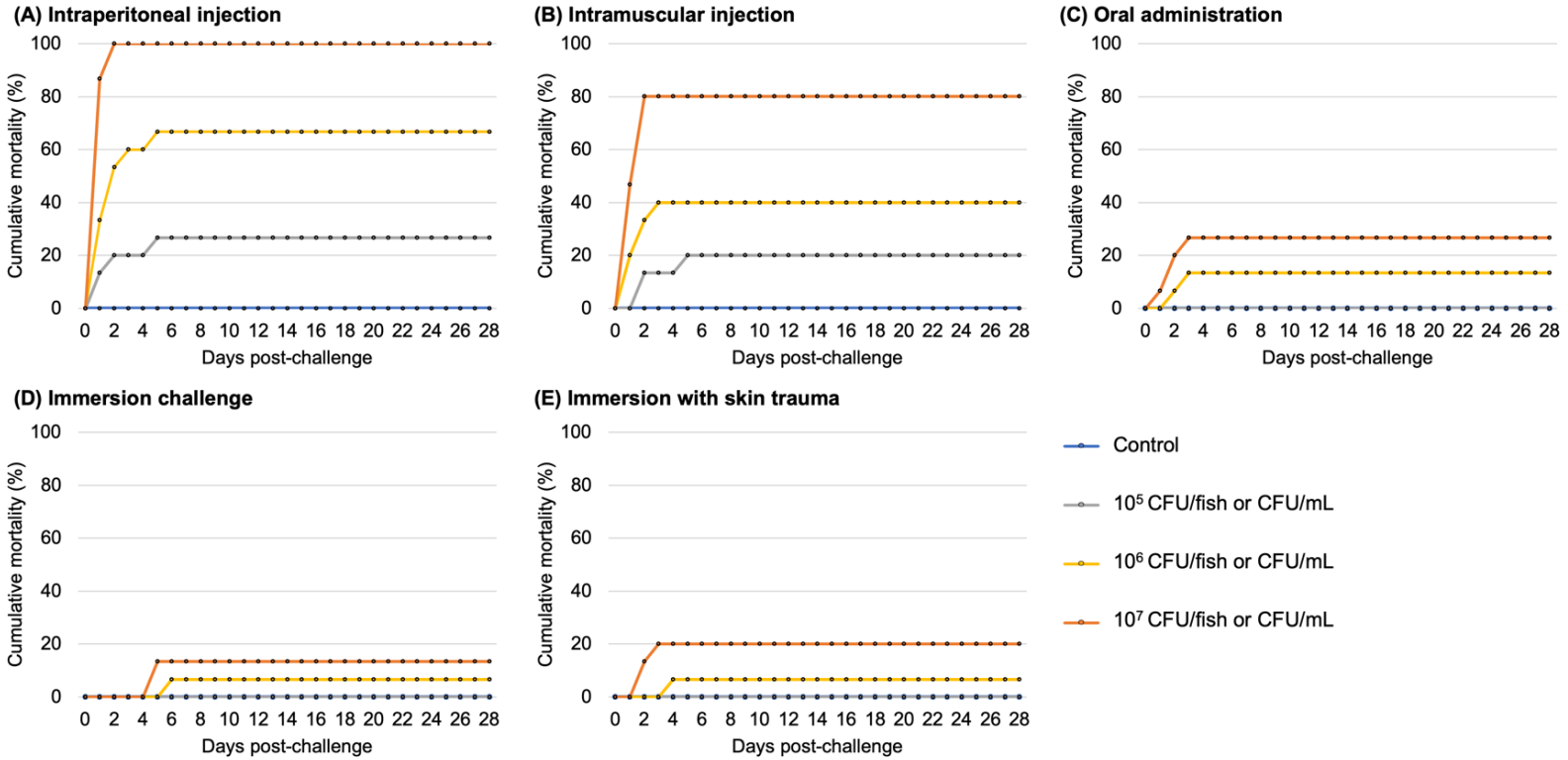

Figure 4.2. Cumulative mortality recored in *Betta splendens* exposed to *Mycobacterium chelonae* by various routes. Acute mortality was observed in fish in both groups challenged via IP injection (A) and IM injection (B). Mortality was very low in fish treated orally (C) and either by immersion (D) or immersion with skin trauma (E).

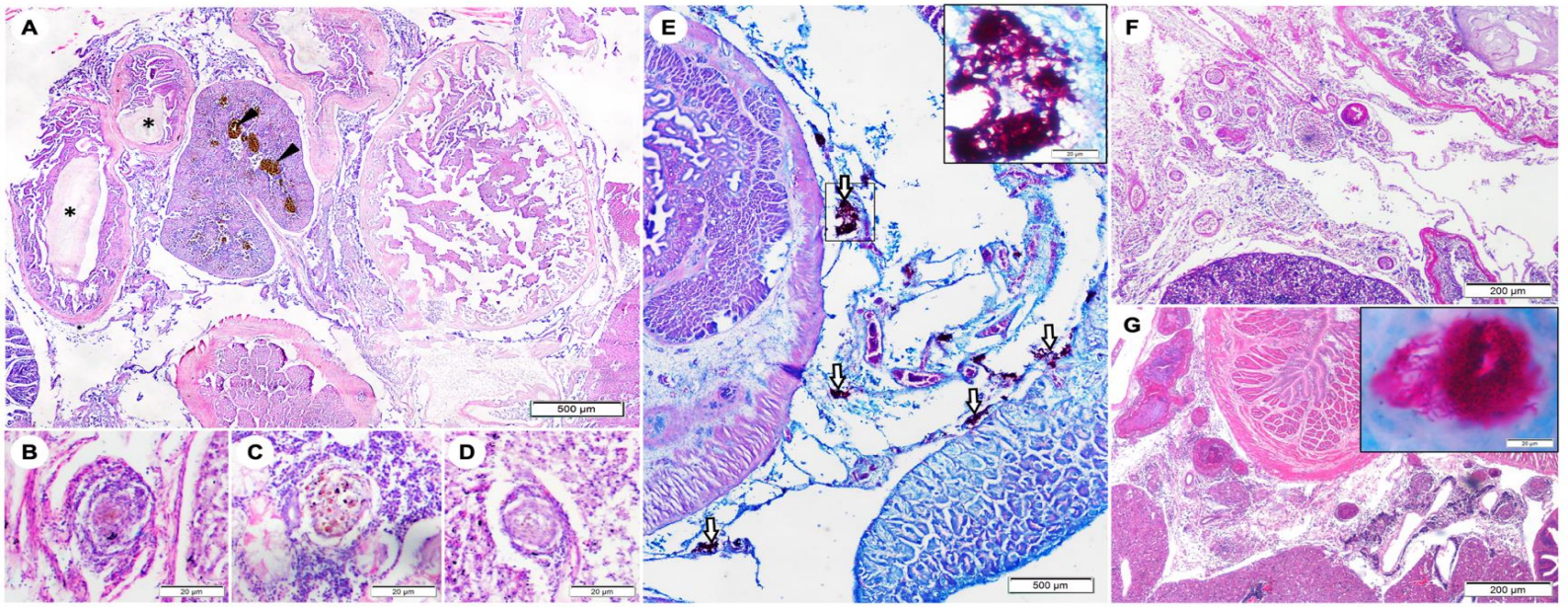

Figure 4.3. Representative histopathology of *Betta splendens* exposed to *Mycobacterium chelonae* by intraperitoneal injection. Acutely deceased fish exhibited severe damage to visceral organs, with marked peritonitis and tissue necrosis (A), accompanied by increased numbers of MMCs in the spleen (arrowheads) and exudate (asterisks). Early granuloma formation in several organs, including in the peritoneum (B), kidney (C), and liver (D). A large number of bacterial cells were detected in the peritoneal cavity (white arrows in Fig. E) and high magnification (inset) revealed numerous acid-fast rod-shaped cells. Surviving fish showed numerous granulomas in the visceral organs, particularly dense formation in the peritoneum (F and G). Acid-fast stained section shows numerous rod-shaped bacterial cells in the core of a granuloma (inset in Fig. G). H&E staining (A, B, C, D, F, and G). Acid-fast staining (E). Scale bars are shown in the pictures.

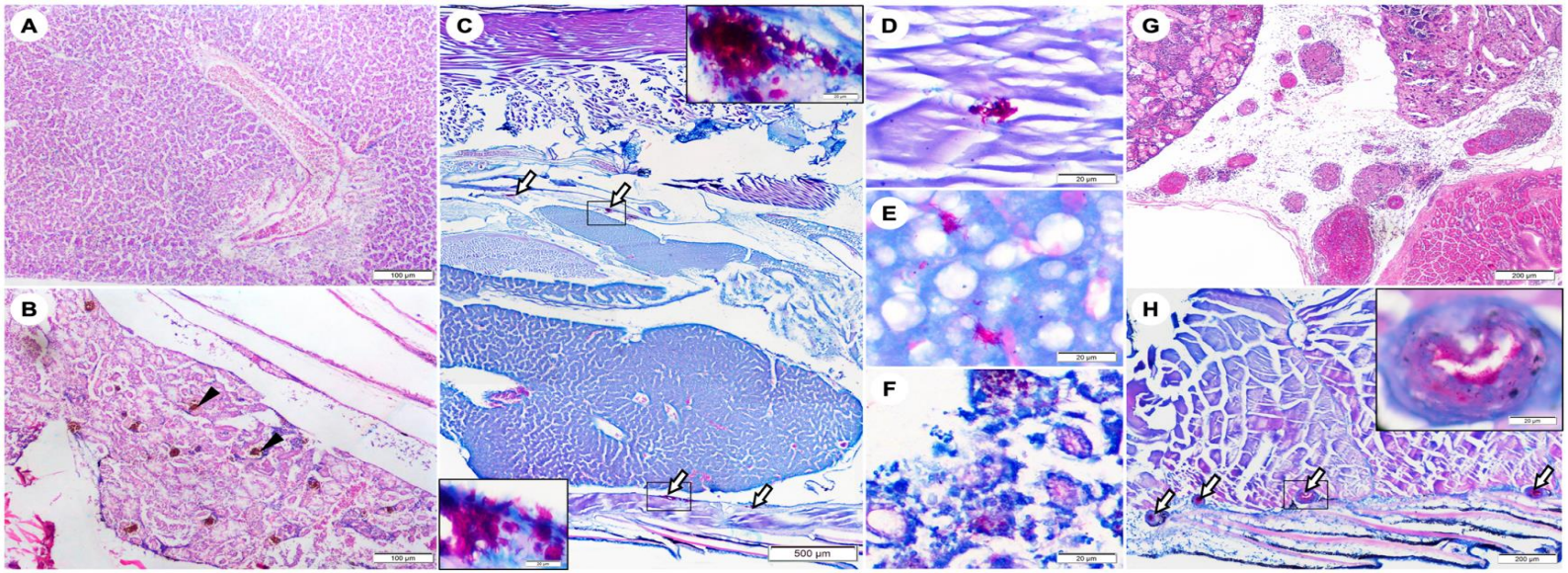

Figure 4.4. Representative histopathology of *Betta splendens* exposed to *Mycobacterium chelonae* by intramuscular injection. Acutely dead fish showed moderate damage with necrosis of several visceral organs including the liver (A) and kidney, accompanied by increased numbers of MMCs (arrowheads in Fig. B). A large number of bacterial cells were detected mainly near the spine and abdominal muscles (white arrows in Fig. C) and high magnification (insets) revealed numerous acid-fast rod-shaped cells. A small number of bacterial cells were scattered in the muscles (D), kidney (E), and liver (F). Surviving fish revealed numerous granulomas in visceral organs (G). Notably, the granulomas were also sporadically found in the muscles (white arrows in Fig. H). Acid-fast stained section showed numerous rod-shaped bacterial cells in the core of a granuloma (inset in Fig. H). H&E staining (A, B, and G). Acid-fast staining (C, D, E, F, and H). Scale bars are shown in the pictures.

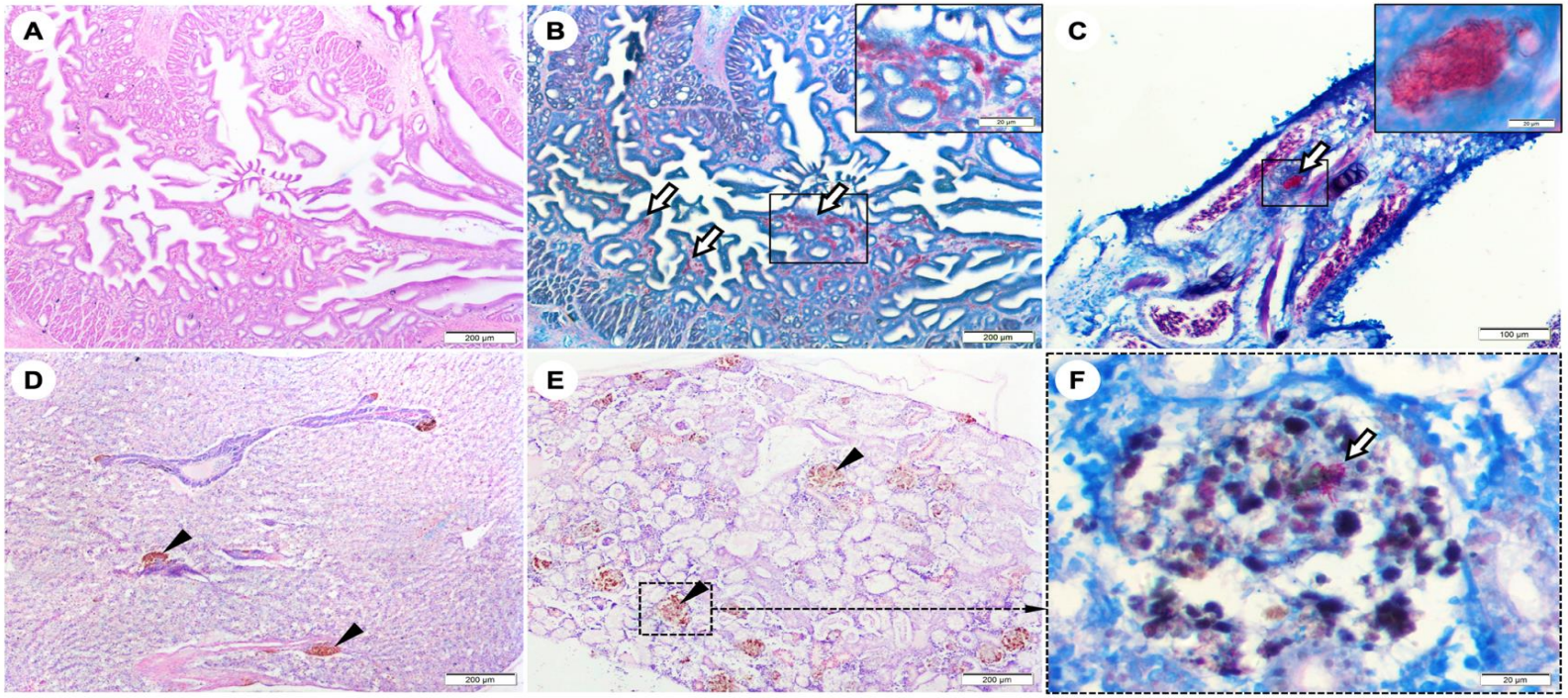

Figure 4.5. Representative histopathology of *Betta splendens* exposed to *Mycobacterium chelonae* by oral exposure. Acutely dead fish showed severe intestinal damage (A) with numerous acid-fast rod-shaped bacteria in the intestinal submucosa (white arrows and inset in Fig. B). The bacteria were occasionally detected in the gill filaments of some acutely dead fish (arrowhead and inset in Fig. C). Surviving fish showed increased numbers of MMCs in several organs such as the liver (D) and kidney (E). Acid-fast rod-shaped bacterial cells were sometimes detected in these MMCs (white arrows in Fig. F). H&E staining (A, D, and E). Acid-fast staining (B, C, and F). Scale bars are shown in the pictures.

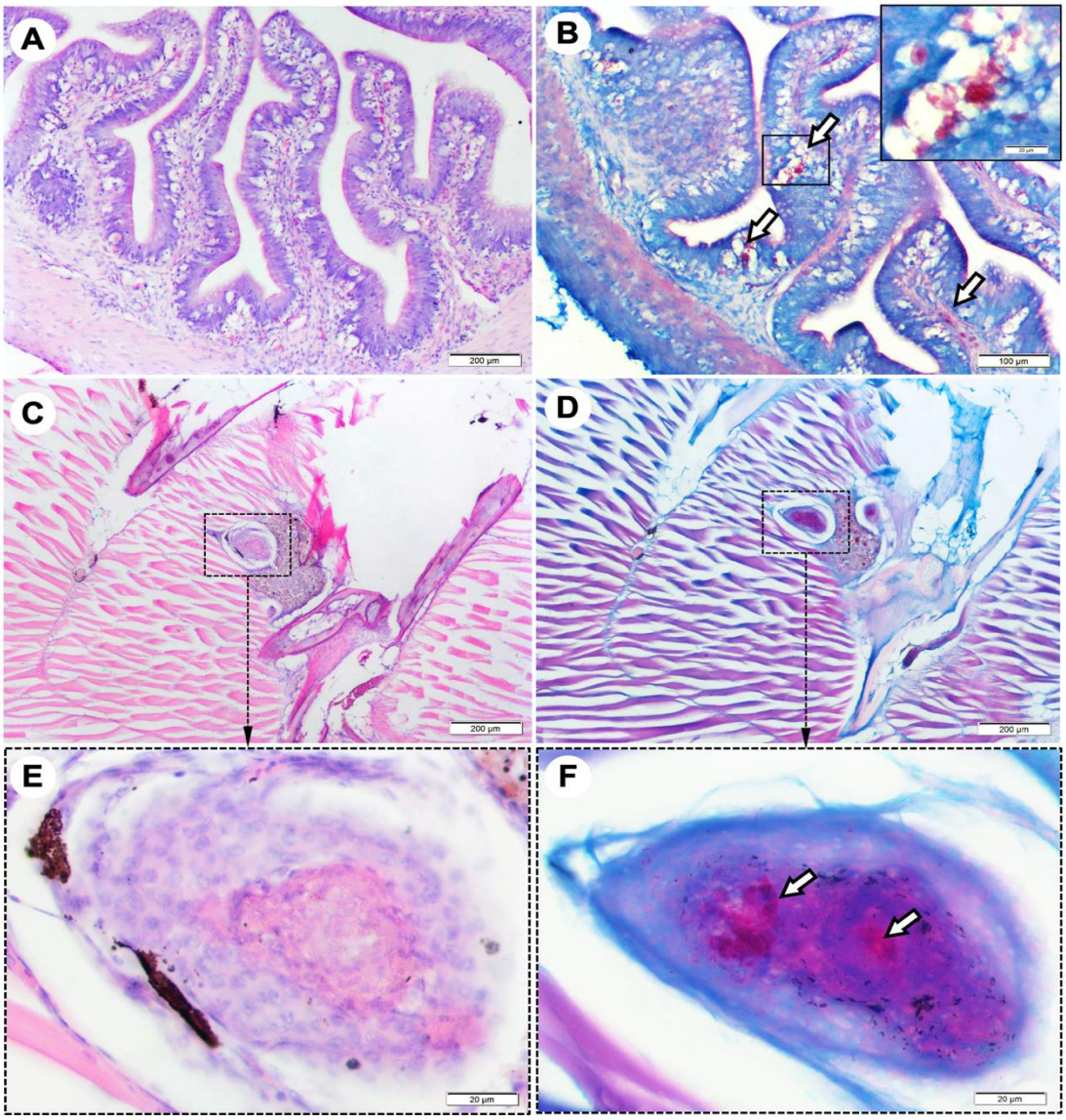

Figure 4.6. Representative histopathology of *Betta splendens* exposed to *Mycobacterium chelonae* by immersion with and without skin trauma. Acutely dead showed mild to moderate intestinal damage (A), with numerous acid-fast rod-shaped bacteria in the intestinal submucosa (white arrows and inset in Fig B). No specific lesions indicative of infection was found in immersion-infected fish, except for granuloma formation at the previous injury site in fish from immersion skin trauma (C and E) with numerous acid-fast rod-shaped cells in the core (white arrows in Fig F). H&E staining (A, C, and E). Acid- fast staining (B, D, and F). Scale bars are shown in the pictures.

4.8. Supplementary data

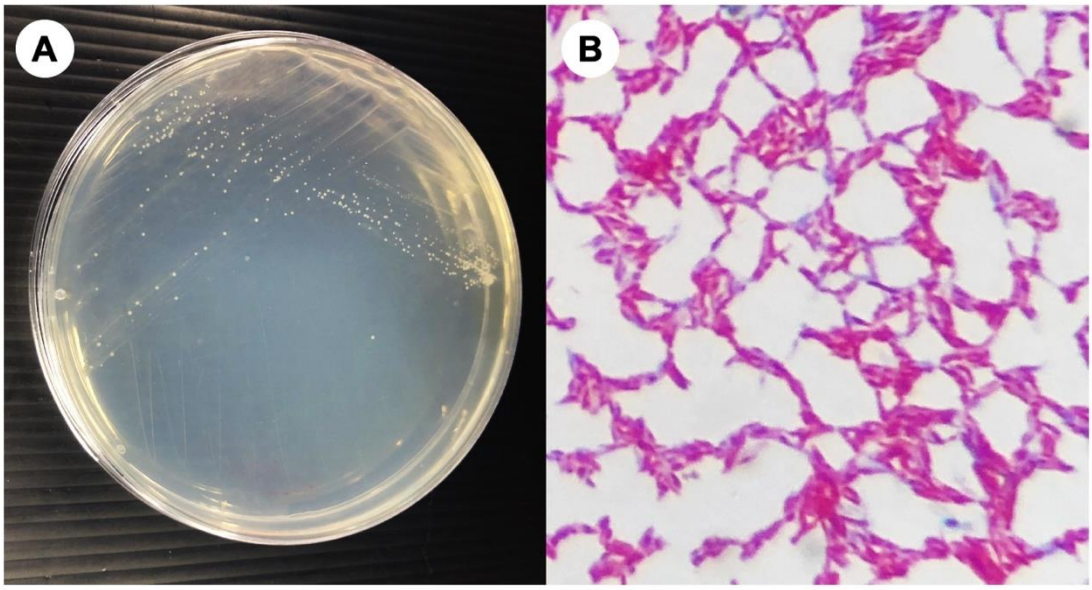

Figure S4.1. Internal organs were aseptically collected from representative fish from all treatment and control groups, spread onto Middlebrook 7H11 agar plates, and incubated at 30°C for up to 4 days (A). Selected bacterial colonies underwent acid-fast staining, and the positive detection of *Mycobacterium chelonae* was confirmed by the bright red staining of the acid-fast bacilli (B).

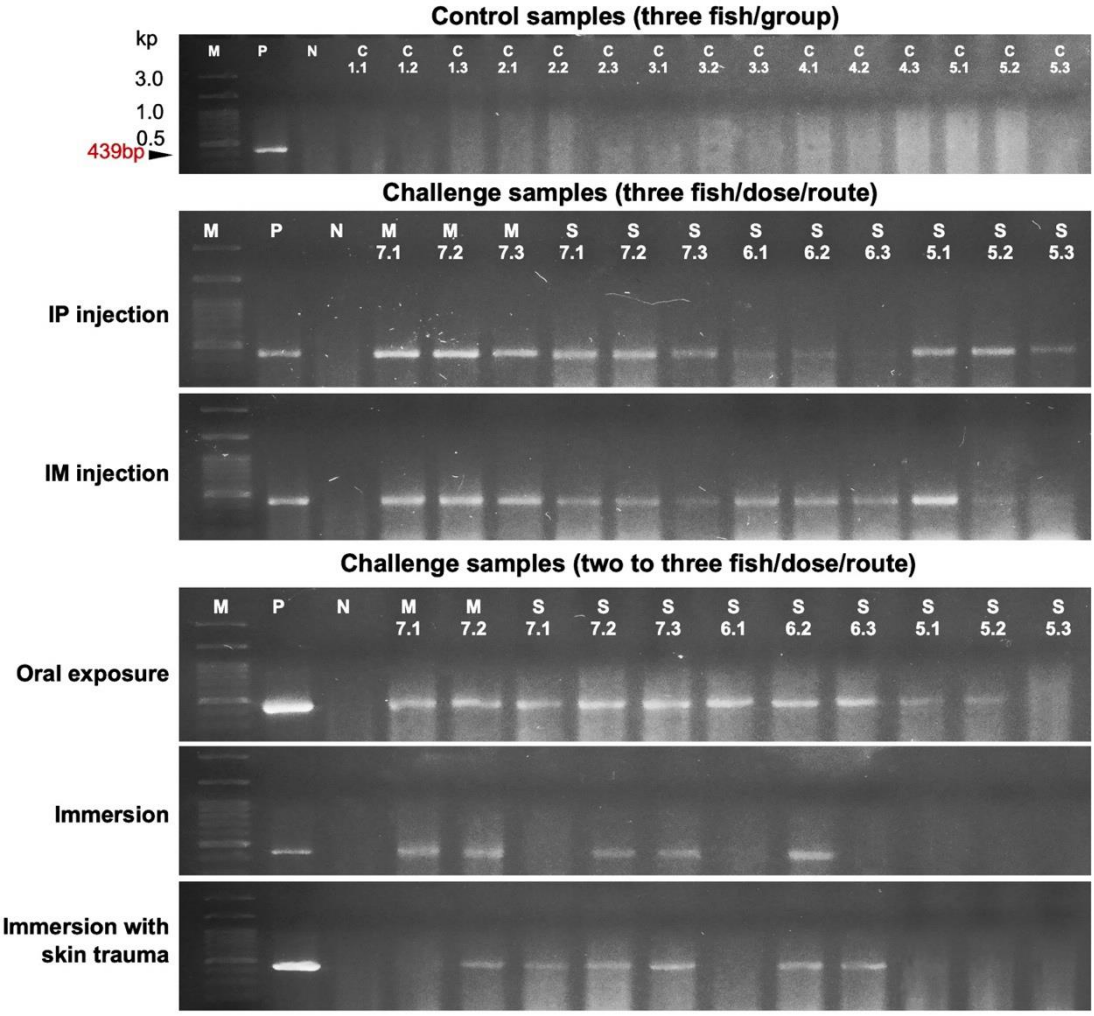

Figure S4.2. PCR was performed on pooled internal organs of representative fish from treatment and control groups. Left from right with M, DNA marker (New England Biolabs, UK); P, positive control with DNA extracted from *Mycobacterium chelonae*; N, no template control. C, control fish treated with PBS; M, sample from moribund fish; S, sample from surviving fish; the first ordinal number represents the log dose of infection, and the following numbers indicate the order of fish samples.

4.9. References

Agius C and Roberts RJ 2003. Melano-macrophage centres and their role in fish pathology. J Fish Dis. 26(9): 499-509.

Arakawa CK and Fryer J 1984. Isolation and characterization of a new subspecies of *Mycobacterium chelonei* infectious for salmonid fish. Helgoländer Meeresuntersuchungen. 37: 329-342.

Awuh JA and Flo TH 2017. Molecular basis of mycobacterial survival in macrophages. Cell Mol Life Sci. 74(9): 1625-1648.

Belas R, Faloon P and Hannaford A 1995. Potential applications of molecular biology to the study of fish mycobacteriosis. Annu. Rev. Fish Dis. 5: 133-173.

Beran V, Matlova L, Dvorska L, Svastova P and Pavlik I 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. J Fish Dis. 29(7): 383-393.

Delghandi MR, El-Matbouli M and Menanteau-Ledouble S 2020. Mycobacteriosis and infections with non-tuberculous mycobacteria in aquatic organisms: A review. Microorganisms. 8(9).

Dinh-Hung N, Dong HT, Senapin S, Pimsannil K, Thompson KD, Shinn AP, Soontara C, Sirimanapong W, Chatchaiphan S and Rodkhum C 2023. Insight into characteristics and pathogenicity of five rapidly growing non-tuberculous *Mycobacterium* species isolated from the Siamese fighting fish, *Betta splendens*. Aquaculture. 739822.

Dinh-Hung N, Dong H.T, Taengphu S, Soontara C, Rodkhum C, Senapin S, Chatchaiphan S 2023b. *Streptococcus suis* is a lethal pathogen in snakeskin gourami, *Trichopodus pectoralis*. *Aquaculture* 739173.

Dong HT, Senapin S, Phiwsaiya K, Techatanakitarnan C, Dokladda K, Ruenwongsa P and Panijpan B 2018. Histopathology and culturable bacteria associated with “big belly” and “skin nodule” syndromes in ornamental Siamese fighting fish, *Betta splendens*. Microb Pathog. 122: 46-52.

Dowdell K, Haig SJ, Caverly LJ, Shen Y, LiPuma JJ and Raskin L 2019. Nontuberculous mycobacteria in drinking water systems – The challenges of characterization and risk mitigation. Curr Opin Biotechnol. 57: 127-136.

Esteban J and García-Coca M 2018. Mycobacterium biofilms. Front Microbiol. 8: 2651.

Falkinham III JO 2021. Ecology of nontuberculous mycobacteria. Microorganisms. 9(11): 2262.

Falkinham JO 2015. Environmental sources of nontuberculous mycobacteria. Clin. Chest Med. 36(1): 35-41.

Francis-Floyd R 2011. Mycobacterial infections of fish. In: Southern Regional Aquaculture Center USA.

Fukano H, Wada S, Kurata O, Mizuno K, Nakanaga K and Hoshino Y 2015. Nontuberculous mycobacteriosis in farmed thread-sail filefish *Stephanolepis cirrhifer*. 魚病研究. 50(2): 68-74.

Gauthier DT and Rhodes MW 2009. Mycobacteriosis in fishes: A review. Vet J. 180(1): 33-47.

Goldstein RJ 2015. The betta handbook. In: Sourcebooks, Inc.

Gómez Manrique W, Claudiano G, Petrillo T, Castro M, Figueiredo M, Belo MA, Moraes J and Moraes F 2014. Response of splenic melanomacrophage centers of *Oreochromis niloticus* (Linnaeus, 1758) to inflammatory stimuli by BCG and foreign bodies. J. Appl. Ichthyol. 30: 1001.

Gudding R, Lillehaug A and Evensen 1999. Recent developments in fish vaccinology. Vet. Immunol. Immunopathol. 72(1): 203-212.

Harriff MJ, Bermudez LE and Kent ML 2007. Experimental exposure of zebrafish, *Danio rerio* (Hamilton), to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: A potential model for environmental mycobacterial infection. J Fish Dis. 30(10): 587-600.

Klesius PH, Shoemaker CA and Evans JJ 2000. Efficacy of single and combined *Streptococcus iniae* isolate vaccine administered by intraperitoneal and intramuscular routes in tilapia (*Oreochromis niloticus*). Aquaculture. 188(3-4): 237-246.

Le Dantec C, Duguet J-P, Montiel A, Dumoutier N, Dubrou S and Vincent V 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl. Environ. Microbiol. 68(11): 5318-5325.

Lichak MR, Barber JR, Kwon YM, Francis KX and Bendesky A 2022. Care and Uue of Siamese fighting fish (*Betta Splendens)* for research. Comp Med. 72(3): 169-180.

Maceda-Veiga A and Cable J 2019. Diseased fish in the freshwater trade: From retailers to private aquarists. Dis Aquat Organ. 132(2): 157-162.

Machida Y, Tang BCC, Yamada M, Sato S, Nakajima K, Matoyama H, Kishihara T, Endo M, Sano M and Kato G 2021. Mycobacteriosis in cultured Koi carp *Cyprinus carpio* caused by *Mycobacterium paragordonae* and two *Mycolicibacterium* spp. Aquaculture. 539: 736656.

Martínez-Lara P, MartínezPorchas M, Gollas-Galván T, Hernández-López J and Robles-Porchas GR 2021. Granulomatosis in fish aquaculture: A mini review. Rev Aquac. 13(1): 259-268.

Monvises A, Nuangsaeng B, Sriwattanarothai N and Panijpan B 2009. The Siamese fighting fish: Well-known generally but little-known scientifically. ScienceAsia. 35: 8-16.

Narendrakumar L, Sudhagar A, Preena PG, Nithianantham SR, Mohandas SP and Swaminathan TR 2022. Detection of *Mycobacterium marinum* and multidrug-resistant bacteria in a chronic progressive disease outbreak among Siamese fighting fish (*Betta splendens*) in India. Biologia. 77(9): 2725-2733.

Orós J, Priestnall SL, & Suárez-Bonnet A 2022. Histopathological description of iridophoromas resembling skin nodule syndrome in Siamese fighting fish *Betta splendens*. Dis. Aquat. Org. 151, 23-27.

Pavlik I, Ulmann V, Hubelova D and Weston RT 2022. Nontuberculous mycobacteria as sapronoses: A review. Microorganisms. 10(7): 1345.

Pleeging C and Moons CPH 2017. Potential welfare issues of the Siamese fighting fish (*Betta splendens*) at the retailer and in the hobbyist aquarium. Vlaams Diergeneeskundig Tijdschrift. 86: 213-223.

Rahmati-Holasoo H, Pedram, Mousavi H, Shokrpoor S, Lewbart G, Azizi A, & Mohammadian RA 2019. Malignant iridophoroma in a male Siamese fighting fish (*Betta splendens* Regan): A clinical, surgical and histopathological study. Bull. Eur. Assoc. Fish Pathol. 39, 106-113.

Rahmati-Holasoo H, Shahbazi M, Mousavi H, Shokrpoor S, Pourmortazavi BM, Azizi A, & Mokhatari A 2020. Polycystic kidney disease in discus (*Symphysodon aequifasciatus*) and Siamese fighting fish (*Betta splendens*): A histopathological study. Bull. Eur. Assoc. Fish Pathol. 40, 2020.

Rajme-Manzur D, Gollas-Galván T, Vargas-Albores F, Martínez-Porchas M, Hernández-Oñate MÁ and Hernández-López J 2021. Granulomatous bacterial diseases in fish: An overview of the host's immune response. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 261: 111058.

Ramakrishnan L 2012. Revisiting the role of the granuloma in tuberculosis. Nat. Rev. Immunol. 12(5): 352-366.

Reed LJ and Muench H 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27(3): 493-497.

Sangari FJ, Goodman J, Petrofsky M, Kolonoski P and Bermudez LE 2001. *Mycobacterium avium* invades the intestinal mucosa primarily by interacting with enterocytes. Infect Immun. 69(3): 1515-1520.

Senapin S, Phiwsaiya K, Laosinchai P, Kowasupat C, Ruenwongsa P and Panijpan B 2014. Phylogenetic analysis of parasitic trematodes of the genus *Euclinostomum* found in Trichopsis and Betta fish. J. Parasitol. 100(3): 368-371.

Sermwatanakul A 2019. Capacitating the local farmers to enhance global marketing of Thailand's national aquatic animal, the Siamese fighting fish. Fish for the People. 17(2): 42-48.

Sirimalaisuwan A, Teeraruk P, Kanjanapitakchai P, Kaewsakhorn T, Potibut P and Pikulkaew S 2017. Detection of *Mycobacterium marinum* in clinically asymptomatic Siamese fighting fish (*Betta splendens*) from ornamental fish shops in Chiang Mai Province, Thailand. Asian Pac. J. Trop. Dis. 7: 344-346.

Skriwan C, Fajardo M, Hägele S, Horn M, Wagner M, Michel R, Krohne G, Schleicher M, Hacker J and Steinert M 2002. Various bacterial pathogens and symbionts infect the amoeba *Dictyostelium discoideum*. Int J Med Microbiol. 291(8): 615-624.

Talaat AM, Reimschuessel R, Wasserman SS and Trucksis M 1998. Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. Infect Immun. 66(6): 2938-2942.

Telenti A, Marchesi F, Balz M, Bally F, Bottger EC and Bodmer T 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol. 31(2): 175-178.

Uribe C, Folch H, Enríquez R and Moran G 2011. Innate and adaptive immunity in teleost fish: a review. Vet Med. 56(10): 486.

U.S. Fish and Wildlife Service 2019. Subject: Siamese fighting fish (*Betta splendens*) ecological risk screening summary (online). Available: https://www.fws.gov/sites/default/files/documents/Ecological-Risk-Screening-Summary-Siamese-Fighting-Fish.pdf.

Weerakhun S, Sukon P and Hatai K 2019. *Mycobacterium marinum* and *Mycobacterium fortuitum* infections in Siamese fighting fish, *Betta splendens* (Regan), in Thailand. Thai J Vet Med. 49(2): 137-145.

CHAPTER 5

Utilizing ozone nanobubbles to mitigate the risk of mycobacteriosis caused by a multidrug-resistant *Mycobacterium chelonae* in Siamese fighting fish (*Betta splendens*)

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5.1. Highlights

- After incubating for 60 min in ozone nanobubbles (NB-O_3) water, the concentration of *Mycobacterium chelonae* decreased by 96.71% to 99.92%.
- Directly treating farm water with NB-O_3 for 10 min reduced both total bacterial counts and presumptive mycobacteria by over 90%.
- Treating water contaminated with *M. chelonae* using NB-O_3 mitigated the risk of mycobacteriosis and improved survivability of betta fish.
- NB-O_3 offers a promising non-chemical and non-antibiotic strategy for disease control in the betta fish industry.

Keywords: *Mycobacterium chelonae;* Mycobacteriosis; Ozone nanobubbles; Prevention; Siamese fighting fish.

5.2. Abstract

Betta splendens, a valuable ornamental fish species, is particularly susceptible to mycobacteriosis which poses a challenge to the sustainability of its culture and trade. Since there are no effective treatments for the disease, betta fish farms must take rigorous preventive measures. This study investigated the efficacy of ozone nanobubbles (NB-O_3) to disinfect water for mitigating the risk of mycobacteriosis in betta fish. Laboratory tests showed a significant disinfecting effect of NB-O_3 against a highly pathogenic, multidrug-resistant strain of *Mycobacterium chelonae* by destroying bacterial cells. After incubation in NB-O_3 water for 60 min, the concentration of *M. chelonae* in distilled water was reduced by 96.71 to 99.92%. In practice, treatment of reserved and cultured water from betta farms with direct NB-O_3 for a single 10 min significantly reduced total bacterial counts and presumptive mycobacteria by over 90%. In experimental infection, sub-adult betta fish (1-month-old) cultured for 14 days in water spiked with *M. chelonae* (~10^6 CFU/mL), which served as a positive control group, had a low survival rate of 38.33%.

In contrast, fish reared in the same water as the positive control group but treated with NB-O_3 three times for 10 min at 20 min intervals had a significantly higher survival rate of 93.93%. Moreover, the relative percent survival (RPS) of the treatment group showed a statistically significant difference, with an RPS of 89.19% compared to only 67.57% in the negative control group (neither exposure to *M. chelonae* nor treatment with NB-O_3). The results highlight the utility of NB-O_3 as a non-chemical and non-antibiotic approach to control the threat of mycobacteriosis, thereby potentially revolutionizing disease prevention efforts in the betta fish industry.

5.3. Introduction

Siamese fighting fish, scientifically known as *Betta splendens*, commonly referred to as betta, is an iconic freshwater fish species native to Southeast Asia (Monvises et al., 2009; U.S. Fish and Wildlife Service, 2019). The vibrant colouration and graceful flowing fins make bettas not only a highly desirable ornamental fish globally but also a species of considerable economic value (Sermwatanakul, 2019). The fish, however, are particularly susceptible to mycobacteriosis which could pose a major challenge to the long-term sustainability of the industry (Puttinaowarat et al., 2002; Beran et al., 2006; Sirimalaisuwan et al., 2017; Weerakhun et al., 2019; Narendrakumar et al., 2022). Unfortunately, there are currently no vaccines or effective treatments for mycobacterial infections in any fish species, including *B. splendens* (see Chong, 2022). It is worth mentioning that mycobacteriosis is an important fish disease and also a zoonosis caused by various species of non-tuberculous mycobacteria (NTM), that the pathogens are ubiquitous and live freely in the aquatic environment (Gauthier & Rhodes, 2009). Furthermore, the emergence of highly pathogenic and multidrug-resistant NTM strains isolated from betta fish has raised concern, both in terms of negative economic impact and public health risks (Dinh-Hung et al., 2023). Thus, the control of mycobacteriosis in fish, including betta fish, underscores the paramount importance of implementing disease prevention

strategies through quarantine measures and water disinfection procedures (Francis-Floyd, 2011). There are, however, currently no non-lethal diagnostic methods for screening mycobacteriosis in betta fish. On the other hand, chemical disinfectants are not always a good option due to their potential to cause residual toxicity (Pandian et al., 2022). Also, the waxy coating in the cell wall of NTM could limit the efficacy of many conventional disinfectants (Francis-Floyd, 2011). There is therefore a need to explore alternative and environmentally sustainable strategies to effectively address such challenges and control mycobacteriosis in betta fish farms.

Ozone (O_3), an effective oxidant, has gained acceptance in various water treatment applications due to its ability to disinfect, deodorize, and remove organic and inorganic contaminants (Langlais et al., 2019). The practical application of ozone in aquaculture, however, is limited by its low stability, poor solubility, and potentially harmful effects on aquatic organisms (Powell & Scolding, 2018). Recent advances in the aquaculture sector have sparked interest in using ozone in the form of nanobubbles (NB), which offer a host of advantages over conventional ozone delivery systems, including improved solubility, increased stability, and prolonged ozone delivery (Gurung et al., 2016; Atkinson et al., 2019; Fan et al., 2020). Nanobubble technology involves introducing nanobubbles (<100 nm) containing a selected gas into water (Agarwal et al., 2011; Atkinson et al., 2019), where these structures exhibit neutral buoyancy and high solubility due to their considerable surface-to-volume ratio (Gurung et al., 2016). Nanobubbles with their minuscule size and negative surface charge enable better dispersion in water due to mutual repulsion (Marcelino et al., 2023). Ozone, when present in nanobubble form, remains stable for a longer time and allows rapid reactions to degrade pollutants while increasing the concentration of dissolved oxygen in water (Seridou & Kalogerakis, 2021; Pal et al., 2022). Moreover, produced oxygen-free radicals have higher reactivity and oxidation potential than widely used disinfectants such as chlorine (Takahashi et

al., 2007). As a result, ozone nanobubbles (NB-O_3) can improve water quality by reducing pathogen loads, optimizing dissolved oxygen levels, and restricting harmful disinfection byproducts (Atkinson et al., 2019; Marcelino et al., 2023). The use of NB-O_3, therefore, may serve as a promising approach to deal with mycobacterial infections in betta fish, although its potential has not yet been explored.

Thus, this study aims to investigate the efficacy of NB-O_3 in treating water for culturing betta fish, focusing on the prevention of mycobacteriosis. At a laboratory scale, we evaluated the disinfectant properties of NB-O_3 against a multidrug-resistant (MDR) *Mycobacterium chelonae* strain, a highly pathogenic species to betta fish, by measuring reduction in bacterial load and observing destruction of bacterial cells. Furthermore, we assessed the actual effects of this treatment method in two betta fish farms by examining the reduction in total bacterial counts and presumptive mycobacterial load in both reserved and cultured water. Simultaneously, we conducted an evaluation of the NB-O_3 potential to mitigate mycobacteriosis risk by studying its effect on *M. chelonae*-contaminated water in terms of reducing bacterial concentrations and resulting impact on fish health between NB-O_3 treated group and control cohorts. All these multi-faceted approaches are expected to provide a comprehensive understanding of the potential and efficacy of NB-O_3 as a water treatment method to mitigate the risk of mycobacteriosis in Siamese fighting fish.

5.4. Materials and methods

5.4.1. Bacterial preparation and nanobubbles system setup

The *Mycobacterium chelonae* isolate used in this study, namely *M. chelone* BN 1983 (Genbank accession no. MG438529), was isolated from betta fish and characterized in our previous studies (Dong et al., 2018; Dinh-Hung et al., 2023). Notably, this isolate was found to be highly pathogenic and multidrug-resistant, resisting 11 out of 18 antibiotics tested, representing seven of nine antimicrobial

classes tested (Dinh-Hung et al., 2023). Briefly, the stored isolate strain at -80°C was thawed and sub-cultured in Middlebrook 7H11 agar (HiMedia, India) containing 10% OADC growth supplement (*i.e.*, Oleic Albumin Dextrose Catalase), and incubated at 30°C for 96 h. Single colonies were then cultivated in Middlebrook 7H9 broth (HiMedia, India) containing 10% ADC (*i.e.*, Albumin Dextrose Catalase) with constant shaking at 250 rpm for 48 h at 30°C. The resulting bacterial suspension was pelleted by centrifugation at 5,000 g for 5 min, washed twice, and adjusted in 1× phosphate-buffered saline (PBS) at pH 7.4 to achieve an OD_{600} of 1.0, corresponding to a concentration of approximately 10^8 CFU/ml (referred to as "bacterial stock solution"). The bacterial stock solution was then diluted with 1× PBS to obtain the appropriate concentrations for the intended use. The nanobubble system (model: aQua+075MO, AquaPro Solutions Pte Ltd., Singapore) was set up as previously described (Jhunkeaw et al., 2021) and as shown in Figure 5.1A. Water parameters, including temperature (T°), pH, dissolved oxygen (DO), and oxidation-reduction potential (ORP), were measured during the experiment using a multiparameter (YSI Professional Plus, YSI Incorporated, USA).

5.4.2. Effect of NB-O_3 on mycobacteria disinfection at laboratory

The disinfectant properties of NB-O_3 against *M. chelonae* were investigated in a laboratory-scale study with three replicates, as illustrated in Figure 5.1B. Given the highly pathogenic and potentially zoonotic nature of the pathogen, the study was conducted by incubating bacterial suspension in 10 L Duran bottles (DWK Life Sciences, Germany) at room temperature. A volume of 50 mL bacterial stock solution was diluted 100 fold in 4,950 mL of distilled water, resulting in a concentration of 2.23×10^6 CFU/mL, as determined by conventional plate counting (referred to as "control" or "before treatment"). The treatment group was prepared in the same manner, but the water used was treated with NB-O_3 for 10 min. Water parameters were measured both during the NB-O_3 run and incubation. At different time points

(*i.e.*, 10, 20, 30, and 60 min), a pooled 3 mL of water was collected after gently shaking the bottle three times (collecting 1 mL at a time) to ensure homogeneity and used for conventional plate count enumeration. The water samples were subjected to ten-fold serial dilutions with 1× PBS and then 100 µL of each dilution was applied in duplicate to Middlebrook 7H11 agar plates containing 10% OADC growth supplement. After incubation at 30°C for 96 h, the dilutions with colony counts between 30 and 300 were selected for enumeration. The mean bacterial concentration determined from two replicate plates was expressed as CFU/mL. The disinfecting properties were evaluated by comparing the bacterial reduction between the samples incubated in distilled water and those in NB-O_3 water. The percentage of bacterial reduction was calculated using the following equation of Jhunkeaw *et al.* (2021):

$$\%\ \text{reduction} = 100 \times \left(1 - \frac{\text{Mean bacterial count } \frac{\text{CFU}}{\text{mL}} \text{ after treatment}}{\text{Mean bacterial count } \frac{\text{CFU}}{\text{mL}} \text{ before treatment}}\right)$$

Scanning electron microscopy (SEM) was used to study the ultrastructure of the bacterial cells. After an incubation period of 60 min, 500 mL of water was collected from both the control and NB-O_3 treated groups, and the bacteria were harvested by centrifugation at 6,000 g for 10 min. The resulting pellet was resuspended in 0.5 mL of 1× PBS solution and spread on poly-L-lysine-coated coverslips (Sigma-Aldrich, USA). After air drying for 3 h, the samples were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, followed by dehydration with a graded ethanol series. The samples were then dried under CO_2 in a critical point dryer (Hitachi, Japan) and coated with a gold layer by sputtering. Ultrastructural comparisons of the bacteria between the control group and the group after NB-O_3 treatment were examined and documented using a scanning electron microscope (Hitachi, Japan).

5.4.3. Effect of NB-O_3 treatment on bacterial disinfection in farms

The present study also aimed to evaluate the disinfectant properties of NB-O_3 in farm-scale settings (see Figure 5.1C, S5.1). The evaluation was performed for both reserved water (*i.e.*, water stored in reserve tanks for later use in fish culture) and cultured water (*i.e.*, water from fish-culture tanks). The cultured water was obtained from tanks housing sub-adult bettas (30-45 days old) and containing organic matter such as fish feces, mucus, food residues, and unknown aquatic bacterial flora. An experiment was performed with two fiberglass tanks, each containing 50 L of water from the reserved and the cultured source, exposed to NB-O_3 for 10 min. Water was sampled before and 20 min after NB-O_3 treatment to determine total bacteria using plate count agar (HiMedia, India) and presumptive mycobacteria using Middlebrook 7H11 agar enriched with 10% OADC growth supplement. The samples were collected from the four corners and center of the tank (1 mL per sampling point), which were then pooled and subjected to a ten-fold serial dilution with 1× PBS, and then 100 µL of each dilution was applied in duplicate to the two types of agar plates. The plates were incubated at 30°C for up to 7 days, and dilutions yielding between 30 and 300 colonies were selected for enumeration. The disinfectant properties were evaluated by comparing bacterial counts before and after treatment. Water parameters were monitored during and after treatment. The study was performed in two separate betta fish farms, with each farm having two replicates of the treatment.

5.4.4. Effect of NB-O_3 on prevention of mycobacteriosis in betta fish

To assess the potential of NB-O_3 in preventing mycobacteriosis, an experimental challenge was conducted in which bettas were reared in water contaminated with mycobacteria. The sub-adult betta fish (30 days old) and reserved water were obtained from the second farm (*i.e.*, Farm 2) studied in Section 5.4.3. The experiment was carried out in a designated area near the farm under the same

environmental conditions, and strict measures were taken to control contamination and prevent the spread of pathogens. Three groups were established, each with 30 fish in a volume of 50 L of water, including negative controls, positive controls, and treatments (Figure 5.1D). The positive control group was prepared by spiking water with an aliquot of the *M. chelonae* stock suspension (~10^8 CFU/mL) at a ratio of 1 bacteria/ 99 water (*v/v*), whereas the negative control group used water without any *M. chelonae* added. For the treatment group, *M. chelonae* was added in the same manner as in the positive control group, followed by three times of 10 min NB-O_3 exposures at 20 min intervals. Experimental fish were introduced 60 min after the water treatment process was completed. This experiment involved multiple direct exposures to NB-O_3 to achieve optimum bacterial reduction and to evaluate suitability of the treated water for fish farming. Enumeration of total bacteria and presumptive mycobacteria before and after NB-O_3 treatment was performed using conventional plate counting method as described above. The cohabitation experiment ran for 14 days with 50% of water in each group replaced on day 10^{th}. Mortality was recorded daily and re-isolation of *M. chelonae* from internal organs of challenged fish was performed using Middlebrook 7H11 agar plates containing 10% OADC. The experiment was conducted in duplicate, and prophylactic efficacy was evaluated by comparing fish survivability between the treatment and control cohorts. The relative percent survival (RPS) was calculated using the following equation described by Amend (1981):

$$\text{RPS} = 100 \times (1 - \frac{\%\ \text{mortality in challenge}}{\%\ \text{mortality in control}})$$

5.4.5. Statistical analysis

Reported values for water parameters, bacterial counts, and fish mortality are given as mean ± standard deviation (SD). Data on the reduction of mycobacteria in the laboratory experiment were analyzed by one-way analysis of variance (ANOVA),

and significant differences between the means of the treatments were confirmed by Duncan's multiple range test with a significance level of *p*-value < 0.05. The Kaplan-Meier method was employed to determine cumulative survival in the experimental challenge. A log-rank test was used to compare survival between the different groups, with *p*-values of 0.05 or less considered statistically significant differences. All statistical analyses were conducted using SPSS version 22 (IBM Corp., USA).

5.4.6. Ethics statement

All water and equipment exposed to *M. chelonae* during the study were treated with chlorine at a concentration of 2% (*v/v*) for at least 60 min before disposal, as recommended in a previous study (Dinh-Hung et al., 2023). The experimental procedures and use of animals were approved by the Institutional Animal Care and Use Committee of Kasetsart University (approval ID: ACKU65-FIS-001).

5.5. Results

5.5.1. NB-O_3 treatment has bactericidal properties against mycobacteria

After treatment with NB-O_3 for 10 min, the ORP of the water reached 948.42 ± 4.28 mV (Figure S5.2) and the treated water was able to reduce the number of *M. chelonae* by 30.41 fold, from an initial 2.23×10^6 CFU/mL to $7.33\times10^4 \pm 2.83\times10^4$ CFU/mL (*i.e.*, 96.71% reduction) after a 10 min incubation period. When the incubation time was extended to 20 and 30 min, the bacterial counts decreased dramatically by 126.23 and 806.02 fold to $1.77\times10^4 \pm 9.29\times10^3$ CFU/mL and $2.77\times10^3 \pm 8.62\times10^2$ CFU/mL, respectively, corresponding to a reduction of 99.21% and 99.88%. Statistically significant differences were observed for incubation times longer than 20 min (see Figugre 5.2). Over time, ORP progressively decreased and finally returned to its baseline value (*i.e.*, < 350 mV) after 60 min. Meanwhile, the

bacterial counts decreased slightly to $1.87\times10^3 \pm 8.33\times10^2$ CFU/mL, representing a 99.92% reduction when the incubation time was extended to 60 min. No significant changes in bacterial counts were observed in the control group during the experiment. The changes in the concentration of *M. chelonae* in the control and NB-O_3 treated groups are shown in Figure 5.2.

Ultrastructural examination of the bacterial cells using SEM clearly showed that most cells suffered significant structural damage after treatment with NB-O_3. This was in stark contrast to the non-treatment phase in which the bacterial cells maintained their intact and unaltered morphological characteristics. The difference between the untreated and treated conditions vividly illustrates the effects of NB-O_3 treatment on bacterial cell structures (Figure 5.3).

5.5.2. NB-O_3 treatment reduced bacteria concentration in water used in betta fish farms

Treatment with NB-O_3 proved to be highly effective in reducing the total bacterial counts and the number of presumptive mycobacteria in both reserved and cultured water of betta farms (see Figure 5.4). Although the percentage reduction varied slightly between the two farms, the general trend showed a significant reduction in the bacterial counts of over 90% after a single 10 min treatment with NB-O_3.

In reserved water, Farm 1 recorded a decrease in total bacterial counts from $1.03\times10^4 \pm 9.89\times10^2$ CFU/mL to 262.5 ± 17.68 CFU/mL, corresponding to a 97.45% reduction, while the counts in Farm 2 diminished from $4.23\times10^4 \pm 2.44\times10^4$ CFU/mL to $6.88\times10^2 \pm 3.01\times10^2$ CFU/mL, achieving a 98.37% reduction. For presumptive mycobacteria, Farm 1 showed a reduction from 60 ± 7.07 CFU/mL to 2.5 ± 3.54 CFU/mL (95.83%), whereas Farm 2 exhibited a decrease from 185 ± 49.05 CFU/mL to 12.5 ± 10.61 CFU/mL (93.24%).

In cultured water, Farm 1 recorded a remarkable decrease in total bacterial counts, from $1.25\times10^7 \pm 2.12\times10^6$ CFU/mL to $6.38\times10^5 \pm 8.84\times10^4$ CFU/mL, representing a 94.90% reduction. Similarly, the total bacterial counts in Farm 2 declined from $1.95\times10^7 \pm 1.20\times10^7$ CFU/mL to $1.37\times10^6 \pm 3.89\times10^5$ CFU/mL, translating to a 92.97% reduction. Concerning presumptive mycobacteria, the counts in Farm 1 fell from 257.5 ± 24.75 CFU/mL to 25 ± 14.14 CFU/mL (90.29% reduction), while the counts in Farm 2 dropped from 292 ± 109.6 CFU/mL to 27.5 ± 3.54 CFU/mL (90.58% reduction).

Water parameters (temperature, DO, pH, and ORP) followed similar trends during and after treatment in all experiments (Figure S5.3), with minor temperature fluctuations of less than 3°C and relatively stable pH. Significant changes occurred in DO levels, rapidly increasing from approximately 5 mg/L to 24-26 mg/L after 10 min of treatment, then gradually declining to around 15 mg/L within 60 min post-treatment. The ORP values for cultured water remained stable at around 300 mV throughout the experiment in both farms. In contrast, ORP values in the reserved water increased rapidly to 968.93 ± 9.23 mV (Farm 1) and 953.23 ± 7.32 mV (Farm 2) within 10 min before returning to near baseline values 60 min after treatment, *i.e.*, 366.64 ± 16.35 mV and 345.15 ± 18.31 mV for Farm 1 and Farm 2, respectively.

5.5.3. NB-O_3 treated water has the potential to prevent mycobacteriosis in culturing betta fish

Bacterial concentrations were determined for all groups before the cohabitation experiment began (Figure 5.5). The negative control sample contained a total bacterial count of $2.52\times10^4 \pm 9.76\times10^3$ CFU/mL, including 145 ± 21.21 CFU/mL of presumptive mycobacteria. Adding the *M. chelonae* suspension (~10^6 CFU/mL) to the negative control to form the positive control group resulted in an increase in total bacterial counts to $1.32\times10^9 \pm 4.95\times10^7$ CFU/mL and presumptive mycobacteria

to 6.15 $\times 10^7 \pm 1.06 \times 10^7$ CFU/mL. The treatment group, prepared in the same manner as the positive control group but then treated with NB-O_3 three times for 10 min each, recorded a 99.99% reduction in both total bacterial counts and presumptive mycobacteria. After treatment, the total bacterial counts had reduced to 75 ± 21.21 CFU/mL and the number of presumptive mycobacteria had decreased to 5 ± 7.07 CFU/mL. Throughout the evaluation of the treatment group, bacterial concentrations remained stable in the negative and positive control groups (see Figure 5.5).

The survival rate of the betta fish in the challenge tests, calculated as the mean of two replicates, is presented in Figure 5.6. The NB-O_3 treatment group had a survival rate of 93.93% compared to 38.33% for the positive control group. Of note, a 20% mortality rate was recorded in the negative control group during the 14-day study period. According to the log-rank test results, the survival rate in the NB-O_3 treatment group differed significantly from both the negative and positive controls, with *p*-values of 0.033 and 0.001, respectively. The relative percent survival (RPS) was 67.57% for the negative control group and 89.19% for the NB-O_3 treatment group. Typical colonies of *M. chelonae* were isolated from the deceased fish in the positive controls, but not in the negative controls or treatments.

5.6. Discussion

5.6.1. NB-O_3 has been proven to be effective in killing mycobacteria

This is the first investigation of the bactericidal properties of NB-O_3 against mycobacteria, particularly *M. chelonae* - a pathogenic and MDR strain. The study represents a significant advance in efforts to develop innovative strategies to control mycobacterial infections in aquaculture. A 10 min NB-O_3 treatment was chosen for this study, building on our previous study (Jhunkeaw et al., 2021), in which the protocol produced approximately 2-3$\times 10^7$ nanobubbles/mL (*i.e.*, each less than 100 nm in diameter). The instability of O_3 makes direct and accurate measurement of O_3

concentration in water difficult, so ORP is commonly used for indirect assessment of O_3 content (Suantika et al., 2001; Lee et al., 2009). According to a method described by Dien *et al.* (2022), ORP values from 503.8 to 941.5 mV linearly correspond to detectable O_3 concentrations between 0.27 and 1.37 mg/L, providing a quantitative framework for estimating O_3 levels using ORP metrics. Under laboratory conditions, our protocol generated NB-O_3 water with an ORP of 948.42 ± 4.28 mV, resulting in a 99.92% reduction in *M. chelonae* load after 60 min of incubation. Our results are consistent with other previous studies conducted under controlled laboratory conditions, such as the study by Imaizumi *et al.* (2018), in which NB-O_3-treated seawater with an ORP of 960 mV effectively eliminated over 99.99% of *Vibrio parahaemolyticus* within one min and achieved complete sterilization after five min. Similarly, Nghia *et al.* (2021) found that a six min treatment of seawater directly with NB-O_3 (with an ORP of 830 ± 70 mV) completely inactivated *V. parahaemolyticus*. Another study by Jhunkeaw *et al.* (2021) showed that a 10 min direct NB-O_3 treatment (ORP of 860 ± 42 mV) effectively reduced both Gram-positive (*Streptococcus agalactiae*) and Gram-negative (*Aeromonas veronii*) pathogenic bacteria in freshwater systems by over 96%. The substantial bacterial reduction in all observed results impressively underscores the bactericidal properties of NB-O_3 treatment and supports its broad-spectrum bactericidal ability in eliminating a wide range of waterborne pathogens.

Mycobacteria are generally characterized by a thick, waxy cell wall that resists penetration by many common disinfectants (Tarashi et al., 2022). As a result, disinfectant agents such as chlorine require higher doses and longer exposure times (Luo et al., 2021). The oxidation potential of O_3 exceeds that of hypochlorous acid, chlorine, chlorine dioxide, and chloramine, being 1.39, 1.52, 1.62, and 1.78 times higher, respectively (He et al., 2023). In line with these findings, a prior study revealed that the disinfectant capacity of ozonated water against *Mycobacterium avium* was

20 to 10,000 times greater than that of chlorine, monochloramine, and chlorine dioxide (Taylor et al., 2000). Through its strong oxidizing properties, O_3 attacks lipoproteins and polysaccharides on cell membranes, leading to the destruction of the cell structure (Seridou & Kalogerakis, 2021). Furthermore, O_3 oxidatively degrades glucose oxidase and macromolecular polymers such as DNA and RNA in bacteria, thereby damaging their vital processes (Fajardo et al., 2022). In the context of our study, DNA damage could not be directly observed, but damage to the cell was detected by SEM. We believe that the detectable mycobactericidal effect is primarily due to the extensive destruction of bacterial cell structures induced by NB-O_3 treatment. Indeed, the changes observed in bacterial cell structures after NB-O_3 treatment can indeed reasonably be attributed to oxidative stress induced by O_3 and its associated reactive species (Pell et al., 1997). Such oxidative stress can trigger a series of destructive events in the bacterial cells, including disruption of cell membrane integrity, induction of protein and lipid oxidation processes, and ultimately cell lysis (Hong et al., 2019). Our results confirm the potent bactericidal activity of NB-O_3 disinfection and highlight its ability to address the disinfectant-resistant properties of mycobacteria.

5.6.2. NB-O_3 promises a practical application to proactively mitigate the risk of mycobacteriosis in betta fish

Given the ubiquity of NTMs in the aquatic environment and their propensity to opportunistically infect fish, there is an urgent need for strategies aimed at reducing the numbers of these bacteria in rearing waters (Delghandi et al., 2020). Although NB-O_3 is recognized as an effective bactericidal method for water disinfection, its use has been largely limited to laboratory-scale experiments primarily designed to prove the concept (Imaizumi et al., 2018; Jhunkeaw et al., 2021; Dien et al., 2021). The practical implementation and commercialization of this technology face significant challenges, primarily due to the complexity of scaling up for larger

aquaculture ponds and the potential risks of direct O_3 exposure to fish. Residual O_3 concentration is critical, as levels between 0.01 - 0.1 mg/L have been found to be highly toxic to fish in both fresh- and seawater (Gonçalves & Gagnon, 2011). Betta fish, however, are typically kept in aquaria with frequent individual water changes due to their unique rearing conditions (Pleeging & Moons, 2017; Lichak et al., 2022). This unique circumstance led us to consider the direct application of NB-O_3 systems at the farm level, with an emphasis on decontaminating the water before it is used for fish rearing. The results showed the potential of NB-O_3 application in the context of water treatment for culturing betta fish with the significant reduction of bacterial populations under different water types. Water that contained organic material (*i.e.*, cultured water) exhibited a lower disinfection effect, possibly due to the rapid oxidation and degradation of O_3 in its presence (Jhunkeaw et al., 2021). This phenomenon is consistent with the unchanged ORP values observed during the NB-O_3 treatment in the cultured water in both farms. This result suggests that water with abundant organic material may need to be treated longer or more frequently. Several studies suggest that ORP values in the range of 300 to 425 mV can ensure the safety of fish, mollusks, and crustaceans (Dien et al., 2021; Seridou & Kalogerakis, 2021). The ORP values return to baseline (*i.e.*, <350 mV) after 60 min for all treatments, indicating that water treated with NB-O_3 is safe to use after this period. Nevertheless, optimization of the protocol for different water inputs, as well as for different farms, is essential for this disinfection process, as it may vary in reality.

Our data also showed that treatment of water contaminated with *M. chelonae* with NB-O_3 reduced the risk of mycobacteriosis and improved survivability of betta fish. To simulate the natural conditions of the rearing system, the water used for the experimental challenge was obtained directly from the farm instead of using tap water from the laboratory. The highly pathogenic *M. chelonae* isolate was introduced at a concentration of approximately 10^6 CFU/mL to ensure successful

infection, as noted in a previous study (Dinh-Hung et al., 2023). The observed mortality rate of 20% in the negative control group is consistent with the expected natural mortality rate of 15-20% during the rearing of subadult fish (as reported by farmers). At this stage, fish are often kept together and water quality deteriorates due to the time interval between water changes (10 days), possibly leading to mortality. The reserved water could also already be contaminated with pathogens, as indicated by the high concentrations of total bacteria. Conversely, it was observed that the *M. chelonae*-contaminated water after treatment with NB-O_3 showed a statistically significant improvement in fish survival rate compared to the negative control group (neither exposure to *M. chelonae* nor treatment with NB-O_3). A recognized limitation of the present study is the lack of a comprehensive analysis of the microbial community in the water, which could have prevented clarity of the contamination levels. While presumptive mycobacteria grew on Middlebrook 7H11 agar, only a small percentage were occasionally confirmed as actual mycobacteria by positive acid-fast staining (data not shown). This outcome does not accurately reflect the prevalence of mycobacteria in water and requires further investigation.

5.6.3. NB-O_3 offers a promising strategy for controlling disease and enhancing the sustainability of the betta fish industry

Information gleaned from farmers indicates that commercial betta fish farms, which yield up to 100,000 fish per rearing cycle, typically experience a mortality rate of 15-20% during the subadult phase (personal communication). Notably, these figures align with the results observed in our experimental challenge study. To illustrate the economic impact, consider a scenario where a single betta fish is valued at roughly $1 when it reaches adulthood. This implies that farms could face potential financial deficits of $15,000 to $20,000 for each rearing cycle. Considering that many farms undergo three to four such cycles annually, the financial implications can be substantial. Our study confirms that use of NB-O_3 reduces the risk

of mycobacteriosis and improves the survivability of betta fish, indicating its potential commercial application on farms. The introduction of NB-O_3 to the betta fish industry, therefore, holds the potential to be game-changing. Not only could it diminish the threat of mycobacteriosis, but it could also revolutionize disease control within the sector.

Although not explicitly addressed in this study, we anticipate that the benefits of NB-O_3 extend beyond water treatment. This innovative approach could be used to disinfect water from farms before it enters the environment, reducing the risk of spreading pathogens and polluting the environment. Additionally, its utility might extend to disinfecting fish aquaria and rearing facilities, as well as curbing biofilm accumulation by soaking these facilities in NB-O_3 water. It is noteworthy that the content of dissolved oxygen (DO) increases after the NB-O_3 treatment, which is probably due to the conversion of O_3 to O_2 molecules (Batakliev et al., 2014). Assuming that this mechanism is correct, the use of NB-O_3 treated water in betta farms could provide additional benefits, such as optimization of DO concentration, which is particularly important for water intended for the trade or transport of fish. Furthermore, the increasing acceptance of NB-O_3 technology, potentially leading to a reduction in associated costs, offers a promising future for its widespread application (Atkinson et al., 2019). This technological progress offers an optimistic perspective in which the fight against pathogens no longer relies on chemicals or antibiotics, but moves toward more efficient and environmentally friendly solutions. Such a transformation could not only improve the health and performance of betta fish but also has the potential to revolutionize the industry.

5.7. Tables and figures

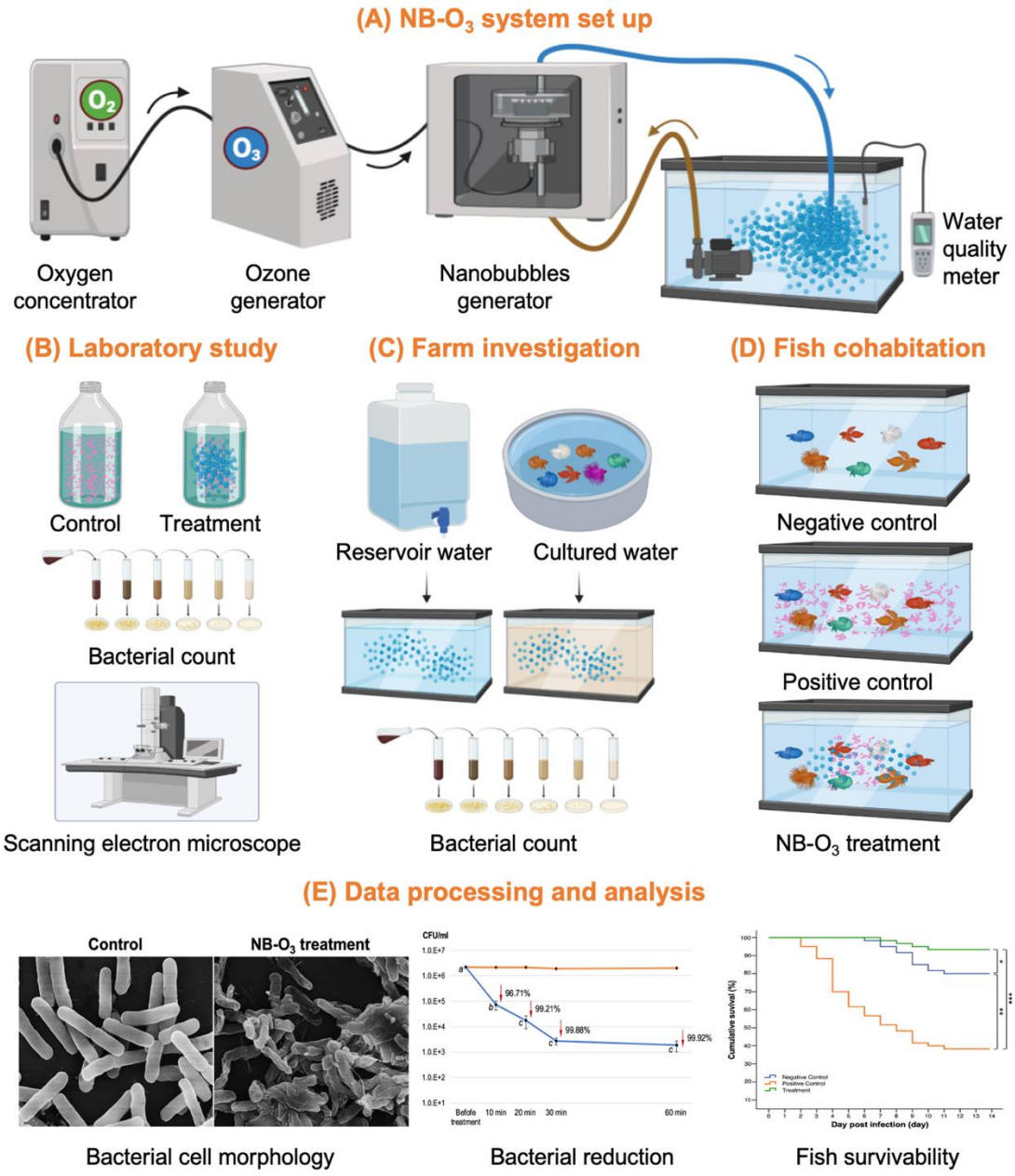

Figure 5.1. An overview of the experimental workflow implemented in this study.

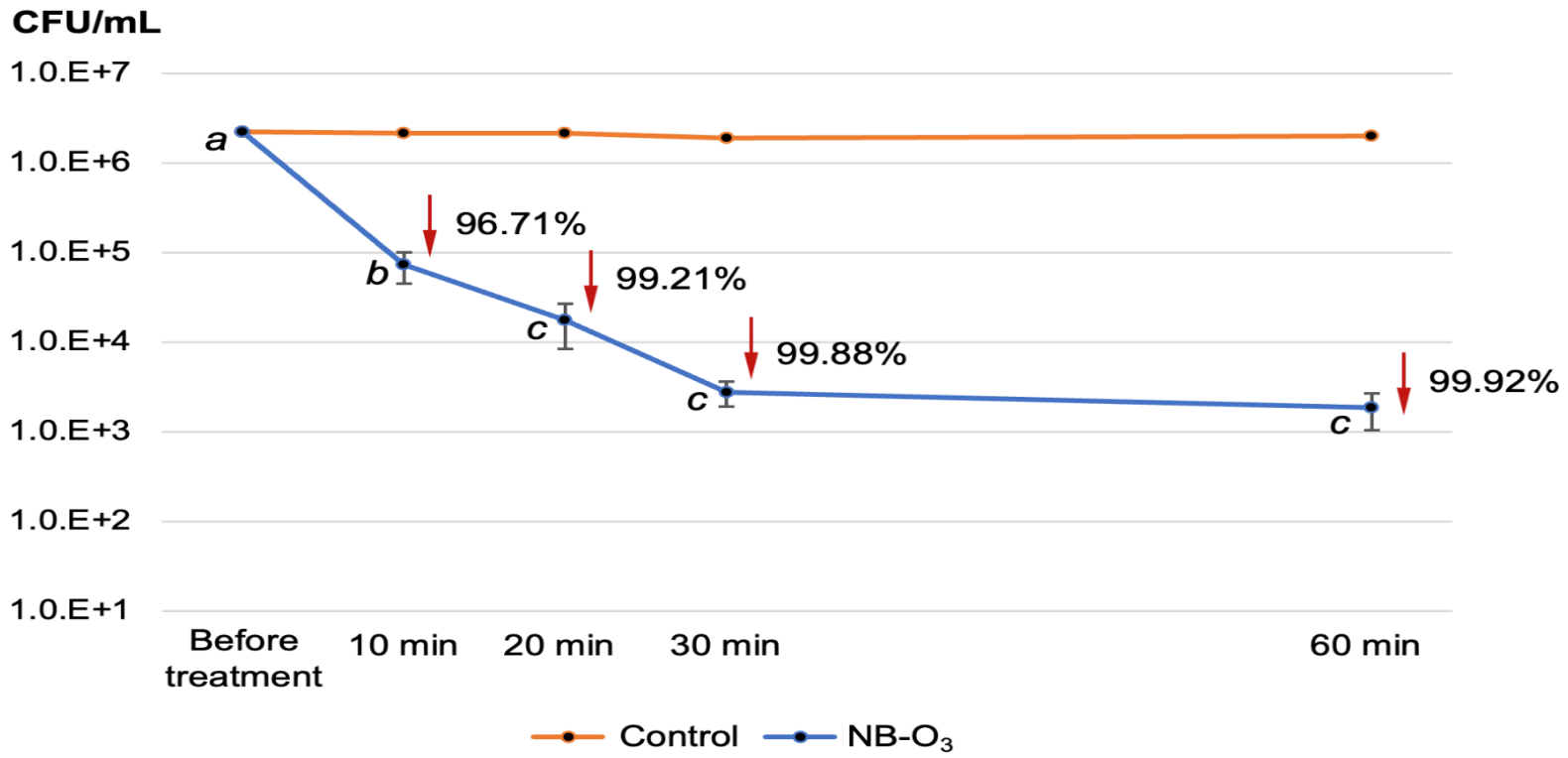

Figure 5.2. Comparison of *Mycobacterium chelonae* bacterial counts after 10, 20, 30, and 60 min incubation in distilled water treated with NB-O_3 for 10 min (blue line) with distilled water without NB-O_3 treatment (orange line). The arrows indicate the percentage reduction in bacterial load compared to the initial concentration. The bars represent the standard deviation based on three replicates. Significant differences between the incubation durations with NB-O_3 water are indicated by the alphabets ($p < 0.05$).

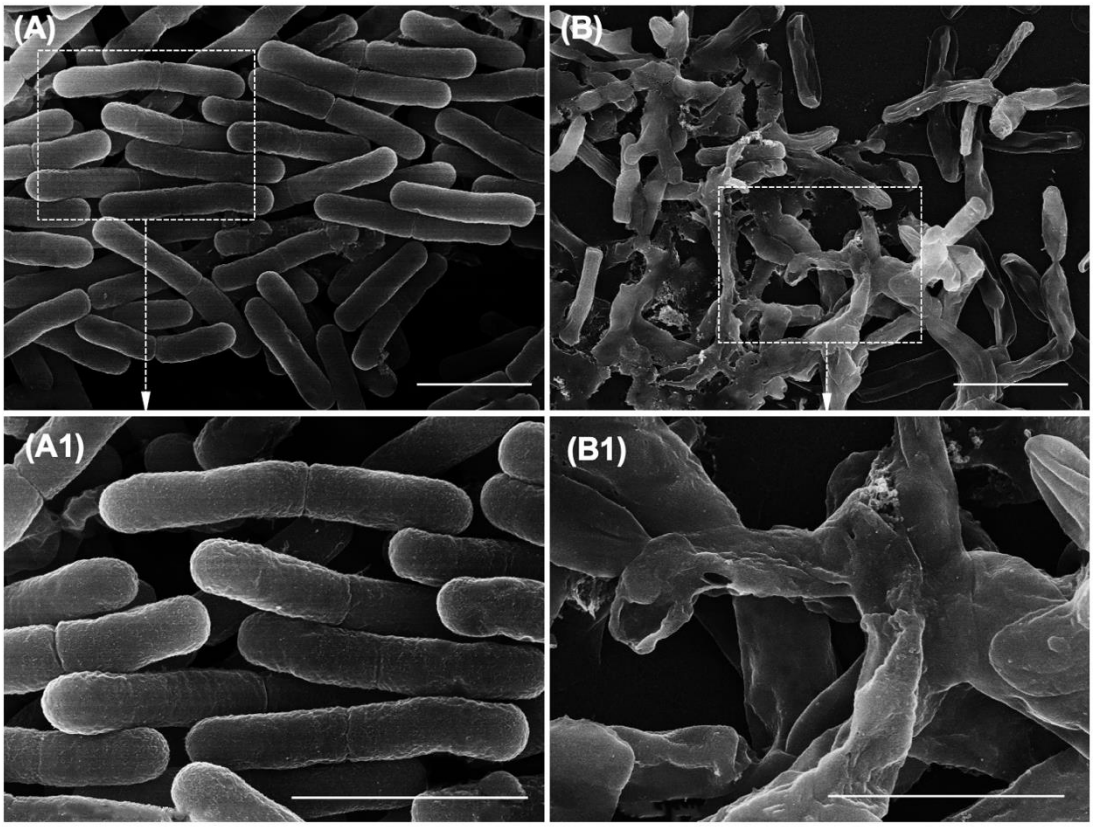

Figure 5.3. Scanning electron micrographs of *Mycobacterium chelonae* before (A, A1) and after (B, B1) incubation in water treated with NB-O_3 for 10 min. Bacterial morphology was normal before treatment, whereas after treatment with NB-O_3, the cells shrank and their original structure was severely destroyed. Scale bar, 5 μm.

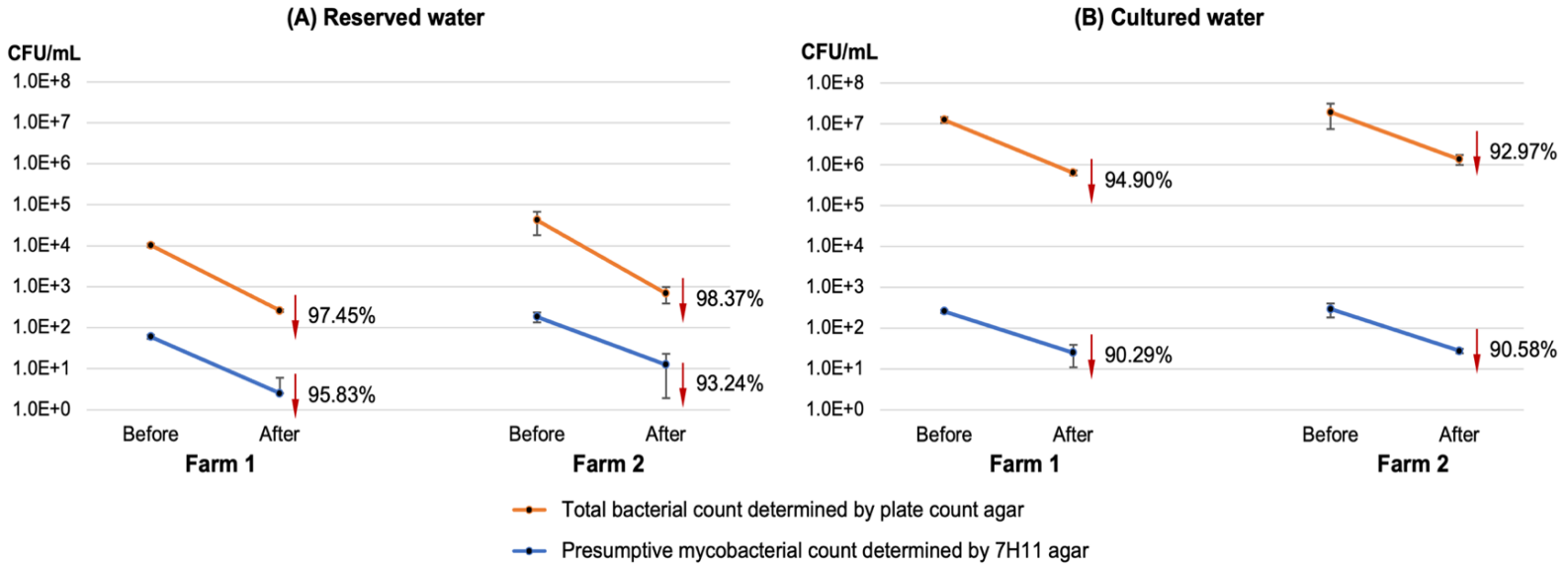

Figure 5.4. Total bacterial counts of reserved and cultured water samples on different agar media from farms after a 10 min exposure to NB-O_3 followed by a 20 min rest period. The arrows indicate the percentage reduction in bacterial load compared to the initial concentration. The bars represent the standard deviation based on two replicates. See Fig. S1 for details of the experimental setup.

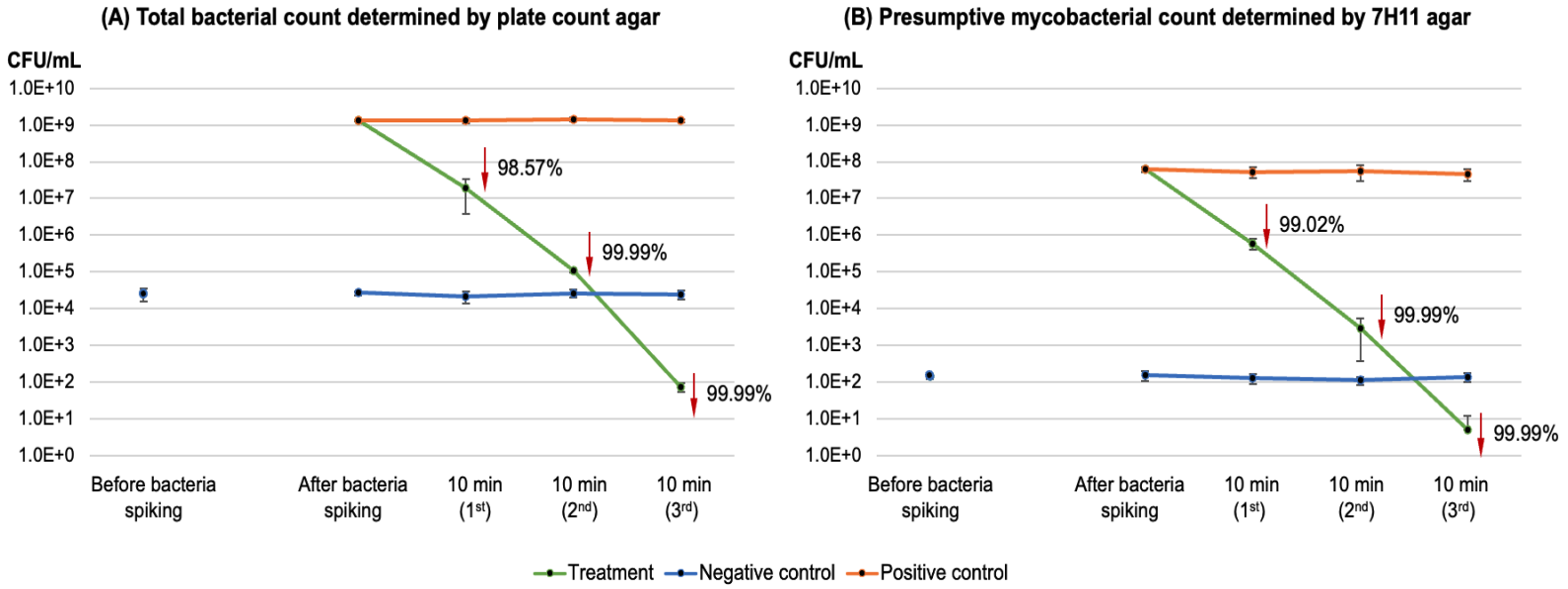

Figure 5.5. Reduction of bacterial counts on different agar media (green lines) of reserved water (from Farm 2) spiked with *Mycobacterium chelonae* (10^6 CFU/mL) and then exposed to NB-O_3 three times for each. The water was used to raise sub-adult betta fish (aged 1-1.5 months) that formed the treatment group in the experiment shown in Fig. 6. The arrows indicate the percentage reduction in bacterial load compared to the initial concentration. The bars represent the standard deviation based on two replicates.

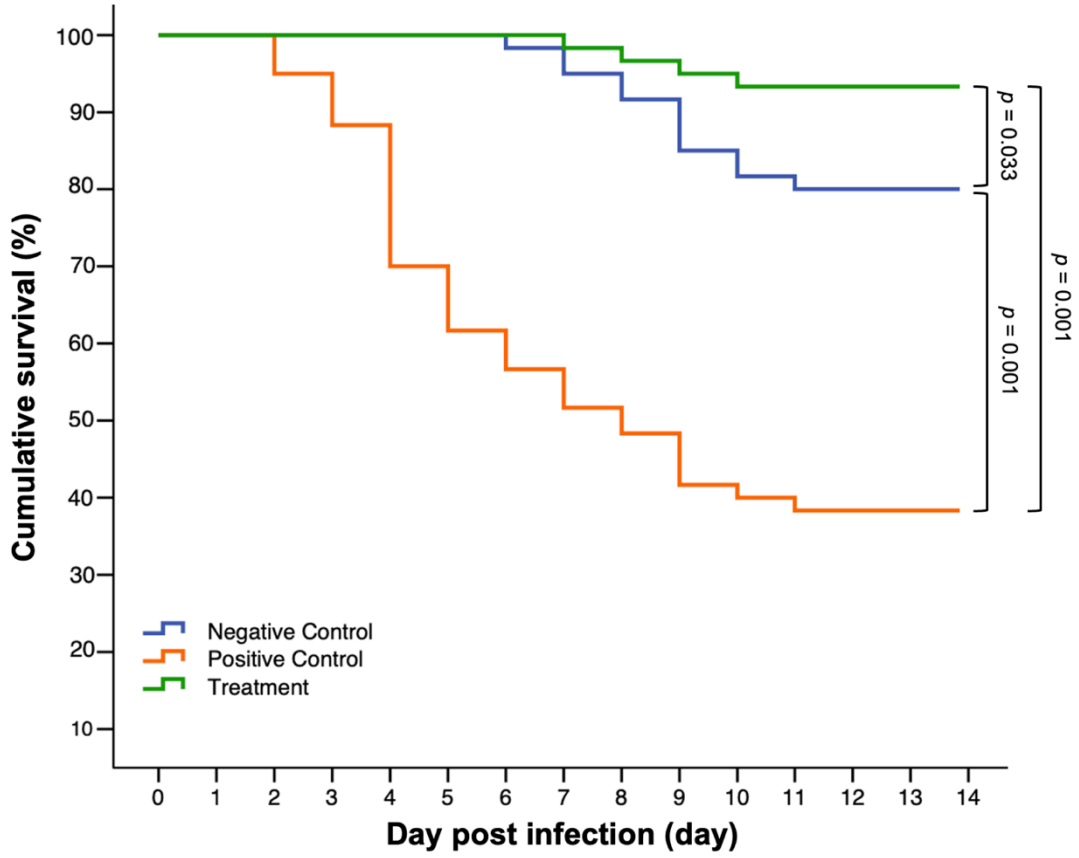

Figure 5.6. Kaplan-Meier analysis of the percent survival of sub-adult betta fish, *Betta splendens*, in the different groups. Negative control, neither exposure to *M. chelonae* nor treated with NB-O_3; positive control, exposure to *Mycobacterium chelonae* but without NB-O_3; treatment, after exposure to *M. chelonae* followed by three treatments with NB-O_3 for 10 min each. Experiments were performed in two independent trials (n = 30). Water was replaced in each group on day 10^{th}. Differences between groups in each experiment were tested using the log-rank test (p-value < 0.05).

5.8. Supplementary data

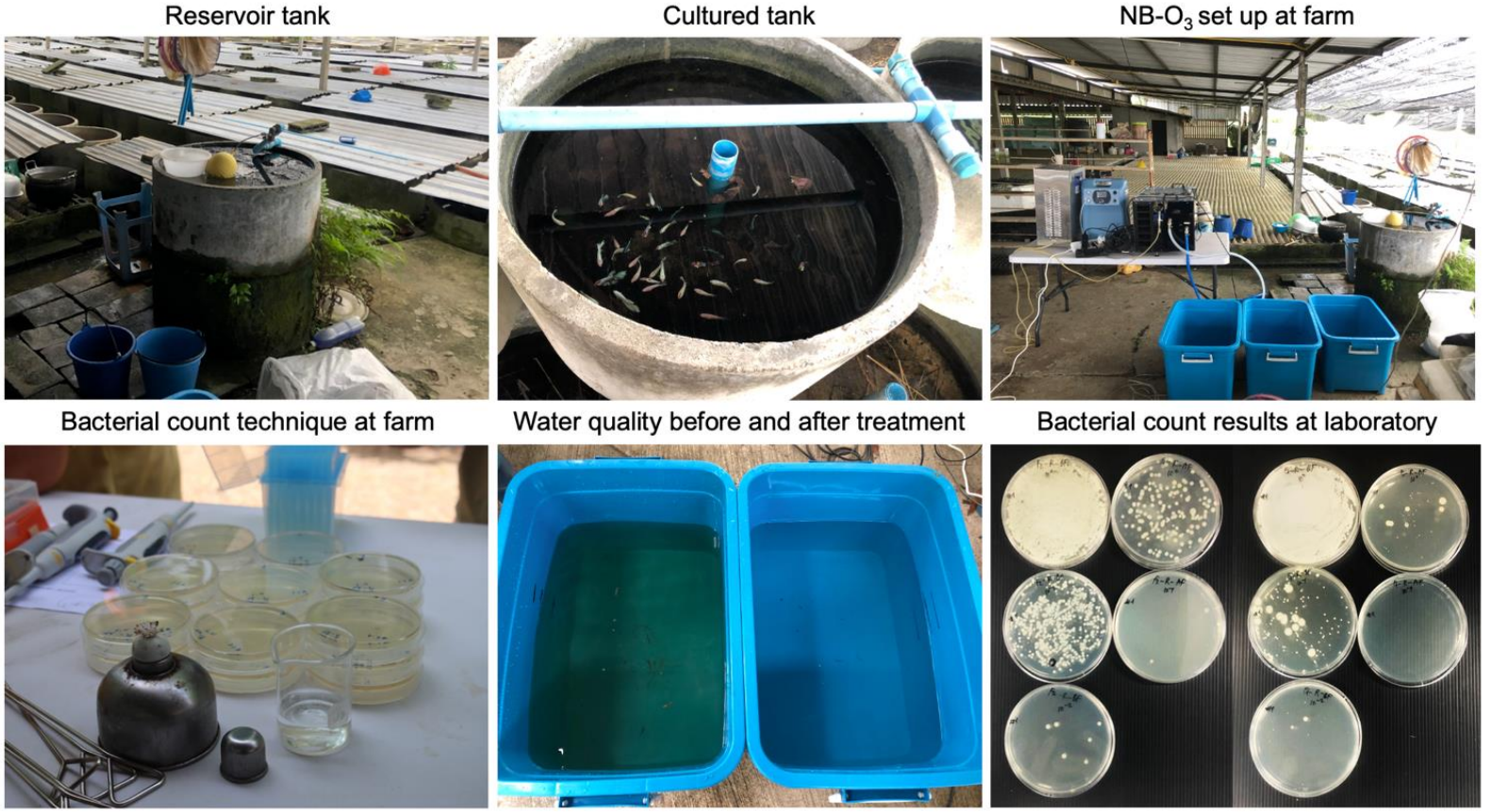

Figure S5.1. The experimental design for testing the application of the NB-O_3 treatment was carried out at two separate farms, each with two replicate trials.

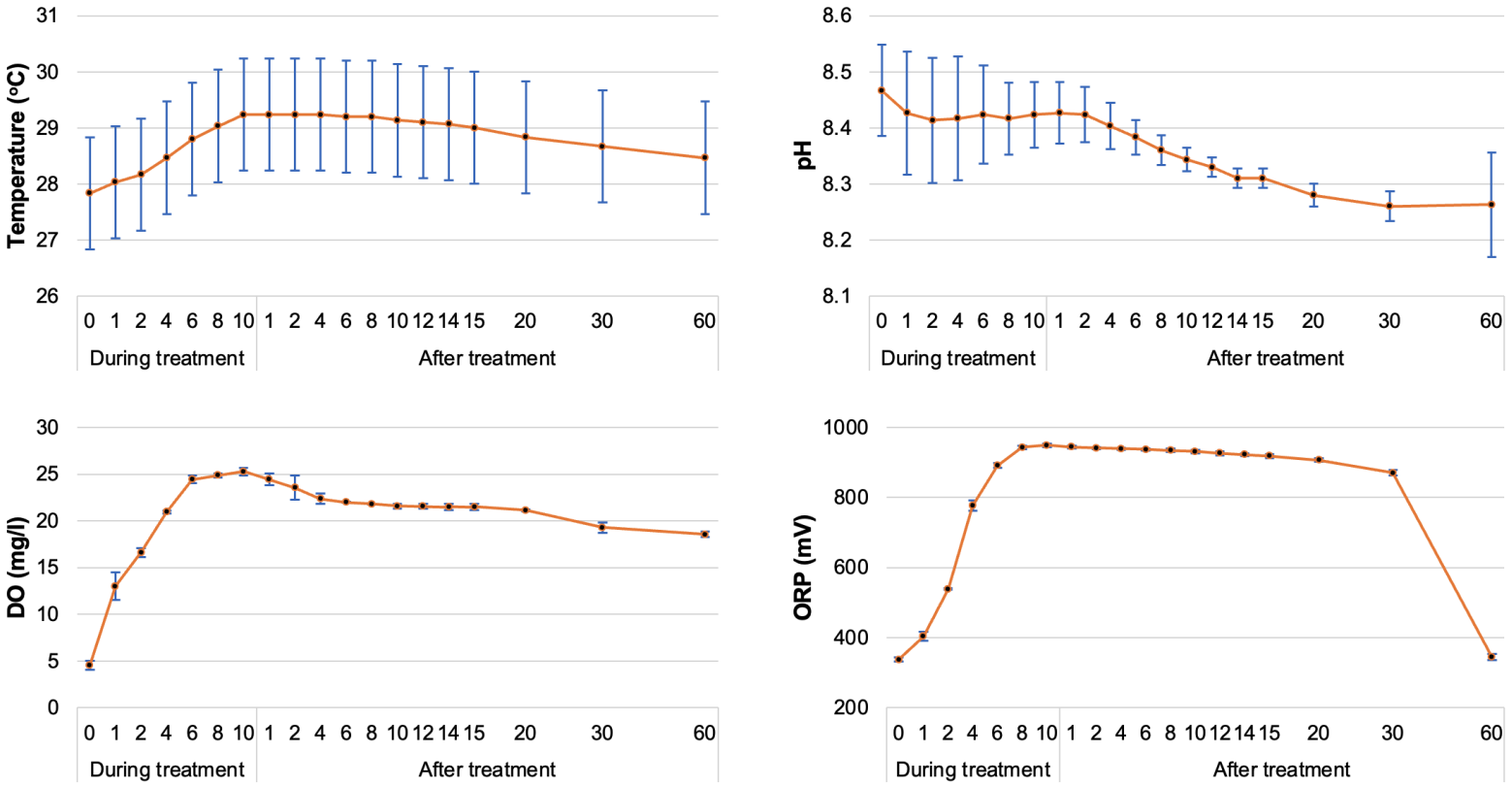

Figure S5.2. Water parameters (temperature, pH, DO, and ORP) during the 10 min treatment and within the 60 min rest period after exposure to NB-O_3 on a laboratory scale with tap water. The bars represent the standard deviation based on three replicates.

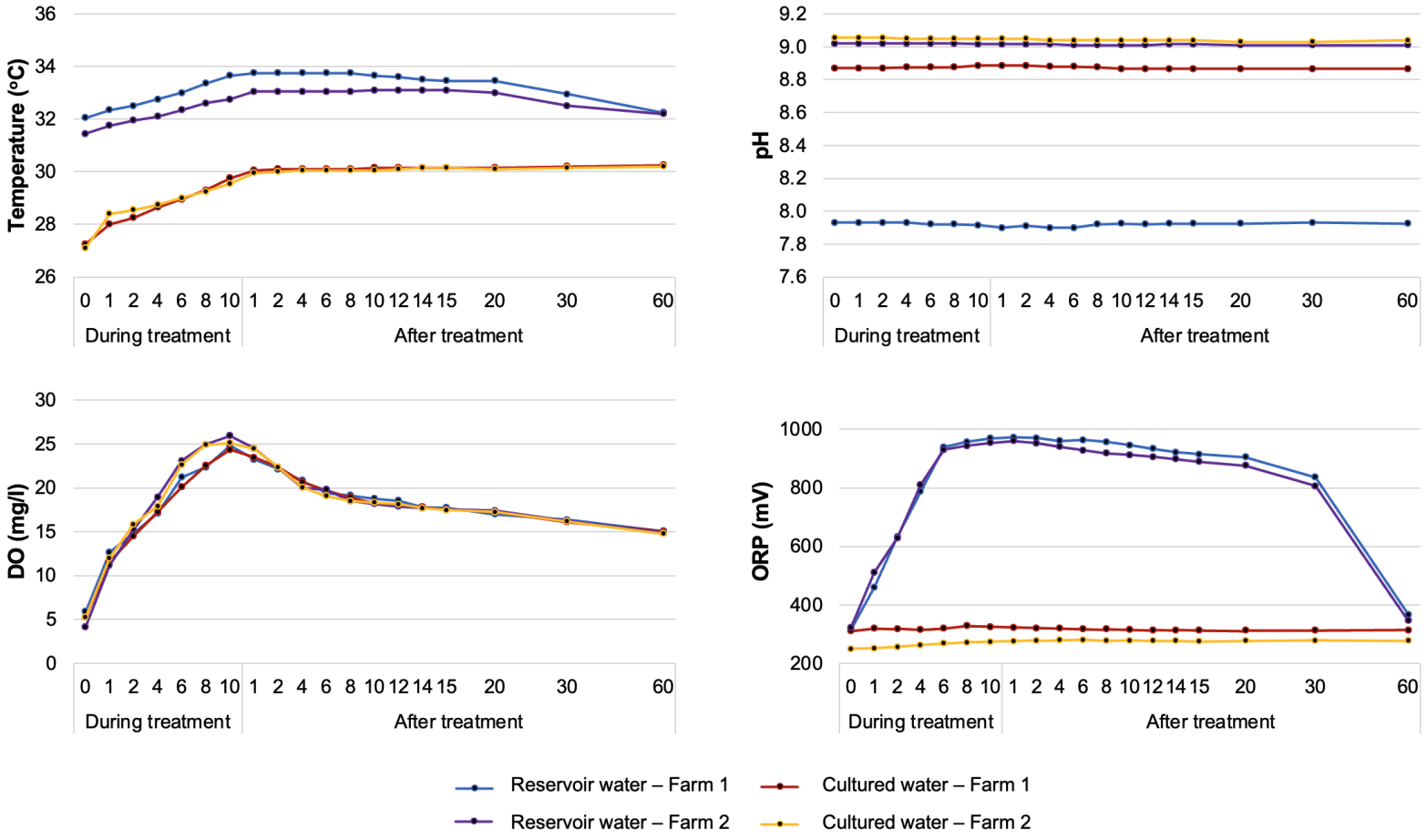

Figure S5.3. Water parameters (temperature, pH, DO, and ORP) during the 10 min treatment and within the 60 min rest period after exposure to NB-O_3 in farms with both reserved and cultured water. The values shown represent the mean of two replicates per farm.

5.9. References

Agarwal A, Ng WJ and Liu Y 2011. Principle and applications of microbubble and nanobubble technology for water treatment. Chemosphere. 84(9): 1175-1180.

Amend DF 1981. Potency testing of fish vaccines. Fish biologics: Serodiagnostics and vaccines. 447-454.

Atkinson AJ, Apul OG, Schneider O, Garcia-Segura S and Westerhoff P 2019. Nanobubble technologies offer opportunities to improve water treatment. Acc Chem Res. 52(5): 1196-1205.

Batakliev T, Georgiev V, Anachkov M, Rakovsky S and Rakovsky S 3914. Ozone decomposition. Interdiscip. Toxicol. 7(2): 47-59.

Beran V, Matlova L, Dvorska L, Svastova P and Pavlik I 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. J. Fish Dis. 29(7): 383-393.

Chong RS-M 2022. Mycobacteriosis. In: Aquac. Int. Elsevier. 407-415.

Delghandi MR, El-Matbouli M and Menanteau-Ledouble S 2020. Mycobacteriosis and infections with non-tuberculous mycobacteria in aquatic organisms: A review. Microorganisms. 8(9).

Dien LT, Linh NV, Mai TT, Senapin S, St-Hilaire S, Rodkhum C and Dong HT 2022. Impacts of oxygen and ozone nanobubbles on bacteriophage in aquaculture system. Aquaculture. 551: 737894.

Dien LT, Linh NV, Sangpo P, Senapin S, St-Hilaire S, Rodkhum C and Dong HT 2021. Ozone nanobubble treatments improve survivability of Nile tilapia (*Oreochromis niloticus*) challenged with a pathogenic multi-drug-resistant *Aeromonas hydrophila*. J Fish Dis. 44(9): 1435-1447.

Dinh-Hung N, Dong HT, Senapin S, Pimsannil K, Thompson KD, Shinn AP, Soontara C, Sirimanapong W, Chatchaiphan S and Rodkhum C 2023. Insight into characteristics and pathogenicity of five rapidly growing non-tuberculous *Mycobacterium* species isolated from the Siamese fighting fish, *Betta splendens*. Aquaculture. 739822.

Dong HT, Senapin S, Phiwsaiya K, Techatanakitarnan C, Dokladda K, Ruenwongsa P and Panijpan B 2018. Histopathology and culturable bacteria associated with

"big belly" and "skin nodule" syndromes in ornamental Siamese fighting fish, *Betta splendens*. Microb Pathog. 122: 46-52.

Fajardo C, Martinez-Rodriguez G, Blasco J, Mancera JM, Thomas B and De Donato M 2022. Nanotechnology in aquaculture: Applications, perspectives and regulatory challenges. Aquac. Fish. 7(2): 185-200.

Fan W, An WG, Huo MX, Yang W, Zhu SY and Lin SS 2020. Solubilization and stabilization for prolonged reactivity of ozone using micro-nano bubbles and ozone-saturated solvent: A promising enhancement for ozonation. Sep. Purif. Technol. 238: 116484.

Francis-Floyd R 2011. Mycobacterial infections of fish. In: Southern Regional Aquaculture Center USA.

Gauthier DT and Rhodes MW 2009. Mycobacteriosis in fishes: A review. Vet J. 180(1): 33-47.

Gonçalves AA and Gagnon GA 2011. Ozone application in recirculating aquaculture system: An overview. Ozone: Sci. Eng. 33(5): 345-367.

Gurung A, Dahl O and Jansson K 2016. The fundamental phenomena of nanobubbles and their behavior in wastewater treatment technologies. Geosystem Eng. 19(3): 133-142.

He Z, Fan X, Jin W, Gao S, Yan B, Chen C, Ding W, Yin S, Zhou X and Liu H 2023. Chlorine-resistant bacteria in drinking water: Generation, identification and inactivation using ozone-based technologies. J. Water Process. Eng . 53: 103772.

Hong Y, Zeng J, Wang X, Drlica K and Zhao X 2019. Post-stress bacterial cell death mediated by reactive oxygen species. Proceedings of the National Academy of Sciences. 116(20): 10064-10071.

Imaizumi K, Tinwongger S, Kondo H and Hirono I 2018. Disinfection of an EMS/AHPND strain of *Vibrio parahaemolyticus* using ozone nanobubbles. J Fish Dis. 41(4): 725-727.

Jhunkeaw C, Khongcharoen N, Rungrueng N, Sangpo P, Panphut W, Thapinta A, Senapin S, St-Hilaire S and Dong HT 2021. Ozone nanobubble treatment in

freshwater effectively reduced pathogenic fish bacteria and is safe for Nile tilapia (*Oreochromis niloticus*). Aquaculture. 534: 736286.

Langlais B, Reckhow DA and Brink DR 2019. Ozone in water treatment: Application and engineering. In: Routledge.

Lee HT, Lee HJ, Terng W, Leu MY and Meng PJ 2009. Optimal Dose of Total Residual Oxidants for Hybrid Tilapia (*Oreochromis mossambicus x O. niloticus*) and Whiteleg Shrimp (*Litopenaeus vannamei*) in Ozone-Treated Sea Water. Isr. J. Aquac. 61: 356-362.

Lichak MR, Barber JR, Kwon YM, Francis KX and Bendesky A 2022. Care and use of Siamese fighting fish (*Betta Splendens*) for research. Comp Med. 72(3): 169-180.

Luo LW, Wu YH, Yu T, Wang YH, Chen GQ, Tong X, Bai Y, Xu C, Wang HB and Ikuno N 2021. Evaluating method and potential risks of chlorine-resistant bacteria (CRB): A review. Water Res. 188: 116474.

Marcelino KR, Ling L, Wongkiew S, Nhan HT, Surendra KC, Shitanaka T, Lu H and Khanal SK 2023. Nanobubble technology applications in environmental and agricultural systems: Opportunities and challenges. Crit. Rev. Environ. Sci. Technol. 53(14): 1378-1403.

Monvises A, Nuangsaeng B, Sriwattanarothai N and Panijpan B 2009. The Siamese fighting fish: Well-known generally but little-known scientifically. ScienceAsia. 35: 8-16.

Narendrakumar L, Sudhagar A, Preena PG, Nithianantham SR, Mohandas SP and Swaminathan TR 2022. Detection of *Mycobacterium marinum* and multidrug-resistant bacteria in a chronic progressive disease outbreak among Siamese fighting fish (*Betta splendens*) in India. Biologia. 77(9): 2725-2733.

Nghia NH, Van PT, Giang PT, Hanh NT, St-Hilaire S and Domingos JA 2021. Control of *Vibrio parahaemolyticus* (AHPND strain) and improvement of water quality using nanobubble technology. Aquac Res. 52(6): 2727-2739.

Pal P, Joshi A and Anantharaman H 2022. Nanobubble ozonation for waterbody rejuvenation at different locations in India: A holistic and sustainable approach. Results Eng. 16: 100725.

Pandian AMK, Rajamehala M, Singh MVP, Sarojini G and Rajamohan N 2022. Potential risks and approaches to reduce the toxicity of disinfection by-product–A review. Sci. Total Environ. 822: 153323.

Pell EJ, Schlagnhaufer CD and Arteca RN 1997. Ozono-induced oxidative stress: mechanisms of action and reaction. Physiol. Plant. 100(2): 264-273.

Pleeging C and Moons CPH 2017. Potential welfare issues of the Siamese fighting fish (*Betta splendens*) at the retailer and in the hobbyist aquarium. Vlaams Diergeneeskundig Tijdschrift. 86: 213-223.

Powell A and Scolding JWS 2018. Direct application of ozone in aquaculture systems. Rev Aquac 10(2): 424-438.

Puttinaowarat S, Thompson K, Kolk A and Adams A 2002. Identification of *Mycobacterium* spp. isolated from snakehead, *Channa striata* (Fowler), and Siamese fighting fish, *Betta splendens* (Regan), using polymerase chain reaction–reverse cross blot hybridization (PCR–RCBH). J. Fish Dis. 25: 235-243.

Seridou P and Kalogerakis N 2021. Disinfection applications of ozone micro-and nanobubbles. Environ. Sci. Nano. 8(12): 3493-3510.

Sermwatanakul A 2019. Capacitating the local farmers to enhance global marketing of Thailand's national aquatic animal, the Siamese fighting fish. Fish for the People. 17(2): 42-48.

Sirimalaisuwan A, Teeraruk P, Kanjanapitakchai P, Kaewsakhorn T, Potibut P and Pikulkaew S 2017. Detection of *Mycobacterium marinum* in clinically asymptomatic Siamese fighting fish (*Betta splendens*) from ornamental fish shops in Chiang Mai Province, Thailand. Asian Pac. J. Trop. Dis. 7: 344-346.

Suantika G, Dhert P, Rombaut G, Vandenberghe J, De Wolf T and Sorgeloos P 2001. The use of ozone in a high density recirculation system for rotifers. Aquaculture. 201(1-2): 35-49.

Takahashi M, Chiba K and Li P 2007. Free-radical generation from collapsing microbubbles in the absence of a dynamic stimulus. J. Phys. Chem. B. 111(6): 1343-1347.

Tarashi S, Siadat SD and Fateh A 2022. Nontuberculous mycobacterial resistance to antibiotics and disinfectants: Challenges still ahead. Biomed Res. Int. 2022: 8168750.

Taylor RH, Falkinham III JO, Norton CD and LeChevallier MW 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium.* Appl. Environ. Microbiol. 66(4): 1702-1705.

U.S. Fish and Wildlife Service 2019. Subject: Siamese fighting fish (*Betta splendens*) ecological risk screening summary (online). Available: https://www.fws.gov/sites/default/files/documents/Ecological-Risk-Screening-Summary-Siamese-Fighting-Fish.pdf.

Weerakhun S, Sukon P and Hatai K 2019. *Mycobacterium marinum* and *Mycobacterium fortuitum* infections in Siamese fighting fish, *Betta splendens* (Regan), in Thailand. Thai J Vet Med. 49(2): 137-145.

CHAPTER 6

General conclusion

6.1. Conclusion

Given the great economic importance of Siamese fighting fish (*Betta splendens*) in Thailand and their importance in the global ornamental fish trade, the threat of mycobacteriosis should not be underestimated. This research elucidates the complex interplay between pathogen characteristics, pathogenicity, pathogenesis, and control strategies associated with mycobacteriosis in betta fish.

Initially, our study highlights the value of phenotypic methods remain useful for distinguishing *Mycobacterium* species isolated from betta fish. Our results may serve as a reliable guide for preliminary detection and identification of certain mycobacteria when molecular methods are not available or to save sequencing costs. The antibiotic resistance and disinfectant susceptibility patterns revealed in this study may help farmers and researchers improve on-farm management to prevent future outbreaks. The data also indicate a concerning level of antibiotic resistance in these isolates from betta and raise public health concerns due to their zoonotic potential. Additionally, information on the virulence of the bacteria underscores the potential economic impact and the importance of ongoing surveillance and investigation to develop affordable treatments and preventive measures against mycobacteriosis in the betta fish industry.

Regarding the interaction between *Mycobacterium* spp. and *B. splendens*, our study represents the first experimental challenges using different infection routes in betta fish with *M. chelonae*, a common and pathogenic isolate in these fish. Our results indicate that the pathogenicity of *M. chelonae* is closely linked to the infection routes, suggesting that the most likely natural routes of infection for *M.*

chelonae involve an injured body surface and/or the digestive tract. This expands our understanding of the pathogenesis of *Mycobacterium* species in betta fish and underscores the need to mitigate risk factors associated with these exposure pathways to effectively control the disease. These valuable insights into host-pathogen interactions (*i.e.*, infection progression, tissue tropism) will aid in the development of strategies to control mycobacteriosis, thereby promoting the long-term sustainability of the betta fish industry.

Another notable finding of our research is highlighting the potential use of NB-O_3 to the betta fish industry, therefore, holds the potential to be game-changing. Not only could it diminish the threat of mycobacteriosis, but it could also revolutionize disease control within the sector. This technological advancement offers an optimistic perspective in which the fight against pathogens no longer relies on chemicals or antibiotics but moves toward more efficient and environmentally friendly solutions. Such a transformation could not only improve the health and performance of betta fish but also has the potential to revolutionize the industry.

In summary, these multi-faceted studies offers valuable insights into mycobacteriosis and have implications that transcend academic boundaries. They highlight the multiple benefits to fish farmers, public health paradigms, economic developments, and technical/scientific innovations, all of which are critical to the flourishing and expansive development of the betta fish industry

6.1. Recommendation

Despite the benefits of the present study, it is recommended that further research efforts be undertaken, which would provide a solid foundation for deciphering and managing mycobacteriosis, thereby ensuring a sustainable and secure future for the betta fish industry.

- **Epidemiological studies of disease prevalence**

- *Importance.* Essential for understanding the prevalence and distribution of mycobacteriosis in betta fish populations.
- *Implication.* Providing insights into infection status highlights the far-reaching impact of the disease.

- **Development of diagnostic techniques**

- *Importance.* Crucial for the development of specific and non-lethal diagnostic techniques.
- *Implication.* Enhancing monitoring capabilities facilitates timely interventions and improves the management of fish populations.

- **Exploring mechanisms of antibiotic resistance**

- *Importance.* Vital for understanding the mechanisms behind resistance in bacteria causing mycobacteriosis.
- *Implications.* Informing the development of novel treatments or prevention methods enhances the effectiveness of therapeutic interventions.

- **Zoonotic implications of mycobacteriosis**

- *Importance.* Essential to grasp the potential zoonotic implications of mycobacteriosis.
- *Implications.* Supporting the establishment of strict guidelines for the husbandry and care of betta fish ensures the safety of both hobbyists and those involved in the sector.

REFERENCES

VITA

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Delamare-Debouteville J, Senapin S, Mohan VC, Dong HT, & Rodkhum C. (2022). Tilapia Lake Virus was not detected in non-tilapine species within tilapia polyculture systems of Bangladesh. Journal of Fish Diseases, 45(1), 77-87. https://doi.org/10.1111/jfd.13537

5. Dinh-Hung N, Dong HT, Soontara C, Rodkhum C, Nimitkuk S, Srisapoome P, Kayansamruaj P, & Chatchaiphan S. (2022). Co-infection of Candidatus Piscichlamydia Trichopodus (Order Chlamydiales) and Henneguya sp. (Myxosporea, Myxobolidae) in snakeskin gourami Trichopodus pectoralis (Regan 1910). Frontiers in Veterinary Science, 9. https://doi.org/10.3389/fvets.2022.847977

6. Mai TT, Kayansamruaj P, Soontara C, Kerddee P, Dinh-Hung N, Senapin S, Costa JZ, del-Pozo J, Thompson KD, Rodkhum C, & Dong HT. (2022). Immunization of Nile tilapia (Oreochromis niloticus) broodstock with Tilapia Lake Virus (TiLV) inactivated vaccines elicits protective antibody and passive maternal antibody transfer. Vaccines, 10(2), 167. https://www.mdpi.com/2076-393X/10/2/167

7. Dinh-Hung N, Dong HT, Taengphu S, Soontara C, Rodkhum C, Senapin S, & Chatchaiphan S. (2022). Streptococcus suis is a lethal pathogen in snakeskin gourami, Trichopodus pectoralis. Aquaculture, 566, 739173. https://doi.org/10.1016/j.aquaculture.2022.739173

8. Kayansamruaj P, Dinh-Hung N, Srisapoome P, Na-Nakorna U, & Chatchaiphan S. (2023). Genomics-driven prophylactic measures to increase streptococcosis resistance in tilapia. Journal of Fish Diseases, 46(6), 597–610. https://doi.org/10.1111/jfd.13763

9. Dinh-Hung N, Dong HT, Shinn AP, Soontara C, Phiwsaiya K, Rodkhum C, Senapin S, & Chatchaiphan S. (2023). Lumpy skin disease of snakeskin gourami: A new record of metacercariae of Posthodiplostomum sp. (Digenea, Diplostomidae) in clinically sick snakeskin gourami, Trichopodus pectoralis Regan, 1910 (Pisces, Osphronemidae). Aquaculture, 573, 739583. https://doi.org/10.1016/j.aquaculture.2023.739583

10. Dinh-Hung N, Dong HT, Senapin S, Pimsannil K, Thompson KD, Shinn AP, Soontara C, Sirimanapong W, Chatchaiphan S, & Rodkhum C. (2023). Insight into characteristics and pathogenicity of five rapidly growing non-tuberculous Mycobacterium species isolated from the Siamese fighting fish, Betta splendens. Aquaculture, 575, 739822. https://doi.org/10.1016/j.aquaculture.2023.739822

11. Dinh-Hung N, Dong HT, Senapin S, Linh NV, Shinn AP, Pirarat N, Hirono I, Chatchaiphan S, & Rodkhum C. (2023) Infection and histopathological consequences in Siamese fighting fish (Betta splendens) due to exposure to a pathogenic Mycobacterium chelonae via different routes. Aquaculture, 579, 740191. https://doi.org/10.1016/j.aquaculture.2023.740191

12. Dinh-Hung N, Dong HT, Senapin S, Shinn AP, Linh NL, Dien LT, Hirono I, Soontara C, Chatchaiphan S, & Rodkhum C. (2023). Utilizing ozone nanobubbles to mitigate the risk of mycobacteriosis caused by a multidrug-resistant Mycobacterium chelonae in Siamese fighting fish (Betta splendens). Under review in Aquaculture

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