INVESTIGATING INFECTIOUS BACTERIAL DISEASES AFFECTING FARMED NILE TILAPIA (OREOCHROMIS NILOTICUS) IN THE PHILIPPINES

THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by
FRANCIS SORIA LEGARIO, BSc, MSc

INSTITUTE OF AQUACULTURE, FACULTY OF NATURAL SCIENCES
UNIVERSITY OF STIRLING
UNITED KINGDOM

July 2021

DECLARATION

I, the undersigned, hereby declare that this thesis has been composed entirely by me and has not been submitted for any other degree. The work presented in this thesis, except where specifically acknowledged, is the result of my own investigations which have been done by me independently.

FRANCIS SORIA LEGARIO
Stirling, UK
July 2021

DR. MARGRET CRUMLISH
Principal Supervisor

PROF. JIMMY F. TURNBULL
Second Supervisor

ACKNOWLEDGEMENTS

I am delighted to express my wholehearted feelings of thankfulness and gratefulness to Dr. Mags Crumlish and Prof James Turnbull, my supervisors and mentors, for all of their help, support, freedom, inspiration, advice and guidance throughout my PhD journey; and for giving me access to their office for a quick discussion about the experiment anytime when I needed it. I could not have wished for better supervisors. I am also indebted to them for their ceaseless endurance for careful reading and reviewing the write-up and also for teaching me fish bacteriology, diagnostics, histopathology and epidemiology.

I would like to express my gratitude to Dr. Johanna Bailey for helping me in my histopathology analysis, and to Dr. Amaya Albalat, who lend precious help and advice in proteomics for the identification of my bacterial isolates. I would also like to thank Dr. Celia L. Pitogo for introducing my supervisors, Dr. Joebert Toledo of Feedmix II Inc. and Dr. Casiano Choresca of Fisheries Biotech Centre-NFRDI, for their assistance during my field work in the Philippines.

I also express my heartfelt thanks to Debbie Faichney for her helping hand in the bacteriology laboratory and for training me in tissue processing for histopathology. Moreover, I am thankful to Rona Werner and Kathryn Grace for their help in bacteriology and molecular biology assays.

I am also thankful to Jacquie Ireland, Dr. John Taggart and Dr. Kerry Bertie for their tips and suggestions in molecular biology work.

I am grateful to my past and present officemates and friends Shankar, Savitree, Chris, Ahmed Ahmed, Ying, Khalid, Elizabeth, Simao, Xu Gong, Claudia, Jessica and Karla without whom life would be boring. I always appreciate their inspiration, suggestion and help in different aspects. I will never forget their

company. Special thanks to Hery Kurniawan and his wife Rhia, their kids Kenzo and Kesha whom I share my food and house.

To Sheila Nisbet, Alasdair and Daniela, who were my Filipino family and help here in Scotland. I am thankful for their generosity and friendship. You all will always have a special place in my life.

To my family in Aylesbury, England Tito Roflo and Tita Susan, and my cousins. Thank you for all forms of help and for adopting me every Christmas holiday. I am also blessed to have students who became friends who took me in their flats in London when I feel bored up here in the North. Thank you, Jaimie, Kae Wendy, Gab and McCoy.

To my DOST Newton PhD co-scholars Rommel and Justine, thank you for the friendship, travels, and memories. The monthly catch up with you guys eases the the sadness of living far away from home. To Dave, my travel buddy, let's do it again when this pandemic is over.

I am also grateful to the DOST-Newton PhD Agham Program of the British Council and the Depertment of Science and Technology, Philippines, and the Iloilo Science and Technology University, for which I will always feel grateful, for the scholarship grant and financial support without which this study would not have been possible.

I am also blessed to have a constant spiritual guidance, conversation, and prayer from my Pastor Carlos and his wife Sis. Adora Finally, to my parents, sisters and nieces Marie & Peonie for the constant video calls, their support and inspiration throughout this long journey which made it worth and enjoyable.

Madamo guid nga salamat (Thank you very much)!

ABSTRACT

Tilapia production in the Philippines has been stagnating if not decreasing for the past decade and several factors have been implicated including infectious diseases. To investigate the reports of disease and mortality outbreaks in 16 tilapia farms in the country, a field survey was performed 2018-2019 where the husbandry and health management practices of tilapia farms were described and biological samples were collected simultaneously, for disease diagnosis and confirmation. The majority of the tilapia farms were freshwater intensive production systems, where analysis of the farm level data showed, a tendency to overstock the systems initially, resulting in low survival levels post-stocking. Although the farming systems varied in their production capacity and management, nearly all farms used commercial feed with little or no comprehensive biosecurity practises being applied. From the survey interviews of this study, data showed that farmers recognised fish diseases using clinical signs and reported mortality outbreaks occurring during March to June, which coincides with increased water temperature.

The fish samples collected exhibited a wide range of gross clinical signs, compatible with bacterial septicaemia. Bacterial isolation and identification recovered Gram-positive *Streptococcus agalactiae* and *S. iniae* strains as well as Gram-negative memebers of the motile *Aeromonas* spp. dominated by *A. veronii*. Histopathological analysis revealed varied pathologies consistent with bacterial infection. These include haemorrhage, congestion, infiltration of inflammatory cells with necrosis of the cells and inflammatory reactions. The observed pathologies supported the observed clinical signs exhibited by the diseased fish samples.

The Gram-positive bacterial recovery data showed a higher prevalence for *S. agalactiae* compared with *S. iniae*, although both species were identified from diseased fish in the farming systems. The higher prevalence of *S. agalactiae* was similar to findings from other Southeast Asian countries. There were two serotypes of *S. agalactiae* identified, namely serotype Ia and serotype Ib which were both

circulating within the tilapia systems investigated. Main histological lesions found in tilapia samples infected with *S. agalactiae* and *S. iniae* were meningitis and encephalitis. The kidney, spleen, heart, and liver were filled with Gram-positive cocci with numerous infiltration of inflammatory cells indicative of bacteremia. The streptococci isolates were all susceptible to globally licensed antibiotics except for oxolinic acid which they have an intrinsic resistance. Additional antibiotic resistance genes were not detected in any strains investigated in this study. Virulence genes necessary for adhesion, invasion and immune evasion were all present in the isolates indicating their virulent nature.

As expected, several species of motile aeromonads were recovered and identified from moribund tilapia. The motile *Aeromonas* spp. were positively identified as *A. veronii*, *A. caviae* and *A. dhakensis*. The histological lesions in fish where the motile aeromonads were recovered were typical of motile aeromonas septicaemia as described in previous studies. The isolates were intrinsically resistant to all β-lactam antibiotics. Resistance to oxytetracycline, tetracycline, erythromycin and sulphamethoxazole-trimethoprim were observed mostly in *A. veronii*. The antbiotic resistant genes *tetE* and *sul1* together with the class 1 integron gene *IntI1* were detected in several *A. veronii* strains. Multpile virulence factors were detected in the isolates but were dominated by the cytotoxic enterotoxin *act* and the aerolysin *aer* which are important in establishment of MAS in fish. There were several genospecies based on presence of virulence genes showing the varied mechanism employed by these pathogens supporting the complexity of the pathogenesis of MAS in fish.

The findings of the present study highlight the contribution of infectious bacterial disease in the stagnating tilapia production in the Philippines. This study established the aetiological agents namely *S. agalactiae*, *S. iniae* and motile *Aeromonas* spp. especially *A. veronii* as the main cause of reported disease and mortality outbreaks.

PUBLICATION, CONFERENCES, AND TRAINING RELATED TO THIS THESIS

F.S. Legario, C.H. Choresca, J.F. Turnbull, M. Crumlish. Isolation and molecular characterization of streptococcal species recovered from clinical infections in farmed Nile tilapia (Oreochromis niloticus) in the Philippines. J. Fish Dis., 43 (11) (2020), pp. 1431-1442, 10.1111/jfd.13247

Scientific conferences and meetings attended

1. F.S. Legario, J.F. Turnbull and M. Crumlish. Investigating infectious bacterial diseases affecting farmed tilapia in the Philippines, PhD research conference, 17 April 2018, Institute of Aquaculture, University of Stirling (Poster Paper).
2. F.S. Legario, J.F. Turnbull and M. Crumlish. Identification and characterisation of bacterial pathogens affecting farmed tilapia in the Philippines, PhD Symposium, 24-25 October 2019, Institute of Aquaculture, University of Stirling (Oral presentation).
3. F.S. Legario. Streptococcosis in Nile tilapia. Complexly Microbial (Episode 3) Webinar. Biological Research and Services Laboratory, National Science Research Institute, University of the Philippines Diliman, Q.C. 6 November 2020. (Speaker).
4. F.S. Legario, J.F. Turnbull and M. Crumlish. Isolation and Characterisation of Streptococcal species from Farmed Nile tilapia in the Philippines. National One Health Antimicrobial Resistance (AMR) Summit Webinar Series Part 4: Research, Development, and Innovation. Webinar.Department of Health and Department of Agriculture, Republic of the Philippines. 23 November 2020. (Speaker).
5. F.S. Legario. Diversity of bacteria recovered from clinical infections in farmed Nile tilapia (*Oreochromis niloticus*) from the Philippines. 2021 MNH Biodiversity Seminar Series. Museum of Natural History, University of the Philippines Los Banos, Laguna. 19 February 2021. (Speaker).

Trainings and workshops attended

1. E1/L ScotPil Training Course. Core modules, Legislation and Ethics. Western Infirmary Lecture Theatre. 22nd October 2018. University of Glasgow, UK.

DEDICATION

To Lola Trining & Nanay Norma

My parents and my family

"Happy is the man who finds wisdom, and the man who gains understanding."

Proverbs 3:13#

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
°C	degree Celsius
°F	degree Fahrenheit
µl	Microliter
µm	Micrometre/micron
α	alpha
β	beta
Abbreviations	Elaboration
AAPR	average annual production rate
ADB	Asian Development Bank
ADH	Arginine dihydrolase
AMD	Amidon
AMR	Antimicrobial resistance
AMY	Amygdalin
ARA	Arabinose
ARG	Antibiotic resistance gene
α-MT	alpha-methyl testosterone
β-H/C	beta-haemolytic/cytolyti
BFAR	Bureau of Fisheries and Aquatic Resources
BIFTOS	Batangas Inland Fisheries Technology outreach Station
bp	Base pair
cAMP	cyclic adenosine monophosphate
CFU	colony forming unit
CHCA	α-cyano-4-hydroxycinnamic acid
CIT	Citrate utilisation
Da	dalton
DO	dissolve oxygen
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
e.g.	Exempli gratia (example)
ESC	Esculin
FAO	Food and Agriculture Organisation
FBC	Fisheries Biotechnology Centre
FCR	feed conversion ratio
fg	femtogram

g	gram
αGAL	α-galactosidase
βGAL	β-galactosidase
GBS	Group B *Streptococcus*
GEL	Gelatinase
GIFT	Genetically improved farmed tilapia
GLU	Glucose
GLYG	Glycogen
GMIT	Genetic manipulation for mproved tilapia
βGUR	B-glucoronidase
h	Hour
ha	Hectare
H & E	haematoxylin and eosin
HGT	horizontal gene transfer
HIP	Hippuric acid
H_2S	Hydrogen sulphide production
i.e.	Id est (that is)
IND	Indole production
INO	Inositol
INU	Inulin
ISKNV	infectious spleen and kidney necrosis
Kb	Kilo base
kDa	kilo dalton
L	Litre
LAC	Lactose
LAP	Leucine aminopeptidase
LDC	Lysine decarboxylase
LPS	Lipopolysaccharide
m	Metre
ml	Milliliter
mg/L	milligrams/Litre
M	molecular marker
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MAN	Mannitol
MAS	motile aeromonas septicaemia
MEL	Melibiose
MT	metric ton
MMC	melanomacrophage centre

NA	Nutrient agar
NaCl	sodium chloride
NFRDI	National Fisheries Research and Development institute
ng/µl	nanogram per microliter
N_2	Nitrogen gas
NO_2	Nitrogen dioxide
ODC	Ornithine decarboxylase
OIE	Office International des Epizooties
OF	oxidation-fermentation
ONPG	β-galactosidase
PAL	Alkaline phosphatase
PCR	polymerase chain reaction
pg	Pictogram
pH	power of hydrogen
ppt	part per thousand
PSA	Philippine Statistics Authority
PYRA	Pyrrolidonyl arylamidase
RAF	Raffinose
RHA	Rhamnose
RIB	Ribose
RFLP	restricted fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
rpoD	sigma 70
s	Second
SA	starch agar
SAC	Saccharose
SCV	small colony variant
SDS-PAGE	Sodium dodecyl sulphate polyactlamide gel electrophoresis
SEA	Southeast Asia
SEAFDEC	Southeast Asian Fisheries Development Centre
SOR	Sorbitol
SPF	specific pathogen-free
ST	Sequence type
STE	sodium-chloride-Tris-EDTA
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system

TAE	Tris-acetate-EDTA
TDA	Tryptophan deaminase
TELV	tilapia larvae encephalitis virus
TFA	trifluoroacetic acid
TiLV	tilapia lake virus
TRE	Trehalose
TSA	tryptone soya agar
TSB	tryptone soya broth
URE	Urease
UV	Ultraviolet
v/v	Volume/volume
VP	Vogues Proskauer
WHO	World Health Organisation
WT	Wild type
w/v	Weight/volume

CHAPTER 1.
General introduction and literature review

1.1 Tilapia Aquaculture

1.1.1 **Tilapia** Biology

Tilapia is the common name for the fishes in the family *Cichlidae*. Many species are broadly euryhaline while others are restricted to fresh or low-salinity waters. They are endemic to Africa, Jordan, and Israel (Popma & Lovshin, 1996), with more than 70 species identified, however, only a few species commercially farmed or have the potential for aquaculture (Table 1.1). Commercially important tilapias are grouped mainly on their reproductive behaviour: *Oreochromis* (maternal mouthbrooders), *Sarotherodon* (paternal and biparental mouthbrooders), and *Tilapia* (substrate incubators) (Watanabe et al., 2002; Lim & Webster, 2006).

Table 1.1 Commercially important tilapias and their characteristics (Modified from Mair, 2001)

Species	Common Name	Characteristics
Oreochromis niloticus	Nile tilapia	Performs well in tropical/subtropical areas; sexual maturity in ponds reach only at age of 5-6 months; suitable for culture in wide range of farming system (extensive to highly intensive system; monoculture and polyculture); high consumer and producer acceptance; least tolerant to cold water
O. aureus	Blue tilapia	Most cold resistant species (can tolerate low temperature of 8-9°C); suitable for culture in countries with seasonal changes in temperature; sexual maturity in ponds reach at age of 5-6 months; commonly used in hybridization for production of monosex tilapias
O. mossambicus	Mozambique tilapia	High saline tolerance (grows well up to 20ppt); early reproduction (attains sexual maturity at 8-9 cm) and high fecundity; poor aquaculture potential except when used for hybridization
O. spirilus	None	Saline tolerant; used in seawater cage culture
O. hornorum (*Tilapia urolepis*)	Zanzibar tilapia	Can tolerate brackishwater
Sarotherodon galilaeus	Galilee	Saline tolerant; slow growth
S. melanotheron	Black-chinned tilapia	Wide salinity tolerance (0-45 ppt but prefers 10-15 ppt; of interest for brackishwater aquaculture; used for extensive aquaculture in some parts of Africa
Tilapia rendalii	Redbreast tilapia	Feeds on macrophytes
T. zilii	Redbelly tilapia	Grows well in full strength seawater
Red tilapia hybrids	Hybrid origin	Suitable for brackishwater and seawater because of salinity tolerance of parental species; commonly used for intensive culture (cages, tanks, raceways) but also reported to be suitable for farming under low-input conditions; initial high consumer acceptance due to colour; sometimes exhibit low fecundity

Tilapias are suitable for aquaculture because they possess impressive range of attributes which include ease of propagation; handling tolerance; good growth on natural food, and adaptability to varied farming conditions, they can be supplemented with low-cost artificial diet; tolerance to wide range of environmental conditions, including changes in salinity, poor water quality and disease outbreaks (Cheverinski, 1982; Lim & Webster, 2006). Moreover, they are widely accepted as food fish because of their good-tasting flesh with mild flavour, and as such they are prepared in many cuisines. Tilapia consumption is not restricted by religious observances making them marketable worldwide (Watanabe et al., 2002).

Tilapias used in aquaculture are mouthbrooders providing high level of parental care. The fry produced from their large eggs are hardy, omnivorous at first feeding, and can accept artificial feeds after yolk-sac absorption. Moreover, they are able to breed in captivity (Watanabe et al., 2002; El-Sayed, 2006). These factors entail only a simple hatchery operation and made their aquaculture a success worldwide (El-Sayed, 2006). As with other fish species, reaching sexual maturity in tilapia is influenced by age, size and environmental conditions, and the timing can vary slightly for different tilapia species. The Mozambique tilapia *O. mossambicus*, in general, reaches sexual maturity at a smaller size and younger age than the Nile tilapia *O. niloticus* and blue tilapia *O. aureus*. This early sexual maturity of Mozambique tilapia led to its unpopularity in the Philippines because mixed-sex fishes resulted to overpopulation and stunting. Moreover, inbreeding led also to loss of favoured genetic traits (Toledo et al., 2008). Additionally, the type of culture environment can affect the rate of sexual maturity of the fish, where tilapia cultured in large lakes mature at a later age and larger size than the same species raised in culture ponds. While those farmed in in ponds usually reaches sexual maturity in less than 6 months and can breed naturally without the need for hormonal induction of spawning. When growth is slow, sexual maturity is also delayed, however, the fish may still spawn at weight as low as 20 to 30 g (Popma & Lovshin, 1996).

The early maturation and frequent spawning were the biological characteristics proved to be important but also caused negative impact in production systems since it causes male and female mixing, overpopulation, and stunting due to overcrowding (Fashina-Bombata & Megbowon, 2012). Additionally, associated with mixing is the non-uniform sizes of fishes during

harvest. An all male monosex tilapia culture is preferred compared with mixed or all females because of the differential growth rates observed in female fish. In males, the metabolic energy is channelled towards muscular growth due to anabolism enhancing androgens (Tran-Duy et al., 2008; Angienda et al., 2010). In females, metabolic energy is reallocated towards reproduction, and the fact that the females incubate the eggs in their mouth so they cannot feed during that period. For tilapia farmers that desire high yields of large-sized fish in a short culture period, all male fry are preferred. Therefore, the production of a male-biased tilapia to meet the industry need is a must for a successful and profitable tilapia farming. Although monosex population can be obtained by manual sexing, it did not gain an impact because it is laborious, time-consuming, and wasteful process. There are several methods used to obtain an all-male tilapia fry namely environmental manipulation, hybridization, sex reversal, and genetic manipulation (Fuentes-Silva et al., 2013). Of the different methods, sex reversal using the hormone methyl testosterone (17-α-MT), a synthetic male hormone which mimics naturally produced testosterone, has been recommended for production of monosex male tilapia because of its simplicity and ease of adaptability (Basavaraja et al. 1991; Pandian & Sheela, 1995; Fuentes-Silva et al., 2013). However, there is a global concern on the effects of this anabolic androgen on consumers, farm workers, and the environment. In some countries, for instance in the European Union and India, marketing of fish treated with 17-α-MT is illegal (White et al., 2006).

Tilapias are considered to be filter-feeders because of their ability to efficiently harvest plankton and other small particulate matters in the water column. It was observed that *O. mossambicus* is less efficient than *O. niloticus*

and *O. aureus* in ingesting planktonic algae, which may account why it grows slower than the two species. The great length of the portions of the intestine provides abundant surface area for nutrient absorption, which is likely one factor in the rapid growth rate characteristic of tilapias (Tengjaroenkul et al., 2000). Moreover, the enzymatic secretion in tilapia has been found to follow a circadian cycle (Montoya-Meijia et al., 2016). The result of the experiment showed that secretion of digestive enzyme activities peaked during nigh time. This is important since it will be useful to establish feeding times to coincide with natural peaks of enzyme secretion thus increasing feed efficiency that will be reflected in the weight gain of the fish providing a more profitable aquaculture production.

Disease outbreaks due to viral (Machimbirike et al., 2019), fungal (Mahboub et al., 2021) and parasitic (Valladao et al., 2016) have been affecting cultured tilapia globally, but by far the biggest infectious disease threat is due to outbreaks from bacterial diseases. In all tilapia farming systems, amongst important bacterial diseases are those outbreaks from *Streptococcus* infections, which affect production worldwide and can occur in most common production systems. The reported clinical signs of streptococcal infection may include skin haemorrhages, ocular alterations, ascites, and abnormal behaviour (Amal & Zamri-Saad, 2011; Suwannasang et al., 2014). In 1997, it was estimated that the yearly global economic loss due to infection with *Streptococcus* was in the order of $150 million (Shoemaker & Klesius, 1997). As well as Streptococcosis, there are several other common infectious diseases reported to cause natural infections in farmed tilapia species. Columnaris disease is caused by the bacterium *Flavobacterium columnare* often shows clinical signs of necrotic gills,

fin rot, skin erosion or necrotic muscle (Figueiredo et al. 2005; Dong et al., 2015). Francisellosis is another bacterial disease caused by *Francisella noatunensis* subsp. *orientalis* and the Gram-negative bacterial species of Edwardsiellosis caused by *Edwardsiella ictaluri* produce clinical signs of visceral white spots in internal organs (Soto et al., 2009, 2012; Nguyen et al., 2016). Haemorrhagic septicaemia is a common clinical sign, often reported from natural infections in tilapia from members of the motile aeromonads (*Aeromonas hydrophila*, *A. sobria*, *A. veronii* and *A. jandaei*) may present clinical signs of haemorrhage, exophthalmia, and ascites (Li & Cai, 2011; Dong et al. 2015; 2017) and mixed clinical signs of complicated multiple infections (Dong *et al*., 2015; Assis *et al*., 2017). As well as bacteria, viruses have been reported to cause natural disease outbreaks resulting in morbidity and mortalities in the tilapine cultured finfish (Crane & Hyatt, 2011; Zhang & Gui, 2015).

In general, it is considered widely that tilapia species can tolerate a wider range of environmental conditions, including factors such as salinity, dissolved oxygen, temperature, pH, and ammonia levels (Table 1. 2) than most cultured freshwater fishes. Most tilapia are highly tolerant of saline waters, although salinity tolerance differs among species. Nile tilapia is thought to be the least adaptable to marked changes in salinity as direct transfer to 18 parts per thousand causes mortality while the Mozambique, blue, and redbelly (*T. zilli)* are the most salt tolerant (El-Sayed, 2006). A study by El Sayed (2006) demonstrated that with exception of Nile tilapia, other tilapia species had an optimal growth and reproductive performance at 19 ppt, but tolerated salinity concentrations of up to 36 ppt. Tilapia are, in general described as a highly

tolerant species for low dissolved oxygen (DO) concentration, even down to 0.1 mg/L (Magid & Babiker, 1975), but optimum growth is obtained at concentrations greater than 3 mg/L (Ross, 2000). Temperature is a major modifier of the metabolic rate in fish. Under farming conditions, optimal growing temperatures are described as typically when the water is between 22° C (72 °F) and 29° C (84 °F); spawning normally occurs at water temperatures greater than 22° C (72 °F). Most tilapia species are unable to survive at temperatures below 10° C (50 °F), and growth is poor below 20 °C (68 °F) (Sarig, 1969; Mires, 1995). Of all the farmed tilapia species, it is the blue tilapia that are described as the coldest tolerant, surviving at temperatures as low as 8 °C (46 °F), while other species can tolerate temperatures as high as 42 °C (108 °F) (Sarig, 1969; Caulton, 1982; Mires, 1995). Other water quality characteristics relevant to tilapia culture are pH and ammonia. In general, tilapia can tolerate a pH range of 3.7 to 11, but best growth rates are achieved between pH 7 to 9 (Ross, 2000). At concentrations of 2.5 and 7.1 mg/L, unionized ammonia is toxic for blue tilapia and Nile tilapia (Redner & Stickney, 1979; El-Sherif et al., 2008) respectively, and depresses feed intake and growth at concentrations as low as 0.1 mg/L (El-Sherif et al., 2008). Optimum concentrations are estimated to be below 0.05 mg/L (El-Sherif et al., 2008). It is toleration of these reported extremes in water quality parameters that has supported the perception that tilapia species are a hardy or robust fish, and yet, as with all farmed species they clearly have an optimal range for maximum performance under farming conditions.

Table 1.2 Tolerance of tilapia on different water quality parameters.

Parameter	Range	Optimum for Growth	Reference
Salinity, parts per thousand	Up to 36	Up to 19	El Sayed (2006)
Dissolved oxygen, mg/L	Down to 0.1	> 3	Magid and Babiker (1975); Ross (2000)
Temperature, °C	8-42	22-29	Sarig (1969); Morgan (1972); Mires (1995)
pH	3.7-11	7-9	Ross (2000)
Ammonia (NH_3), mg/L	Up to 7.1	< 0.05	El-Shafey (1998); Redner and Stickney (1979)

1.1.2 Global Status of Tilapia Aquaculture

According to the Food and Agriculture Organization of the United Nations (FAO), the global production of tilapia was estimated at 7.02 million tons (MT) in 2021 (Figure 1.1), with the top three producers being the People's Republic of China (1.78 MT), Indonesia (1.12 MT) and Egypt (0.88 MT) (FAO, 2017). Other leading producers included Bangladesh, Vietnam, and the Philippines (FAO, 2017).

Farming of tilapia has increased in the last three decades as they are easy to grow and market. More than one hundred countries now farm tilapia (FAO, 2013), and 98% of them are grown outside their original habitat (Shelton, 2002). After carps, tilapia are the second most farmed freshwater fish in the world, with 3.96 million metric tons (MmT) of fish produced, comprising 6.3% of global aquaculture production (FAO, 2011). Tilapia are consumed globally in countries where they are considered as traditional as Africa and Asia as well as

those that consider them exotic such as USA, Canada, Europe, Central and South America as well (Gupta & Acosta, 2004).

Production of the farmed tilapia continues to grow. The production in 2019 reached 6.5 MmT, a 4 % growth compared with values from 2018, despite significant disease losses (around 300,000 metric tons, significantly due to *Streptococcus* spp. infections) reported for Asia and costing perhaps as much as $500 million in lost value (Aquaculture Alliance, 2019).

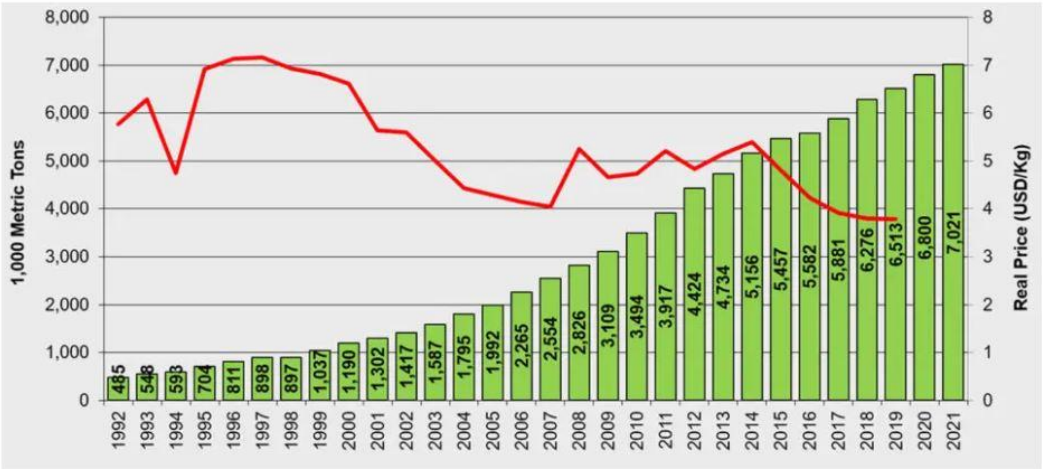

Figure 1.1 Global production of farmed tilapia, with representative U.S. import prices for frozen fillets (red line), 1992 to 2021. (Sources: FAO: 1990-2016. Miscellaneous sources: 2017-2019. NFMS: Prices US imported frozen fillets.) (Taken from Tveteras et al., 2020).

Producers of farmed tilapia in the world. Tilapia is currently the most popularly cultured aquatic animal group in the world in freshwater, brackishwater and seawater, with 145 countries or regions reporting tilapia production to FAO in 2018 (FAO, 2019). It is presently cultured in different culture environments such as freshwater, brackishwater and seawater. Although tilapia originated in Africa, Asia has dominated its production ever since the fish was introduced to aquaculture. Asia produced 4.2 million tonnes

of farmed tilapia in 2018, which accounted for 68.8 % of the world total. However, Asia's share of in the world production has declined significantly compared with 84.4 % in 1998 (FAO, 2020). In contrast, Africa, the original source of tilapia, has successfully increased its share from 7.5 % in 1998 to 21.8 % in 2018. The share of the Americas slightly increased to 9.3 % in 2018 from 8.1 % in 1998. China remained the largest producer of farmed tilapia in 2018, with a production of 1.62 million tonnes (Table 1.3). However, its share in the world production declined from 52.3 % in 1998 to 26.9 % in 2018. Meanwhile, in 2013, Indonesia surpassed Egypt to become the world's second-largest producer of farmed tilapia. It produced 1.22 million tonnes of farmed tilapia in 2018, which accounted for 20.3 % of the world total. In 2018, Egypt was the only African country among the top-ten producers of farmed tilapia, contributing 17.4 % of the world total. Thailand and the Philippines used to be among the top-four producers of farmed tilapia, however, their shares in the world production of farmed tilapia declined to 3.5 % and 4.6 %, respectively in 2018 (Miao & Wang, 2020) (Table 1.3).

Table 1.3 Major producers of farmed tilapia in the world (individual production above 50,000 tonnes in 2018). (Taken from Miao & Wang, 2018).

Country	Production (1,000 tonne)		% in Global production	
	1998	2018	1998	2018
China	471.8	1,625.5	52.59	26.93
Indonesia	65.9	1,222.7	7.35	20.27
Egypt	52.8	1,051.4	5.88	17.43
Bangladesh	-	344.8	-	5.72
Brazil	24.1	317.1	2.68	5.26
Philippines	72.0	277.0	8.03	4.59
Vietnam	-	260.0	-	4.31
Thailand	73.8	211.4	8.23	3.51
Colombia	17.7	77.9	1.97	1.29
Ghana	1.4	70.6	0.15	1.17
Uganda	0.2	70.1	0.02	1.16
Taiwan POC	36.1	62.6	4.03	1.04
Mexico	5.4	52.7	0.60	0.87

Cultured tilapia production by species. Globally, 23 species and species groups in the family *Cichlidae* were included in FAO aquaculture production statistics by 2018. Among these, Nile tilapia has dominated global farmed tilapia production (Table 1.4). World production of farmed Nile tilapia reached 4.53 million tonnes in 2018, which accounted for 75 % of the total farmed tilapia production. However, the share of Nile tilapia in total production of farmed tilapia has declined significantly from 83.4 % in 1998. The production of tilapia that is not elsewhere included accounted for 17.1 % in the total farmed tilapia production, which suggests a significant proportion of farmed tilapia

production could be reported down to species level. In 2018, production of the hybrid of blue tilapia and Nile tilapia reached 0.41 million tonnes and accounted for 6.7 % of total farmed tilapia production (FAO, 2020). The production is primarily from China, where technology has been adopted to take advantage of all male offspring (>95 %) from the hybridisation of blue tilapia and Nile tilapia without hormone manipulation. The share of Mozambique tilapia in the total farmed tilapia production significantly declined to less than 1 % in 2018.

Table 1.4 Global production of ten major farmed tilapia species in 2018 (individual farmed production above 1,000 tonnes). (Table modified from Miao & Wang, 2020)

Species	**Production (tonne)**		**% of total tilapia production**	
	1998	**2018**	**1998**	**2018**
Nile tilapia (*O. niloticus*)	748,040	4,525,431	83.38	75.03
Tilapias nei (*Cichilidae*)	103,564	1,030,004	11.54	17.08
Blue-Nile tilapia, hybrid	0	406,048	0.00	6.73
Mozambique tilapia, (*O. mossambicus*)	40,652	53,754	4.53	0.89
Shire tilapia, (*O. shiranus*)	0	5,036	0.00	0.08
Blue tilapia (*O. aureus*)	844	3,182	0.09	0.05
Three spotted tilapia (*O. andersonii*)	2,689	2,147	0.30	0.04
Redbreast tilapia (*Coptodon rendalii*)	839	1,903	0.09	0.03
Longfin tilapia (*O. macrochir*)	207	1,800	0.02	0.03
Tanganyika tilapia (*O. tanganicae*)	0	1,690	0.00	0.03

1.1.3 Current Issues and Problems in the Philippine Tilapia Sector

In the Philippines, tilapia is the second most important fish species that is farmed, next to milkfish, to improve food security and alleviate poverty (Toledo et al., 2008). The introduction of strains with improved growth rates when raised in freshwater environments has contributed to the increased tilapia production in the country. Additionally, farmed tilapia has demonstrated itself to be a food that is particularly relevant to the poor, due to its stable, low price and its income elasticity of demand among poorer populations. Continuous breeding using the same tilapia population have resulted in genetic deterioration which affected growth traits in successive generations (Eknath & Acosta, 1998). Thus, the Genetic Improvement of Farmed Tilapia (GIFT) project was launched in Asia and succeded in producing tilapia with faster growth rates, higher survival rates, and a shorter harves time, thus increasing fish yields dramatically (Yosef, 2009). It is estimated that 19.3−22.6 million Filipinos have benefited from GIFT and GIFT-derived strains (Yosef, 2009).

While Asia is the main exporter of farmed tilapia, the Philippines lags definitively behind other Asian competitors (El-Sayed, 2006). This is because historically, the national marketable size of 143– 200 grams has been much smaller than the international marketable size of 400-500 grams for live fish and 700 to 1,000 grams for fish that will be filleted (Dey et al., 2000; World SeaFood Market 2005). While American consumers demand a large fish that can be filleted, the Philippine consumers of domestic produced tilapia traditionally preferred a smaller fish (World SeaFood Market, 2005).

Over the years, Philippine aquaculture has become diverse in terms of species, culture systems and environment, type of operation and scale, and intensity of practice (i.e., stocking density and number of cages in lakes) (FAO, 2010). However, the main species contributing remains stable: milkfish, tilapia, penaeid shrimp, seaweeds, and mussels. Of these, tilapia ranks third in terms of production volume following seaweeds and milkfish (Figure 1.2).

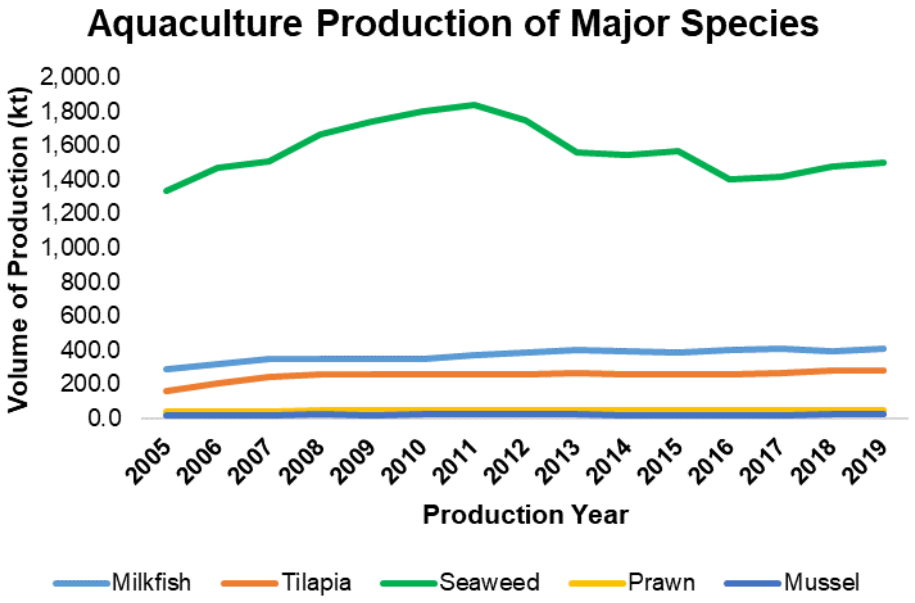

Figure 1.2 Volume of production of top five aquaculture commodities (2005-2019, Philippine Statistical Authority).

The culture of tilapia has become a major industry in the Philippines next to milkfish in terms of production and economic importance (Baliao & Dosado, 2011). As stated previously, Nile tilapia is the most widely cultured tilapia species produced in the freshwater systems in the Philippines as it has the most rapid growth compared with other tilapia species. This accounts for around 90% of total production (Baliao & Dosado, 2011). On the other hand, the Mozambique tilapia (*O. mossambicus*) are highly tolerant of variations in salinity

(euryhalinity) but are slow growing and accounts for the remaining 10% production of the total amount of tilapia production for both fresh and brackishwater systems farmed in the Philippines. The popularity of tilapia in the Philippines extends from a perpetual or consistent high domestic demand for this food fish combined with the relative ease and low-cost production.

The introduction of Nile tilapia paved way for the progress of freshwater aquaculture in the Philippines, and the introduction of monosex culture made it a profitable venture. Three major lakes in the Philippines host numerous tilapia cages and pens, *i.e.,* Laguna de Bay, Taal Lake, and Lake Sebu. The production intensity has increased over the years and different production methods have been tried in freshwater lakes and brackishwater ponds with encouraging results (ADB, 2005). The current productions in the freshwater systems are a combination of semi-intensive and intensively farmed grow-out ponds, and primarily intensive in floating cages. Particularly net cage culture of tilapia (Figure 1.3) has been introduced, and it requires a relatively low investment, is quick and easy to start up, allows more production cycles per year and gives opportunity to expand into tilapia farming business. In net cages, the stocks are contained thus providing ease in the feed management, adequate supply of good water and growth conditions, and protection of stocks against predators and competing animals (Baliao et al., 2000; Romana-Eguia et al., 2005). However, tilapia net cages are primarily used in freshwater and brackishwater ponds and presently a small number in seawater.

Figure 1.3 Tilapia floating cages in Taal Lake, Batangas, Philippines. (21 March 2018).

To supply the persistent domestic demand for tilapia, new breeds were developed like the GIFT, Genetic Manipulation for Improved Tilapia (GMIT), National Inland Fisheries Institute (NIFI), Israel strain, FAC Selected Strain (FAST), SEAFDEC-selected strain (Eguia & Taniguchi, 2006). However, even with the improved tilapia strains, production volume of tilapia remained the same if not sometimes decreasing. These breeds were developed in response to the decreased production in the 1980s because of the dwindling quality of tilapia stocks primarily on growth and survival characteristics due to inbreeding, genetic and founder effects, and gene introgression (Toledo et al., 2008). This is due to the fact that Philippines has limited freshwater areas for large scale farming. Whilst teasing apart the varied reasons for the lack of sustainable development it has become clear that any substantial expansion of tilapia farming in the Philippines should include and consider the development of saline-tolerant tilapia mariculture, which the Bureau of Fisheries and Aquatic

Resources (BFAR) and some private sectors are now field testing. The viability of tilapia culture in brackishwater ponds has already meet with high degree of success as it is co-cultured with prawn. However, the production from brackishwater ponds and freshwater cages can meet only domestic demand but not the increasing international market demand. For the Philippines to join as one of the major exporters of tilapia, it has to increase the current production volume. However, the tilapia production from 2002-2020 (Figure 1.4) showed that it has stagnated if not decreased over the years.

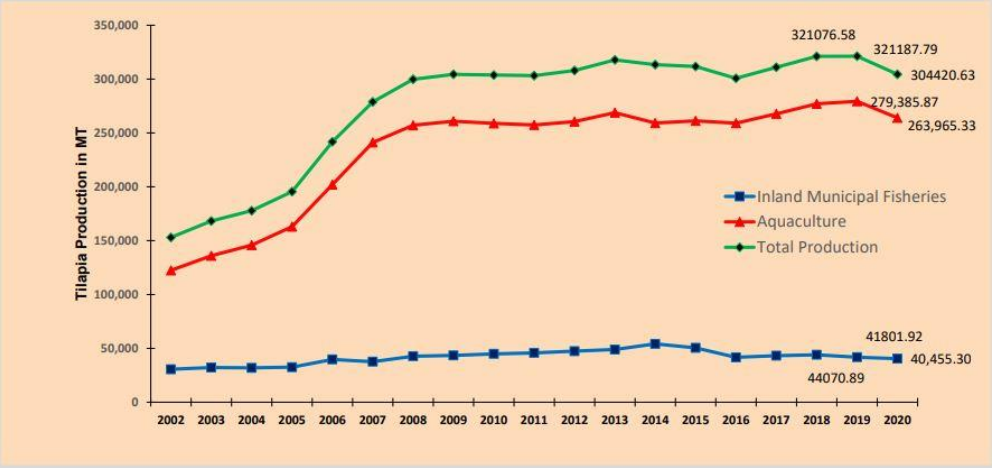

Figure 1.4 Tilapia production in the Philippines over a ten year period. (Source: Fisheries Statistics of the Philippines 2002-2020). (Taken from Fisheries Statistics of the Philippines 2002-2020).

The total tilapia production as seen in Figure 1.4 is only slightly higher from tilapia production from the production due to aquaculture. The slight difference is due to the tilapia production from inland municipal fisheries which constitutes minimally.

In the study of Guerrero (2019), data from the Philippine Statistics Authority (PSA) showed that from 2014-2016 there was a 0% increase in the production

of tilapia. From the fisheries data of PSA, the average annual production rate (AAPR) was 0.7%. From the total annual productions of the various culture systems for 2007–2015 (Table 1.5), the AAPRs found were 4.8% for brackishwater ponds, 1.5% for freshwater pens, 1.3% for freshwater cages, and 1.1% for freshwater ponds.

Table 1.5 Tilapia productions (MT) by culture systems and AAPRs (%) for 2007-2015. (Adapted from Guerrero, 2019).

Year	Brackishwater Pond	Freshwater Pen	Freshwater Cage	Freshwater Pond
2007	12,155	19,215	79,001	130,456
2008	14,957	21,120	81,748	138,682
2009	14,921	21,512	83,749	140,422
2010	13,999	21,533	102,622	142,913
2011	14,194	22,268	98,452	142,645
2012	14,380	21,379	100,682	144,261
2013	15,213	21,905	105,329	142,852
2014	18,449	21,358	87,742	143,336
2015	18,378	21,460	94,723	142,339
AARP	4.8	1.5	1.3	1.1

From the data in Table 1.4, it clear shows that only the brackishwater pond system has seen an increasing trend in production while the rest of the freshwater systems were either stagnating or decreasing. This increasing trend of production in brackishwater ponds could be due to the shift in black tiger shrimp to tilapia farming or their co-culture (Cruz et al., 2008). The limited freshwater area and intensification of culture indicates that the holding capacity of these environments has already reached its limit.

In the previous study of Toledo et al. (2008), they have identified several factors that contributes to the weakness of the commercialization of tilapia industry. These includes: (1) maintenance of genetic integrity in improved tilapia

stocks, (2) sustainable production of improved genetic stocks, (3) formulation of guidelines on how to manage this new diversity, (4) controlling environmental degradation caused by aquaculture activities in lakes and other freshwater bodies where tilapia are raised, and (5) improving rural access sector to financial resources.

In the study of Guerrero (2019), focus group discussion and key informant interviews with tilapia farmers in the Philippines identified the cause of low tilapia production were: (1) high water temperature, (2) lack of government assistance, (3) poor breed of tilapia, (4) high cost of production, and (5) lack of capital. There was no mention of diseases as one of the main factors that can affect production which is contrary to the current situation where global tilapia aquaculture is plagued by diseases like streptococcosis (Amal & Zamri-Saad, 2011; Suwannasang et al., 2014) and the most recent tilapia lake virus (TiLV) (Dong et al., 2017; Surachetpong et al., 2017). This TiLV has also been reported in the Philippines affecting farmed Nile tilapia (OIE, 2017).

Tilapia productions in the freshwater pens and floating cages in major freshwater lakes in the country appear to be holding up with relatively low AAPRs despite the reported fish kills that have occurred in them which was suggested to be due to cage congestion and water pollution (Luistro 2008; Escandor, 2015; MindaNews, 2017). The number of fish pens in Laguna de Bay has been reduced due to concerns regarding their negative ecological and social impacts (Palma, 2016). Likewise, the number of cages in Taal Lake has been lessened from 14,000 to 7,000 to minimize fish mortalities during the occurrence of lake overturns (Guerrero, 2019). There is a need to regulate the number of tilapia pens and cages in water bodies within their carrying capacities

to sustain the tilapia production, however, further increase in the production for such culture systems is limited.

It is said that the real expansion of production will come only with the development of saline-tolerant tilapia since Philippine aquaculture industry is synonymous with brackishwater production. The potential to further increase tilapia production in brackishwater ponds can only be achieved with the development of tilapia strains and hybrids (e.g., Molobicus) of the BFAR that are saline-tolerant, the culture of tilapia in brackishwater ponds has been promoted (Guerrero, 2018). Additionally, the area for farming is not an issue since country has more than 200,000 ha of brackishwater ponds that are mainly used for the culture of milkfish and shrimps (*Penaeus* spp.). Other tilapia species can also be considered like the euryhaline black-chin tilapia (*Sarotherodon melanotheron*), which thrives in Laguna de Bay and Manila Bay (Bigalbal et al., 2018). This introduced tilapia species in Taal Lake is now one of the top five most abundantly caught fishes with good acceptance by consumers (Guerrero, 2019).

The viability of tilapia culture in marine cages is also seen as another strategy that can increase production in the coming years. This marine cage culture of saline-tolerant tilapia is highly plausible since the Philippines has expansive coastal area which are currently underutilized for mariculture activities. Currently, there are only very little fish farmers that ventures into marine cage culture of tilapia due to some problems that have been encountered (Salayo et al., 2012). However, with the new culture environment, it will be expected that the expansion could possibly be constrained with the emergence of new diseases, sustainable supply and acclimatization of

fingerlings, and nutrition. Moreover, some local biologists and environmentalist have apprehensions due to fear of tilapia escapees that may disrupt ecosystem balance.

Despite the technological advancements in husbandry and improved tilapia strains, diseases directly jeopardize aquaculture production by causing mass mortality in farming area, or indirectly by deteriorating reproduction performance and feed conversion efficiency (Kayansamruaj et al., 2020). The economic impact of diseases on global aquaculture has been estimated by FAO to be US$ 6 billion annually, however, in Southeast Asia (SEA) the economic impact of diseases on freshwater finfish production unfortunately has not been systematically estimated to date (Brummett et al., 2014). Important major disease outbreaks caused by bacterial pathogens including *Streptococcus* sp., *Flavobacterium* sp., *Edwardsiella* spp. and *Aeromonas* spp. have been intermittently reported in every major freshwater finfish species of the region (Kayansamruaj et al., 2020). In the Philippines, reports of mortality and disease outbreaks in farmed tilapia due to bacterial infection is a present health problem of the sector. Diagnostic reports from the Bureau of Fisheries and Aquatic Resources (BFAR) indicates that the major causative agents of these infections are the streptococci and motile aeromonads, however, specific species and strains of the bacterial pathogens were not verified. The distribution of major bacterial diseases in three important freshwater fishes of SEA is demonstrated in Figure 1.5.

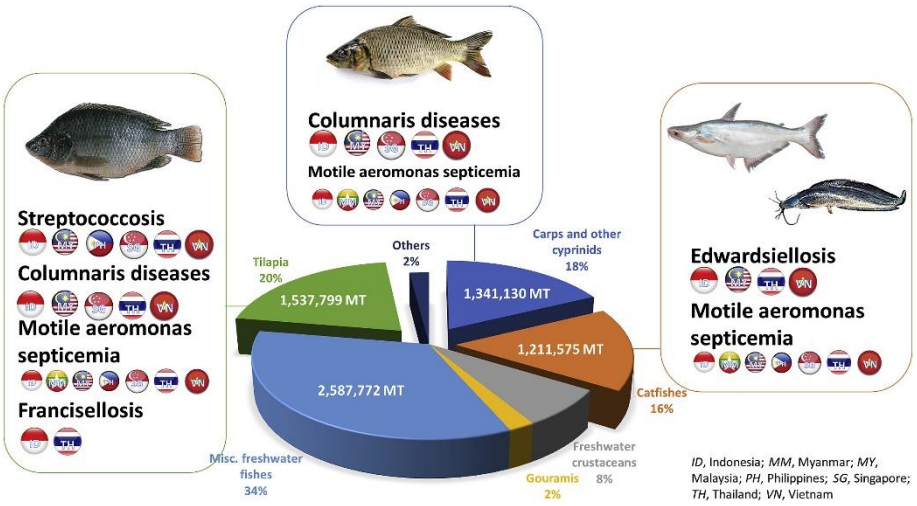

Figure 1.5 Production of the aquaculture species and distribution of major bacterial diseases in Southeast Asian countries (Image taken from Kayansamruaj et al., 2020).

In order to control disease outbreaks, sanitization of cultivation system might not be able to handle majority of the small-scale finfish farms SEA. Morever, the use of antibiotics is not encouraged in compliance with the food safety and food security policies. Therefore, the activation of self-immunity in fish by vaccination is the most practical and reliable method to control infectious diseases. However, effective vaccines can only be made by establishing first the specific type of bacterial strains in the area and characterise their pathogenic or virulent properties.

1.2. Streptococcosis in Tilapia

Streptococcosis is an infectious disease in fish that is caused by several bacterial species such as *Streptococcus parauberis, Streptococcus iniae*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae* regardless of geographical region (Toranzo et al., 2005; Vendrell et al., 2006; Agnew & Barnes, 2007; Nho et al., 2009) (Table 1.6). It is a multifactorial disease in fish, depending on host variety, age, immune status, type of pathogen (species and strain), and environmental conditions (Ghittino et al., 1999; Ravelo et al., 2001; Vendrell et al., 2006).

Table 1.6 Streptococcus bacterial agents and detailed information of affected fish species, locations, hosts, and clinical criteria. (Adapted from Mishra et al., 2018).

Species	Host	Fish species	Clinical criteria	Geographical Location
Streptococcus iniae	Fish, Human	Hybrid striped bass, Nile tilapia, Hybrid tilapia, Rainbow trout, Red drum, Rabbitfish, Sea bass, Olive flounder, Barramundi, Wild fish	Haemorrhage, exophthalmia, abdominal distension, ascites, lesions (liver, kidney, spleen, and intestine)	Canada, Americas, Bahrain, Israel, Thailand, China, Japan, Singapore, Taiwan, Korea
Streptococcus parauberis	Fish, Cow	Olive flounder, Rainbow trout, Cultured turbot, Hybrid striped bass	Chronic wasting syndrome, haemorrhagic septicaemia, exophthalmia, meningitis with abnormal swimming	Israel, Italy, Japan, Spain, USA, China, Iran, Korea, Malaysia, India
Streptococcus agalactiae	Fish, Cow, Human, Chickens, Camels, Dogs, Horses, Cats, Frogs, Hamsters, Monkeys	Nile tilapia, Barcoo grunter, Golden pompano, Giant Queensland grouper, Ya-fish, Silver pomfret	Erratic swimming, appetite, lethargy, uncoordinated movements, exophthalmia (uni- or bi-lateral), intraocular haemorrhage, opaqueness of cornea, ascites	Europe, Turkey, China, Indonesia, Malaysia, Japan, Korea, Vietnam, Philippines, Americas
Lactococcus garvieae	Fish, Cow, Human, Cat, Dog, Water buffalo	Rainbow trout, Yellowtail, Tilapia, Japanese eel, Grey mullet, Black rockfish, Catfish, Wild wrasse, Giant fresh water prawn, Olive flounder,	Melanosis, lethargy, erratic swimming, disorientation, fins, exophthalmia (uni- or bi-lateral), swollen abdomens, anal prolapses, haemorrhages (periorbital, perianal, buccal	Turkey, Australia, South Africa, England, Portugal France, Balkans, Israel, Korea

		Amberjack, kingfish	regions)	
Streptococcus dysgalactiae	Fish, Calves, Lamb, Human, Sheep, Dogs, Pig, Lamb, Cats	White spotted snapper, Kingfish, Grey mullet, Cobia, Hybrid red tilapia, Pompano, Basket mullet, Pompano, Golden pomfret, Amur sturgeon, Nile tilapia, Yellow tail, Amber-jack	Abnormal swimming, loss of orientation, exophthalmia	Brazil, Indonesia, Malaysia, Taiwan, China, Japan
Vagococcus salmoninarum	Fish	Rainbow trout, Atlantic salmon, Brown trout	Loss of equilibrium, exophthalmia, melanosis, bleeding (jaw, eye, mouth, abdomen, fins, and anus), necropsy, transparent fluid accumulation, fibrinous deposits (heart, liver, spleen)	France, Italy, Spain

Of the pathogenic *Streptococcus* spp. *S. agalactiae* and *S. iniae* are recognised as major bacterial pathogens affecting cultured and wild populations of fresh and marine water fish species globally (Agnew & Barnes, 2007; Mian et al., 2009). Mortalities are often high with as much as 80% being reported due to *S. iniae* in commercial tilapia farm (*O. aureus*) in Mexico (Ortega et al., 2018). In addition, mortalities exceeding 80% have been reported in red tilapia (*Oreochromis* spp.) farms infected with *S. agalactiae* (Zamri-Saad et al., 2010). Due to the high mortality of this infectious disease, it led to massive economic losses to the tilapia industries worldwide (Liu et al., 2016).

1.2.1 Genus Streptococcus

Streptococcosis is a disease that is caused by the infection of *Streptococcus* sp. *Streptococci* are Gram-positive, spherical, or ovoid in shape and 0.5-2.0 µm in diameter which can occur in pairs or chains when grown in liquid media, are non-motile, non-spore-forming and Gram-positive. It is facultatively aerobic, requiring nutritionally rich media for growth and commonly

attacks red blood cell to produce greenish discolouration (α-haemolysis) or complete clearing (β-haemolysis) on blood agar. In addition, it is also a type of bacteria that are homofermentative in metabolism, producing mainly lactic acid, but without gas and catalase negative (Holt et al., 1994).

A bacterial disease outbreak of *Oreochromis* spp. and rainbow trout (*Oncorhynchus mykiss*) in Israel in 1986 led to the isolation and naming of *S. shiloi* and *S. difficile* (Eldar et al., 1994). These were considered as newly described species of fish pathogens causing meningoencephalitis in cultured fish; *S. difficile* was additionally described as serologically non-typeable (Eldar et al., 1994). However, it was demonstrated through whole-cell protein electrophoresis that the type strain of *S. difficile* was indistinguishable from *S. agalactiae* strains when recovered from various host sources (Vandamme et al, 1997). Comparative sequence analysis using the 16S-23S ribosomal DNA intergenic spacers of *S. difficile* and *S. agalactiae* revealed their genetic relatedness (Berridge et al., 2001). High levels of genetic similarity between these putative species were also found by Kawamura et al. (2005) and suggested that although there are biochemical differences between them, *S. difficile* and *S. agalactiae* are synonyms. The specific epithet of *S. difficile* was subsequently emended to *S. difficilis* by Euzeby (1998). Similarly, it was also found out that *S. shiloi* and *S. iniae* strains were phenotypically identical and through DNA-DNA hybridisation the level of homology between strains was 70-100%. Thus Eldar et al., (1995) declared that *S. shiloi* should be considered as junior synonym to *S. iniae*.

The beta-haemolytic strains of *Streptococcus* describe by Japanese researchers (Minami, 1979; Kitao et al., 1981; Ohnishi & Jo, 1981;

Nakatsugawa, 1983; Sakai et al, 1986) should be classified as a subspecies of *S. iniae* (Kitao, 1993). Kitao (1993) further stated that the *Streptococcus* species described by Robinson and Meyer (1966), Plumb et al. (1974), and Rasheed and Plumb (1984) all belong to the same category as non-haemolytic or gamma-haemolytic, and Lancefield ‘s B group. Since *S. agalactiae* is classified as Group B *Streptococcus* (Lancefield, 1933), the Streptococcus species in reported these studies are considered *S. agalactiae*.

The *S. iniae* bacterium is classified into two serotypes based on their arginine dihydrolase activity. Outbreaks of streptococcosis in rainbow trout farms in Israel which were vaccinated against *S. iniae* occurred from 1995 to 1997 (Barnes et al., 2003). These were associated with a variant of the bacterium which, unlike the previous isolates, was arginine dihydrolase negative (AD–ve) (Zlotkin et al. 1998) and could be differentiated using the rapid amplified polymorphic DNA (RAPD) technique (Bachrach et al., 2001). Furthermore, serological analyses using rainbow trout antisera indicated antigenic differences and the earlier isolates (AD+ve) were classified as Serotype I and the later isolates (AD–ve) as Serotype II. Antisera to Serotype I did not agglutinate Serotype II, but some cross-reactivity was observed in the opposite direction (Bachrach et al., 2001).

S. agalactiae can be sub-divided into ten serotypes (Ia, Ib and II to IX) based on the composition of the capsular polysaccharide (Imperi et al., 2010). Serotypes Ia and Ib are the most prevalent in seafood (van der Mee-Marquet et al., 2009). In diseased tilapia, serotypes Ia, Ib and III are the most commonly isolated strains (Evans et al., 2008; Suanyuk et al., 2008; Rodkhum et al., 2011). In Brazil, serotype Ib is the most prevalent, although strains belonging to

serotype Ia and non-serotypeable strains have been isolated in streptococcosis outbreaks in tilapia (Godoy et al., 2013). Serotype III has four subtypes, and mainly the subtype 4 has been described as an emergent pathogen in fish farms in Thailand and China (Delannoy et al., 2013; Li et al., 2013) and is related with high zoonotic potential (Ip et al., 2006; Delannoy et al., 2013). Furthermore, in experimental challenge, serotype III showed much higher virulence than serotype Ia (Areechon et al., 2016).

1.2.2 Transmission

The transmission of *S. iniae* and *S. agalactiae* is primarily by the newly introduced diseased fish into the farm (Nguyen et al., 2002). The bacteria are excreted in the faeces of infected fishes, survive in the water, and become infectious to other healthy fish (Nguyen et al., 2002). The horizontal transmission of the pathogens between fish is believed to be the most common route of dissemination as cohabitation of dead or infected fish with healthy fish resulted in infection or disease (Amal & Zamri-Saad, 2011). Moreover, in the study by Xu et al. (2007) streptococcal infection could occur through wounds and abrasions of the skin. This mechanism is usually involved in fish that were cultured in high densities. Furthermore, the transmission of *Streptococcus* between different species of wild and cultured fish, within the same aquatic environment, is likely to occur (Evans et al., 2002). This is supported by the study of Colorni et al. (2002) where they found that wild fish and fish cultured nearby have been found to be infected with the same *S. iniae* strains in Israel. Similarly, Bromage & Owen (2002) reported that the fish cohabiting barramundi

pens had the same *S. iniae* strains as the barramundi. In addition, the transmission among the species of reef fish has also been reported in the Caribbean (Ferguson et al., 2000).

1.2.3 Pathogenesis

Infection by *Streptococcus* leads to various clinical signs, which include haemorrhages at the gill plate, loss of appetite, spine displacement, haemorrhages in the eye, corneal opacity, and haemorrhages at the base of the fins and in the opercula. The most frequently reported signs are uni- or bi-lateral exophthalmia, also known as “popeye”, and distended abdomen. The post-mortem examinations of the affected fish revealed the presence of blood-tinged fluid in the body cavity, enlarged and reddened spleen, pale but enlarged liver, as well as inflammations around the heart and kidney while haemorrhagic lesions were observed on the skin (Bullock, 1981; Yanong & Floyd, 2002; Salvador et al., 2005). Other clinical signs include darkening of the skin and erratic swimming, which is either spiralling or spinning just below the surface of water. In some cases, however, the affected fish showed no obvious clinical signs before death and the mortality is mainly due to septicaemia and infection of the brain and nervous system (Barham et al., 1979; Yanong & Floyd, 2002).

A comprehensive comparative histopathological investigation by Chen et al. (2007) into tilapia infected naturally and experimentally with either *S. agalactiae* or *S. iniae* found that both pathogens can cause pericarditis, epicarditis, myocarditis, endocarditis, and meningoencephalitis. Additionally, the *S. agalactiae* infected tilapia showed large numbers of cocci present in tissues

and in circulation, which was not observed in *S. iniae* infected tilapia. The authors suggest that *S. iniae* infected tilapia develops a chronic form of streptococcosis during a natural disease outbreak as the fish is more effecting in controlling the infection, whereas in *S. agalactiae* infected fish an acute type of streptococcosis is produced. In experimental *S. iniae* infection, lymphohistiocytic, leptomeningitis, meningoencephalitis, encephalitis and meningitis were also described in infected tilapia (Baums et al., 2003).

According to Hernandez et al. (2009), *S. agalactiae* has a predilection for organs such as the brain, eyes, and heart. Moreover, Zamri-Saad et al. (2010) noted that during a natural disease outbreak of *S. agalactiae* the liver, spleen and kidney showed the presence of marked congestion and the endothelial lining of the blood vessels for the liver and spleen swollen and vacuolated. Histological findings from experimental and natural disease outbreaks of *S. agalactiae* have been previously described by and Filho et al. (2009), Ali et al. (2010), and Azad et al. (2012).

The pathogenesis of *S. iniae* is a multistep process which is believed to occur through the colonisation of external tissue, followed by local spread and subsequent invasion of the bloodstream (Zlotkin et al., 2003). Once in the bloodstream, the bacteria pass through the blood-brain barrier of the host either as free bacteria or within hijacked monocytes or phagocytes and then inhabit the central nervous system (CNS) (Zlotkin et al., 2003; Agnew & Barnes, 2007). The latter process is described as the “Trojan horse effect.” *S. iniae* loaded within the macrophage are able to withstand macrophage bactericidal activities and can trigger apoptosis to facilitate their release around the host’s body (Zlotkin et al., 2003; Agnew & Barnes, 2007). Such a process is also thought to

prevent the triggering of the host's defence mechanisms. In the work of Locke et al. (2007a) they found that certain strains possess a polysaccharide capsule which are more virulent in fish than their non-encapsulated since these encapsulated strains were resistant to phagocytic killing and macrophage clearance. These results indicate that phagocyte colonisation and survival may not be the principal infection strategy utilised by *S. iniae* (Agnew & Barnes, 2007) since the capsule plays an important role in preventing opsonophagocytosis by the host defense mechanisms.

Less is known about the pathogenesis of *S. agalactiae* infection in fish. Virulent GBS that contributed to sepsis onset would usually proceed through three consecutive steps as follows: (i) adhesion and colonization on a host surface, (ii) invasion across epithelial cells, and (iii) immune evasion to prevent bacterial clearance by the host immune response (Lin *et al*. 2011; Kenzel & Henneke, 2014). However, this bacterium is also thought to utilise macrophages to cross the blood-brain barrier and disseminate in organs and tissues (Bowater et al., 2012). In the study of Guo et al., (2014), their results showed that *S. agalactiae* could be phagocytosed in large numbers by murine macrophages in the absence of complement and antibodies in a dose dependent manner which is similar to *S. iniae*. Moreover, previous study have also shown that GBS enters macrophages very efficiently and can survive intracellularly for more than 24 h, a period that *in vivo* would be more than sufficient to maintain a bacteraemia required for developing meningitis (Valenti-Weigand et al., 1996). Like *S. iniae*, *S. agalactiae* may induce apoptosis or necrosis in macrophages but it is hypothesized that haemolysin may contribute to phagocyte killing (Guo et al., 2014). The target organs of *S. agalactiae* are the brain, eye and kidney,

all similar to *S. iniae*, and vasculitis and septicaemia are the major pathogenic effects of *S. agalactiae* (Abdullah et al., 2013).

Our understanding of the pathogenesis of *S. agalactiae* and *S. iniae* infections remain limited, particularly concerning the mechanisms by which these bacterial species travel and disseminate in the bloodstream to reach the central nervous system. As pathogenesis is attained by expression of virulence factors, many studies have attempted to identify and characterise these. Both bacterial species produce and secrete a variety of products that contributes to adherence, colonisation, invasion, and protective immunity from the host. The available information regarding virulence factors of *S. agalactiae* and *S. iniae* in the published literatures are shown in Table 1.7 and Table 1.8, respectively.

Table 1.7 Known virulence factors of *Streptococcus agalactiae*. All *S. agalactiae* strains isolated from mammals and the virulence factors were verified using mammalian based models. (Modified from Feathersone, 2014)

Virulence factor	Related genes	Function	Reference
Fibrinogen-binding protein	*fbs*	A fibrinogen protein that contributes to adhesion to host surfaces, protects from opsonophagocytosis and elicits a fibrinogen-dependent aggregation of platelets	Pietrocola et al., 2005; Schubert et al., 2002
C5a peptidase	*scpB*	A surface-associated serine protease which cleaves C5a, a major neutrophil chemoattractant, and facilitates adherence to fibronectin	Beckmann et al., 2002
Hyaluronate lyase	*hylB*	Degrades hyaluronan, the main polysaccharide component of the host connective tissues and facilitates bacterial invasion	Mello et al., 2002
B-haemolysin-cytolysin	*cyl*	A surface-associated toxin with the ability to promote intracellular invasion and neutrophil recruitment, triggers apoptosis of cells and cause cytolytic injury	Liu & Nizet, 2006
CAMP factor	*cfb*	Pore-forming toxin that causes lysis of red blood cells and binds to the Fc fragments of immunoglobulin	Lang & Palmer, 2003
Capsule	*cps*	Polysaccharide capsule that reduced complement deposition and phagocytosis by the host's immune system	Hanson et al., 2012; Yamamoto et al., 1999
Serine protease	*cspA*	Protease that inactivates chemokines and aids the capacity to resist opsonophagocytic killing by neutrophils	Bryan & Shelver, 2009
Pili	*PI-1, PI-2a and PI-2b*	Mediates resistance to cathelicidin antimicrobial peptides	Maisey et al., 2008; Papasergi et al., 2011
Penicillin binding protein	*PBP1a-ponA*	Surface protein that promotes resistance to phagocytic killing independent of capsular polysaccharide	Jones et al., 2002

Table 1.8 Known virulence factors of *Streptococcus iniae*. All *S. iniae* strains isolated from fish and the virulence factors were verified using fish based models. (Modified from Feathersone, 2014)

Virulence Factor	Related genes	Function	Reference
Capsule	*cpsD*	Surface capsular polysaccharide that lowers the rate of phagocytosis by host immune cells	Locke et al., 2007a
Streptolysin S	*sagA*	Expression contributes directly to cytolytic injury to cells and tissues	Locke et al., 2007b
Sim protein	*simA/simB*	Contributes to bacterial adherence, invasion of fish epithelial cells and macrophage resistance	Locke et al., 2008
Polysaccharide deacetylase	*pdi*	Virulence proteins involved in adherence and invasion lysozyme resistance and survival in blood	Milani et al., 2010
Phosphoglucomutase	*pgm*	Contributes to normal cell morphology, surface capsule expression and resistance to innate clearance mechanisms	Buchanan et al., 2005
C5a peptidase	*scpI*	Surface proteins that impairs the ability of the host to fight an S. iniae infection	Locke et al., 2008

1.2.4 Diagnostic approaches for piscine *Streptococcus* spp.

Disease diagnosis in fish remains a challenge due to the multitude of potential pathogens within the aquatic environment. As such, there remains a reliance on gathering multiple sources of information including farm history and outbreaks history combined with biological samples to confirm the cause of the mortality or morbidity event. It is accepted practise for those working in the aquatic diagnostics, that a combination of clinical presentations with recovery and/or detection of the bacteria is practised to provide a presumptive identification of the disease.

Currently, the immediate and inexpensive diagnosis of streptococcosis in infected fish is difficult as fish exhibit similar clinical symptoms regardless of the etiological agent (Muzquiz et al., 1999; Baeck et al., 2006).

Clinical sign is the primary signature of bacterial infections that depends on various factors and could also be similar to non-infectious conditions, so it is

difficult to understand the precise cause of the disease presentation. A study of tilapia fish showed that clinical signs and degree of lesions depend on several factors such as *S. agalactiae* strain variations, their infectious dose, water conditions, temperature, and handling procedures (Chang & Plumb, 1996). Misidentification or unidentification of isolates are common due to the complexity of bacteria and their interrelations (Lau et al., 2006). Moreover, asymptomatic fish serve as a pathogen reservoir and pose challenges for correct identification due to the absence of clinical signs (Bromage et al., 1999). Presently, diagnosis of subclinical infections in fish is a major concern (Mishra et al., 2018).

Bacterial identification methods based on culture, morphology, or biochemical reactions are time- and resource-consuming. Some pathogen databases (RAPID Strep strip, VITEX systems, API 20E STREP, Rapid Strep 32 and ATB Expression System) are incomplete or incorrect, and result in improper identification of bacteria (Dodson et al., 1999; Facklam et al., 2005; Lau et al., 2006). Additionally, other challenges for accurate identification include the mixed nature of the aquaculture environment, low numbers of biological samples, or unknown tissue location in carriers (Klesius et al., 2006). Identification of *S. agalactiae* based on biochemical features (i.e., capacity to hydrolyze hippurate) or phenotypic characteristics (acidification of tagatose, ribose, and sucrose) are not effective due to high levels of biochemical heterogeneity among strains (Ravelo et al., 2001).

Molecular methods are based on several candidate genes (Table 1.9), that have been well characterized for diversity, including *16S rRNA*, heat-shock genes (*groESL*), and tRNA gene intergenic spacer regions (ITSs) (Clarridge et

al., 2001; Teng et al., 2002). Comparative studies have discussed various methodologies and found molecular methods to be most effective for bacterial identification (Bosshard et al., 2006). Gene sequence analysis based on the *16S rRNA*, *sodA*, and *tuf* gene identified *S. dysgalactiae* as a fish-specific pathogen (Abdelsalam et al., 2013).

Table 1.9 Candidate genes used for differentiation and diagnosis of various Streptococcal bacterial agents. (Adapted from Mishra et al., 2018).

Candidate Gene	References
Manganese-dependent superoxide dismutase (*sodA*)	Kitten et al., 2012; Poyart et al., 2000
Heat shock protein (*groESL*)	Hung et al., 2013; Teng et al., 2014
Ribosomal protein (*rpoB*)	Drancourt et al., 2013
Recombination and repair protein (*recN*)	Hung et al., 2013
Repair protein (*recN*)	Glazunova et al., 2013
Lactate oxidase gene (*lctO*)	Zlotkin et al., 1998
rRNA (eg. 16S rRNA or 16-23S rRNA)	Clarridge et al., 2002
RNA polymerase	Drancourt et al., 2004
D-alanine-D-alanine ligase	Garnier et al., 1997
b-subunit of the elongation factor	Picard et al., 2004
Polysaccharide capsules gene (*cps*)	Lowe et al., 2007
Invasion associated gene (*iag*)	Rajagopal, 2009
Surface immunogenic protein (*sip*)	Springman et al., 2009
C5a peptidase (*scp*)	Springman et al., 2009
Serine protease (*csp*)	Springman et al., 2009
tRNA gene intergenic spacer region (*ITS*)	Tung et al., 2007

The multilocus sequence typing (MLST; analyses of multiple genetic loci or housekeeping genes) is another molecular method that is considered the "gold standard" of typing for many bacterial species (Maiden, 2006; Jolley et al., 2012). This is method is used to study the epedimiology of *S. agalactiae* genetic populations where specific sequence type is associated with a specific disease syndromes (Delannoy et al., 2013). MLST is an important tool for molecular epidemiology because the MLST databases for individual pathogen species

currently cover far more isolates than have been characterized based on whole genome sequencing (Spratt, 2011). However, insufficient resolution among very closely related bacteria can be a problem (Achtman, 2008).

1.2.5 Disease Prevention and Control

Streptococcosis is a disease which requires an integrated health management approach to prevent and control because many factors are involve in it pathogenesis such as age of the fish, type of host, strain of the bacteria etc. (Wendover, 2009). Preventative measures include reduction in fish stocking density, ensuring good water quality, diligent removal of dead/moribund fish, implementing stringent bio-security protocols and minimising stressors such as fish handling and transportation.

Development and application of streptococcal vaccine for streptococcosis has been widely researched in an attempt to prevent disease outbreak. Experimental *S. agalactiae* vaccines composed of (a) concentrated extracellular products (ECP) (Evans et al., 2004b), (b) formalin-killed whole cells (Evans et al., 2004b), (c) bacterial antigens (Sheehan, 2009), and (d) live attenuated bacteria (Pridgeon & Klesius, 2013) have all been described. Merck Animal Health Inc. developed a commercially available oil adjuvant injection vaccine, AQUAVAC® Strep Sa, however, this only prevents *S. agalactiae* Biotype 2 (serotype Ib) infections and is only for use in fish > 15 g (Pridgeon & Klesius, 2013). This means that younger fish remain vulnerable to infection. An ideal vaccine should produce a broad range of protection against *S. agalactiae* infections, however, developing a polyvalent *S. agalactiae* vaccine that protect

against all strains of *S. agalactiae* will be nearly impossible due high antigenic diversity (Pridgeon & Klesius, 2013). A recent study by Hayat et al. (2021) showed a feed-based formalin-killed *S. iniae* vaccine gave greater level of protection and increased antibody response to fish when challenged with *S. iniae*, however, this high level of protection was observed in fish that received booster feed vaccine on the 14 and 21 days. Although the result is promising, an exclusive shift to feed-based vaccination is still a challenge due to its sutiability in ponds and cages where the amount of feeds eaten by the fish may vary. Moreover, result showed that 3 doses are required for a 70% protection from infection.

Several drugs have been tested for the treatment of streptococcosis. Among other, Darwish & Griffin (2002) found that oxytetracycline incorporated into the feed was effective in controlling *S. iniae* in blue tilapias (*O. aureus*). Some reports concluded that erythromycin is effective against streptococcal infections in cultured yellowtails (Shiomitsu et al., 1980) and rainbow trout (Kitao et al., 1979) while doxycycline, oxytetracycline, kitasamycin, oleandomycin, josamycin, and lincomycin have also been used to control streptococcosis in the cultured yellowtail in Japan (Kitao et al., 1979). Similarly, sodium nifurstyrenate, a fisheries drug, has been proven to be successful in Japan in treating streptococcosis when incorporated with feed (Kashiwagi et al., 1977). Meanwhile, streptococcal infections respond to antibiotic therapy, but the disease cannot be legally controlled with antibiotics all the way to the market because the withdrawal period for all effective antibiotics is longer than it takes for the streptococcal infection to return. Furthermore, it is only a matter of time before *Streptococcus* develops resistance to the antibiotics. In fact,

streptococcal strains at several facilities have already developed resistance to some antibiotics (Darwish & Hobbs, 2005). Therefore, the best approach would be the use of an efficacious vaccine to prevent outbreaks thus reducing the reliance on antibiotics. (Klesius et al., 2000). In this way, antibitiocs will be more effective in controlling the disease.

1.3 Motile Aeromonas Septicaemia in Tilapia

1.3.1 Motile Aeromonads

At present, the genus Aeromonas comprises 31 species (Chen et al., 2016). Among them, *A. hydrophila*, *A. sobria*, *A. caviae*, *A. allosaccharophila*, *A. veronii* biogroups *sobria* and *veronii* and *A. encheleia* are motile and does induce disease in fish. From all motile aeromonads, A*. hydrophila* is the most commonly recovered and identified pathogen in fish (Camus et al. 1998; Nielsen et al. 2001) though questions on the validity of the identification are being raised as molecular methods have confirmed other motile aeromonad species were involved. *Aeromonas dhakensis* is another motile Aeromonas species that causes disease in fish (Carriero et al., 2016) and that has been wrongly identified as *A. hydrophila* (Chen et al. 2016). According to Saikot et al. (2013), *A. hydrophila* is a Gram-negative motile rod, widely prevalent in aquatic sources (Garcia et al., 2007; Odeyemi et al. 2012; Stratev & Odeyemi, 2016) and pathogenic for fish, amphibians, reptiles and mammals, including men (Dias et al. 2016). The *A. hydrophila* is an opportunistic pathogen causing disease in fish under stress (Saavedra et al., 2004) induced by high water temperature, high levels of ammonia and nitrites, changes in water pH, low

levels of dissolved oxygen, parasitic infections, high stocking density and rough handling and transportation (Camus et al., 1998). Other researchers outline *A. hydrophila* as the main pathogen causing disease in fish farms accompanied with high death rates and great economic losses (Pridgeon & Klesius, 2011a). In the literature, the disease is termed “motile *Aeromonas* septicaemia” or MAS (Cruz-Papa et al., 2014), “haemorrhagic septicaemia”, “ulcer disease”, “red-sore disease” (Thiyagarajan et al., 2014) and “tail and fin rot” (Nielsen et al., 2001). In the view of Das et al. (2013), septicaemia is due to the presence or action of 2 toxins extracellular haemolysin and aerolysin, which are the primary virulence factors involved in establishing the infection. Susceptible species as per Aoki (1999) are brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), ayu (*Plecoglossus altivelis*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), clariid catfish (*Clarias batrachus*), Japanese eel (*Anguilla japonica*), American eel (*Anguilla rostrata*), gizzard shad (*Dorosoma cepedianum*), goldfish (*Carassius auratus*), golden shiner (*Notemigonus crysoleucas*), snakehead fish (*Ophiocephalus striatus*) and tilapia (*Tilapia nilotica*). Also, *A. hydrophila* was reported to induce disease in freshwater ornamental species as the dwarf gourami (*Colisa lalia*), giant gourami (*Osphronemus goramy*), discus cichlids (*Symphysodon spp.*), black tetra (*Gymnocorymbus ternetzi*), platy (*Xiphophorus maculates*), tiger barb (*Barbus pentazona hexazona*), guppy (*Poecilia reticulata*) and silver catfish (*Pangasius sutchi*) (Musa et al., 2008). The MAS in fish is characterised with superficial lesions, haemorrhages, ulcerations, abscesses, exophthalmia, ascitic fluid and liver and kidney lesions (Stratev & Odeyemi, 2017).

1.3.2 Transmission

Aeromonas spp. cause haemorrhagic and ulcerative diseases in fish (Austin & Adams, 1996). *A. caviae, A. sobria* and *A. schubertii* have all been implicated in diseases of aquatic finfish and crustaceans as well as infections in humans, whereas *A. salmonicida* is a fish pathogen, and has not been associated with infections in any terrestrial animal species (Janda & Abbott, 1996). Clinical sign of infections in fish are varied depending on the species of the fish and disease status. Ulcerative lesions of the skin around the base of the fins and the anus, raised scales, abdominal distension, and exophthalmia are clinical signs that are common with disease caused by other bacteria. Depending on the severity of infection, anaemia, hepatomegaly, and ascites may develop in affected fish (Lowry & Smith, 2007). *Aeromonas* infections in fish are often secondary to other stresses, such as poor water quality, parasitism, and nutritional deficiencies.

Members of the genus *Aeromonas* have been known as important emerging human pathogens, which are responsible for numerous diseases including gastroenteritis, localized wound swelling, septicaemia, meningitis, and pneumonia (Lehane & Rawlin, 2000; Galindo et al., 2006; Galindo & Chopra, 2007). Infections in people working in aquaculture or keeping fish as pets and following injuries from handling and direct contact with mucus and tissues from infected or carrier fish have also been reported (Lehane & Rawlin 2000). One detailed investigation in Spain spanning 1997 to 2006 found that *Aeromonas* spp. ranked fourth among the causes of total gastrointestinal diseases reported each year (Epidemiological Surveillance System, 2007).

1.3.3 Pathogenesis

Aeromonas infections are complex and multifactorial. Many species are believed to be pathogenic since they satisfy most of the requirements of pathogenic bacteria which are *A. hydrophila*, *A. dhakensis*, *A. veronii*, *A. sobria* and *A. caviae*. Severity of disease is influenced by a number of factors, including putative virulence factors, the type and degree of stress exerted on a population of fish, the physiological condition of the host, and the degree of genetic resistance inherent within specific populations of fish. Motile aeromonads vary in their relative pathogenicity (De Figueiredo & Plumb, 1977). Several virulence factors are required for the bacterium to colonize, enter, and produce damage in host tissues. Other virulence factors are also involved in evading the host defense systems, the ability to spread within tissues, and the ability to kill the hosts (Smith, 1995). The main pathogenic factors associated with *Aeromonas* are surface polysaccharides (capsule, lipopolysaccharide, and glucan), S-layers, iron-binding systems, exotoxins, extracellular enzymes, secretion systems, fimbriae and other nonfilamentous adhesins, and flagella (Figure 1.6) (Janda & Abbot, 2010; Tomas, 2012). To determine the potential virulence and pathogenicity of *Aeromonas* spp., both phenotypic virulence traits and virulence genes have been used (Tomas, 2012; Sreedharan et al., 2013; Arslan & Kucuksari, 2015).

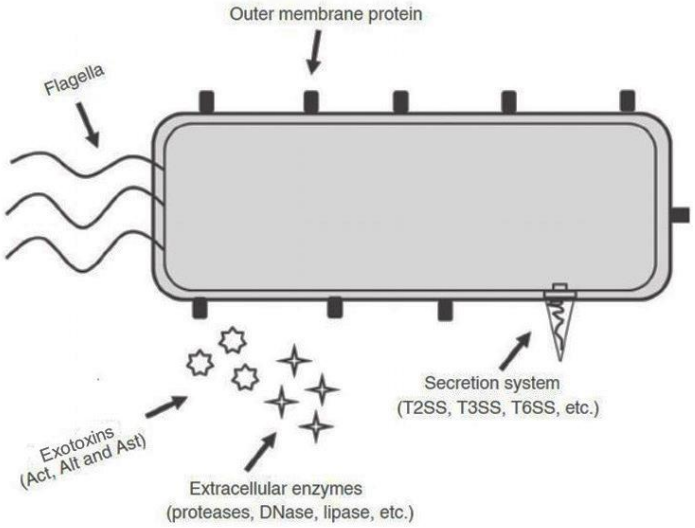

Figure 1.6 Major virulence factors of piscine motile *Aeromonas* spp. (Taken from Hossain & Heo, 2020)

Capsule. The capsule is composed of polysaccharides that covers the outer membrane of the bacterial cell and participates in the bacteria interactions with the environment. In consequence, capsules have been described as a major virulence factor (Merino & Tomas, 2010) of many pathogens, as it provides protection from host phagocytosis and its induction and expression increased the level of virulence for fish and provided resistance to serum killing (Magarinos et al., 1996; Merino et al., 2012). Mesophilic *Aeromonas* spp., *A. hydrophila* AH-3 (serogroup O:34), PPD134/91, JCM3980 strains of *A. hydrophila* (serogroup O:18), *A. veronii* bv. *sobria* (serogroup O:11) and *A. salmonicida* are all able to form a capsular polysaccharide when grown in a glucose-rich medium (Garrote et al., 1992; Gardufio et al., 1993; Martínez et al., 1995). *Aeromonas salmonicida* and *A. hydrophila* serogroup O:34 strains grown under conditions promoting capsule production showed significantly higher ability to attack fish cell lines compared with those grown under conditions, which did not promote capsule formation (Merino et al., 1996; 1997).

Aeromonas hydrophila group II capsule (IIA and IIB) producing strains have been reported to possess resistance to serum and phagocyte killing (Zhang et al., 2003). It has also been shown that purified capsular polysaccharides and O-antigen of *A. hydrophila* PPD134/91 have the ability to confer resistance to serum-mediated killing (Zhang et al., 2002).

Exotoxins. Like other bacteria, *Aeromonas* spp. can secrete a wide range of exotoxins. There are three major types of exotoxins identified in *Aeromonas* spp., such as cytotoxic enterotoxin, cytotonic enterotoxin and aerolysin. These toxins play important roles in establishing infections in *Aeromonas* spp. (Chopra et al., 1996; Sha et al., 2002; Soler et al., 2004). Enterotoxins are major virulence factors of *Aeromonas* spp. produced and secreted by several species targeting the intestines. They consist of two categories: cytotoxic and cytotonic enterotoxins. Cytotoxic enterotoxins are described as heat-labile (*Act*). These toxins are pore-forming channel haemolysins able to modify cell permeability (Castilho et al., 2009). Haemolysins belong to two major classes; one class is the aerolysins (*aerA*), which are usually ß-haemolysins that produce clear zones of haemolysis on blood agar. The *aerA* genes specifying the aerolysins were cloned and sequenced from *A. hydrophila* (Howard et al., 1987) and *A. veronii* biovar *sobria* and *A. caviae* (Singh & Sanyal, 1992). The α-haemolysin is the second class of haemolysins and were first cloned and sequenced from *A. hydrophila* ATCC7966 (Janda, 1991; Hirono et al., 1997). Both aerolysins and related β-hemolysins have the ability to lyse red blood cells in addition to enterotoxic activity, and cause tissue damage in a variety of eukaryotic cell lines, including Hep-2, HeLa, Chinese hamster ovary, Vero, and erythrocytes (Asao et al.,

1984; Chopra et al., 1993; Fujii et al., 1998; Wong et al., 1998). In contrast, cytotonic enterotoxins differ from aerolysins and aerolysin-related β-haemolysins both genetically and in biological action (Janda, 2001). They do not produce a cytopathic effect in eukaryotic cells. Instead, their action results in cell elongation or rounding (Janda, 2001).

Two types of cytotonic enterotoxins have been reported. The first type is the heat-labile enterotoxin (*alt*) at 56°C, and is also known as lipase, extracellular lipase, or phospholipase (Chopra & Houston, 1999). The *alt* protein consists of a single 44 kDa polypeptide (Chopra et al., 1994; 1996). Heat-stable enterotoxin (*Ast*) is the second type of the cytotonic enterotoxin reported in *A. hydrophila* and *A. sobria and* is stable at 56°C (Chopra & Houston, 1999). Both cytotonic enterotoxins (*alt* & *ast*) are unrelated to cholera toxin (Albert et al., 2000; McCarter, 1995) and cause higher intracellular cAMP and PGE2 levels in cultured CHO cells (Chopra et al., 1992; 1996).

Extracellular Enzymes. *Aeromonas* species secrete a wide range of extracellular proteins, including proteases, serine proteases, elastases (e.g., *AhpB*), lipases, DNAses, amylases, chitinases, xylanase, lecithinases and gelatinases. These secreted proteins are important molecules involved in a variety of pathogenicity mechanisms including tissue damage, evading host defences, and deriving essential nutrients from the host for survival (Chopra & Houston, 1999; Figueras, 2005; Scoaris et al., 2008; Figueras et al., 2009). The extracellular enzymes of *Aeromonas* spp. are usually produced by the type II secretion system or general secretory pathway for establishing the pathogenicity (Pemberton et al. 1997). There are several types of extracellular enzymes, such as proteases, lipases, DNase, gelatinase, elastase, nucleases,

gelatinases and glycerophospholipid-cholesterol acyltransferase (Hossain & Heo, 2020).

Aeromonas spp. secrete a group of proteases, which degrade many diverse proteinaceous compounds, such as albumin, fibrin, gelatin, and native elastin molecules (Janda, 1985). Two major proteases are produced by *Aeromonas* strains. One enzyme, a thermolabile serine protease that is inactivated by ethylenediaminetetraacetic acid (EDTA), appears to belong to the general class of thermostable metalloproteases; the other protease is the thermostable (56°C, 30 min) metalloprotease that is EDTA-sensitive or insensitive (Rivero et al., 1991; Ellis et al., 1997). The role of proteases in pathogenesis is not clearly understood. They are implicated in colonization, invasion of host tissues during host-pathogen interaction and mediate tissue lysis during pathogen invasion by helping the bacteria to take essential nutrients from the host environment for further survival, multiplication, and distribution inside the host (Bjornsdottir et al., 2009)**.** Researchers have confirmed that the toxic fraction of extracellular products (ECP) secreted by *A. hydrophila* is linked with the haemolytic activity and protease activity (Allan & Stevenson, 1981; Kanai & Wakabayashi, 1984; Sakai, 1985; Subashkumar et al., 2006).

Lipases contribute to bacterial nutrition and are involved in erythrocyte lysis (Pemberton et al., 1997; Chopra & Houston, 1999). *Aeromonas* spp. are capable of secreting several lipases, which hydrolyse esters of glycerol with long-chain fatty acids (Jaeger et al., 1994). An extracellular lipase purified from the culture supernatant of *A. hydrophila* H3 showed both esterase and lipase activities (Anguita et al., 1993). Later, *A. hydrophila* strains PLA1, LipE, Lip and

Apl-1 were found to secrete a group of lipases with high homology (Ingham & Pemberton, 1995; Merino et al., 1999).

Glycerophospholipid-cholesterol acyltransferase (GCAT) is a well-known phospholipase isolated from both *A. hydrophila* and *A. salmonicida* (Thornton et al., 1988; Eggset et al., 1994). GCAT can digest plasma membranes of host cells, and leads to their lysis (Thornton et al., 1988). However, generation of an *A. salmonicida* GCAT isogenic mutant did not result in decreased virulence, demonstrating that they may be accessory rather than essential virulence factors (Vipond et al., 1998). Three groups of chitinases A, B and C are known to be secreted by *Aeromonas spp.* (Watanabe et al., 1993).

Nucleases have been long recognized as possible virulence factors, but their exact role in virulence is unclear compared with the role of other degradative enzymes (Pemberton et al., 1997). DNases are considered as possible nutritional enzymes in *Aeromonas* (Scoaris et al., 2008). They have been involved in bacterial infections, given that DNases are enzymes that directly attack DNA, a macromolecule essential for the function of any host cell. In addition, the degradation of DNA yields carbon and nitrogen molecules to the bacteria (Podbielski et al., 1996).

Secretion systems. Gram-negative bacteria have developed different secretion systems to transport proteins to the cell surface. Secretion systems in bacteria have been classified as type I, II, III, IV, V, and VI systems (Henderson *et al*., 2004). T2SS is responsible for the transport of aerolysin, DNases, amylases and proteases. Type III secretion system is associated with the injection of effector proteins into the cytosol of host cells (Li & Cai, 2011). Type III (T3SS) and Type VI secretion systems (T6SS) have been recognized to play

a significant role in the virulence of many Gram-negative bacteria and are often activated upon contact with target cells and carry their toxin proteins directly into the host cell cytosol. In *Aeromonas* spp., the functioning of T3SS has only been described in *A. salmonicida* (Burr et al., 2002) and in *A. hydrophila* strains AH-1, AH-3 and SSU (Yu et al., 2004; Sha et al., 2005), while the functioning of T6SS has been described in *A. hydrophila* strains SSU, AH-3 and ATCC 7966 (Suarez, et al., 2008). T4SS plays an important role as a carrier of different virulence factors to the eukaryotic host cells and performs conjugal genetic transfer between bacteria, which is also related to the spread of antimicrobial resistance genes (Rangrez et al., 2006).

Other Virulence factors. Bacterial adherence to the host may involve either specific interactions between a receptor and a ligand or hydrophobic interactions. The receptors are usually specific carbohydrate or peptide residues on the eukaryotic cell surface, and the ligands termed adhesins are bacterial surface proteins or polysaccharides. Fimbriae/pili are micro-filamentous structures protruding from the bacterial surface. They are formed by individual subunits identified as pilin. The pili may be present singly or in bundles. They are generally described as adhesive organelles and are also involved in some other bacterial processes, as well as phage binding, DNA transfer, biofilm formation, cell aggregation, host cell invasion, and twitching motility (Tomás, 2012). *Aeromonas* spp. contain two different types of fimbriae identified based on morphology. They are short, rigid fimbriae (S/R) and long, wavy fimbriae (L/W). The S/R fimbriae are the predominant type in aeromonads with high pili numbers (Kirov et al., 1995).

Depending on the environmental conditions, bacteria are able to move in a free way by swimming, swarming, gliding, twitching, and sliding among others or stay in the same place to form colony groups and colonize surfaces. Motile *Aeromonas* spp. has both polar and lateral flagella. A polar flagellum is used for moving on solid surfaces and functions for adhesion, whereas multiple lateral flagella are mainly used for swarming over surfaces and serve as colonization factors. About 60% of mesophilic *Aeromonas* spp. contain lateral flagella (Kirov et al., 2002). *A. caviae* is able to produce polar and two lateral flagellins, whereas *A. hydrophila* produces two polar and only one lateral flagellin (Rabaan & Shaw, 2002; Canals et al., 2006), which are mostly responsible for the adhesion, invasion of the fish cell and also the formation of biofilm (Merino et al., 1997; Gavín et al., 2002).

1.3.4 Diagnostic approaches in piscine Motile *Aeromonas* spp.

Diagnosing disease and identifying the infectious agents are important for managing any disease situation. It has been recommended that detecting a bacterial fish pathogen re-quires analysis of samples from 4 to 10 infected fish and between 10 and 60 samples from apparently health populations (Noga, 2010). The target organs are generally the kidney, spleen, skin, and ovarian fluid. Sometimes, pathogens are detected in mucus, blood, faeces and skin (Gustafson et al., 1992; Byers et al., 2002; Beaz-Hidalgo et al., 2008; Kulkarni et al., 2009). The kidney and intestine are the recommended sites when screening for asymptomatic carriers (Beaz-Hidalgo & Figueras, 2012).

A number of methods have been reported for the detection/cultivation and identification of aeromonad pathogens, including traditional (phenotypic characteristics), immunological and molecular techniques. Non-selective culture media used for the isolation of *Aeromonas* strains include tryptone soya agar (TSA), brain heart infusion agar (BHIA) or Columbia blood agar with incubation at 15-25°C for up to 7 days (Bernoth, 1997; Hiney & Olivier, 1999; Austin & Austin, 2007; 2012).

Numerous biochemical methods have been proposed for the identification of *Aeromonas* species, but they mainly recognize three phenotypic groupings i.e. *A. hydrophila* (including *A. hydrophila, A. bestiarum, A. salmonicida* and *A. popoffii), A. caviae (A. caviae, A. media* and *A. eucrenophila)* and *Aeromonas sobria (A. sobria, A. veronii, A. jandaei* and *A. trota*) (Borrell, et al., 1998; Kozińska et al., 2002; Abbott et al., 1992; 2003; Martin-Carnahan & Joseph, 2005; Martínez-Murcia et al., 2005). However, identifications based on phenotyping lack accuracy and tend to result in inconsistencies due to misinterpretation in some characters and because of issues with lack of reproducibility when they are carried out under different laboratory conditions, such as varying temperatures and incubation times (Figueras et al., 2011). In addition, the expression of biochemical characteristics between different isolates of the same taxon, e.g., *A. hydrophila,* are not always the same (De Figueiredo & Plumb, 1977; Figueras et al., 2011). Therefore, with the issues surrounding the use of phenotyping, there has been a move towards molecular based identification.

Sequencing of the *16S rRNA* gene is considered a strong taxonomic tool and is now widely used in bacterial taxonomy (Cascón et al., 1996; Khan &

Cerniglia, 1997). However, research has indicated that phylogenetic analyses based on *16S rRNA* gene sequencing may lead to difficulty with distinguishing between closely related taxa (Martínez-Murcia et al., 1999; 2005). Also, the data are compared with entries in the BLASTN data base, which are entered by authors and for which there is not any external verification DNA probes and restriction fragment length polymorphism (RFLP) profiles designed from the *16S rRNA* gene are useful to distinguish *Aeromonas* at the species level (Ash et al., 1993; Dorsch et al., 1994; Borrell et al., 1997; Khan & Cerniglia, 1997; Figueras et al., 2000; Lee et al, 2002). However, some strains have produced unpredicted or different restriction patterns making their identification uncertain (Alperi et al., 2008). Sequencing analyses based on the gene sequences of *gyrB* and *rpoD* are invaluable for the recognition of *Aeromonas* species and for the proper identification of novel closely related isolates (Soler et al., 2004; Yanez et al., 2003). Finally, the use of a combination of 16S rDNA-RFLP analyses (Borrell et al., 1997; Figueras et al., 2000) and housekeeping genes (*gyrB, rpoD*), (Martínez -Murcia et al., 2005; Alperi et al., 2008; Tena et al., 2008; Figueras et al., 2009) make suitable molecular markers for assessing the phylogeny of closely related *Aeromonas* species.

1.3.5 Disease Prevention and Control

A commercially available vaccine against the motile aeromonad infection is not available for farmed fishes. Motile aeromonas septicaemia in fish is controlled by antibiotics, but their extensive use increases the resistance of *A. hydrophila* (Fang et al., 2004). According to Chen et al. (2012), aeromonads

produce four classes of chromosomally mediated β-lactamases. Class A broad-spectrum β-lactamases determine the resistance to all penicillins, cephalosporins and monobactams and those from class B metallo-β-lactamases to penems and carbapenems. Beta-lactamases from class C cephalosporinases determine the resistance to cephamycins and third-generation cephalosporins, while enzymes from class D β-lactamases to penicillin. A study performed by Fosse et al. (2003) demonstrated that *A. hydrophila* produces β-lactamases from classes B, C and D. According to Aravena-Roman et al. (2012), the resistance of aeromonads to β-lactams could be also attributed to plasmids and integrons. Kadlec et al. (2011) investigated the resistance of motile aeromonads to trimethoprim and trimethoprim-sulfamethoxazole, coded by an integron. *A. hydrophila* isolated from diseased fish were sensitive to aminoglycosides, third-generation cephalosporins, lincosamides, macrolides, nitrofurans, fluoroquinolones, sulfonamides, tetracyclines, chloramphenicol and rifampicin, but the isolation of antibiotic-resistant *A. hydrophila* from diseased fish is also well documented (Popovic et al., 2000; Guz & Kozinska, 2004; Belem-Costa & Cyrino, 2006; Adanir & Turutoglu, 2007; Daood, 2012; Jeeva et al., 2013; Laith & Najiah, 2013; Samal et al., 2014). Table 1.10 provides data about the treatment of motile *Aeromonas* septicaemia in fish by antibiotics.

Table 1.10 Treatment of motile *Aeromonas* septicaemia in fish. (Taken from Stratev et al. 2016)

Antibiotic	**Dose**	**Period of treatment (days)**	**Administration**	**Fish**	**Reference**
Oxytetracycline (Terramycin®)	50-75 mg/kg of fish per day	10	Feed additive	Pond fishes, channel catfish, salmonids	Cipriano et al. (1984)
Sulfadimethoxin-ormetoprim (Romet 30®)	50 mg/kg of fish per day	5	Feed additive	Catfish, salmon, trout	Swan & White (1991)
Trimethoprim-sulphamethoxazole	10 g/t of pool water	3	Immersion	Carp	Adanir & Turutuglo (2007)
Enrofloxacin	20 g/10kg food, two times a day	7	Feed additive	Caro	Chirila et al. (2008)
Ciprofloxacin	5 ppm	5	Immersion	Discus	El-Ghany et al. (2014)

The frequent use of antibiotics in the therapy of bacterial fish diseases could entail the development of antimicrobial resistance in many pathogens. This necessitates alternative methods for control of fish diseases, such as vaccination for prevention of motile *Aeromonas* septicaemia (Pridgeon & Klesius, 2011b). Different vaccines have been developed against motile *Aeromonas* septicaemia in fish from whole cell, outer membrane protein, extracellular products, lipopolysaccharide and biofilms. Despite that, no commercial vaccine against *A. hydrophila* is yet available (Poobalane et al., 2010). This is due to the extreme biochemical and serological heterogeneity of *A. hydrophila* - the main barrier to the development of efficient commercial vaccine (Wang et al., 2013).

Disinfection is a common means for disease control in fish farms. It is applied in biosecurity programmes for prevention of a specific disease, for reduction of morbidity rates or for eradication of diseases (OIE, 2009). In cases of disease in fish farms, Torgersen and Hastein (1995) recommend disinfection of pools, tanks, ducts, premises and adjacent areas, transport containers and

vehicles. With this respect, the sensitivity of *A. hydrophila* to glutaraldehyde, formaldehyde, quaternary ammonium salts, benzalkonium chloride, iodine, ethanol, potassium permanganate (Stratev & Vashin, 2014) and chlorine (Sisti et al., 1998) was acknowledged.

1.4. Rationale and Study Aim

Tilapia, the second most important cultured fish after milkfish, is the most consumed farmed fish in the Philippines, with an average yearly consumption of 4.6 kg per person, yet the tilapia farming sector has stagnated if not decreasing in the last ten years, due to several factors such as limited freshwater areas, high water temperature, high cost of production, poor breed of tilapia, lack of government assistance, and lack of capital (Guerrero, 2019). Reports of disease epidemics especially infectious bacterial diseases have been observed for the past ten years, however, its role to current production trend is unknown and has not been given full attention because of the prevailing belief that tilapia is a hardy and disease-resistant species. Before the start of my study, tilapia farmers in Luzon were reporting outbreaks of diseases and high mortality in the farms during the warm months of the year which is from March to June. From anecdotal reports and personal communications, the survival rate for the tilapia was only 30%, and the farmers were raising this issue with the concerned government agencies to help them address this problem.

Published studies or reports on tilapia diseases in the Philippines is scanty if not absent. These reported disease problems have been long suspected as streptococcosis and motile aeromonas septicaemia, but clinical

data were fragmented or not current. Considering these disease problems, a diagnostic investigation was conceptualised to establish the primary aetiological agents involved in these disease and mortality outbreaks, determine the factors that may have contributed to the problem, and their role in the tilapia production trend in the country. Given the limited area for production and the rising of water temperature due to climate change, this disease problem will only exacerbate in the coming years if not addressed. Thus, there is a considerable threat not only to the livelihoods of farmers and fisherfolk, but also to the country's food security.

The general objective of this study was to determine the causative infectious bacterial agents associated with the disease and mortality outbreaks observed in tilapia farms in the Philippines.

The main aims of each chapter in this study are as followed:

- To survey tilapia farms to characterise and describe current husbandry and health management practices that may have contribute or is contributing to reports of disease and mortality outbreaks.

- To establish that the reported disease and mortality outbreaks are infectious in nature.

- To characterise the antibiotic response profile, antibiotic resistance genes, and virulence factors of the *S. agalactiae* and *S. iniae* isolates that may contribute to their pathogenic potential.

- To characterise the antibiotic response profile, antibiotic resistance genes, and virulence factors of other different motile *Aeromonas* species strains that are involved in their pathogenesis.

CHAPTER 2.

Current Status of Tilapia Farming in the Philippines

2.1 Abstract

A field-based survey was performed in March 2018 to June 2019 to describe the status of tilapia farming and provide data on infectious diseases encountered within the range of production systems, representative of the Philippine tilapia sector. Tilapia farms sampled were primarily intensive grow-out farms and hatcheries in various production environments namely freshwater, brackishwater and seawater. Sixteen farms (N =16), 12 grow-out and 4 hatcheries, were studied and sampled. Results from a questionnaire identified infectious disease as one of the main causes of fish health issues encountered in all production systems included in the study. Data from the questionnaire showed that farmers were able to successfully identify external clinical signs in their stocks during disese outbreaks. Tilapia farmers reported higher levels of disease outbreaks occurring in March to June which coincided with increased water temperature. In floating cages in Taal Lake, fish mortality during the 1st month of stocking was approximately 70%, and a further 7.5% of the remaining stocks were affected by disease thereafter. Although high mortality was observed after initial stocking, the remaining fish density was still beyond the recommended level. This high stocking density and the vast quantity of tilapia cages with increased use of commercial feeds has increased nutrient pollution in the waters. Seventy percent of farmers used some form of

treatment or interventions which included salt (NaCl) and the use of antibiotics although around 30% of the farms included did not use any treatment.

The present study provides evidence that infectious diseases is currently a problem and fish losses due to clinical disease outbreaks are likely to have contributed to the stagnating growth of tilapia production in the Philippines. Moreover, valuable data on farming or husbandry management can be used to develop policies for health strategies in the tilapia sector.

2.2 Introduction

2.2.1 Tilapia Farming: Development and Status

Tilapia farming in the Philippines started in the 1950s with the introduction of the Mozambique tilapia (*Oreochromis mossambicus*), however, this tilapia species became unpopular with local fish farmers and resulted to reduce the interest in commercial tilapia culture at that time. The introduction of the Nile tilapia (*Oreochromis niloticus*) from Thailand and Israel in the 1970s renewed the interest in tilapia farming in the Philippines. Better growth traits of the Nile tilapia resulted in expansion of the sector, especially in pond and cage culture systems which then contributed to the development of the country's tilapia production. Although production was initially in ponds, local cage farming techniques (Figure 2.1) were developed and adapted, and the Philippines became a pioneer in Asia for Nile tilapia cage culture in lakes and reservoirs (Guerrero, 2002; BFAR-PHILMINAQ, 2007). The culture of red hybrid tilapia (*Oreochromis* spp.) started in the 1980s, however the majority of the tilapia

production within the Philippines is still based on the naturally coloured Nile tilapia.

Figure 2.1 Tilapia floating cages in Taal Lake, Batangas, Philippines (https://www.manilatimes.net/2021/04/15/news/regions/taal-volcano-registers-more-tremors/864037/).

The limited number of freshwater bodies, and the ecological concerns primarily regarding the degradation of lakes, has pushed the tilapia sector in the Philippines to expand farming into brackishwater ponds. Additionally, the potential to culture tilapia in seawater is being explored due to the availability of an extensive coast around the Philippines. The tilapia farms in Luzon are mainly freshwater and is the biggest producer in the country with 92% of the total farmed tilapia production (261,210 MT), followed by Mindanao (freshwater and

seawater) with 6%, and the Visayas (mainly brackishwater) with 2% (BFAR 2016).

For more than a decade tilapia production has stagnated (Figure 2.3). A comprehensive study has not yet been performed to identify all contributing factors influencing the potential growth of the tilapia farming sector in the Philippines, however, a study by Guerrero (2019) did identify the limiting factors as high water temperature, lack of government assistance, poor genetic potential of tilapia due to inbreeding, high cost of production, and lack of capital.

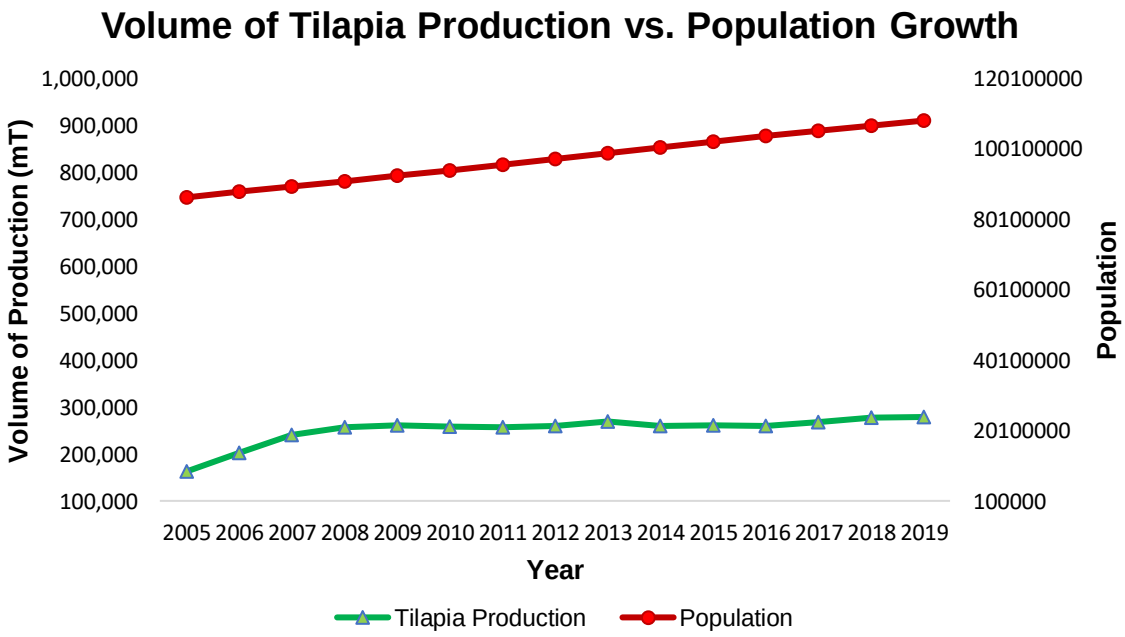

Figure 2.2 Volume of production of tilapia versus population growth of the Philippines. (2005-2019, Philippine Statistical Authority; World Bank).

Tilapia are often described or considered as a hardy fish, more resistant to poorer water quality and able to tolerate a wider range of environmental conditions (Prabu et al., 2019). This is often in comparison with other fish

species such as milkfish, which are farmed in similar conditions and regarded as more sensitive to their environmental surroundings. Due to this perceived robustness of tilapia, they were once considered to be more resistant to bacterial, parasitic, fungal, and viral diseases as compared with other cultured fishes (Amal & Zamri-Saad, 2011). In more recent times, however, tilapias have been found to be susceptible to diseases. Recent increases in production have largely been achieved through intensification of existing farming systems, resulting in higher risks of disease outbreaks (Henriksson et al., 2018) and mortality episodes. In the Philippines, tilapia farms with limited water exchange and circulation, no aeration, high stocking and feeding rates, and high organic load can cause sudden death of fish in cages (Bagariano & Lantin-Olaguer, 1999). Infectious disease outbreaks, particularly of bacterial aetiology, remain one of the main limiting factors that threaten tilapia production, specifically when cultured under intensive conditions (El-Sayed, 2019). In the Philippines, the tilapia farming sector has reportedly suffered high levels of fish mortalities primarily attributing to seasonal upwelling in lakes and of other unknown causes, however, infectious diseases are also suspected. Whilst anecdotal reports have identified bacterial diseases occurring in the Philippine tilapia farming sector since 2009, no confirmed studies have been reported as to date. Additionally, the emergence of tilapia lake virus (TiLV) disease has added to the burden of annual and recurring disease problem in farms in the country (OIE, 2017).

Like other farming sectors, the likelihood of new, emerging, and re-emerging diseases increases as aquaculture activities intensify and expand (Bondad-Reantaso et al., 2005; Walker & Winton, 2010; Oidtmann et al., 2011).

One way to improve health management is to understand the production system events, possible risks and pathways for pathogen transmission, and to identify interventions that may lead to improvements in the health status of fish farms (Bondad-Reantaso et al., 2005).

2.2.2 Study Aim

This overall aim of this chapter was to describe the husbandry, health management practices, and reports of disease/mortality outbreaks in tilapia grow-out farms and hatcheries. Additionally, biological samples were gathered for subsequent disease investigations (Chapter 3).

2.3. Materials and Methods

2.3.1 Survey

A cross-sectional survey was designed and performed together with staff from the National Fisheries Research and Development Institute-Fisheries Biotechnology Centre (NFRDI-FBC). The primary raw data were collected using questionnaire-based interviews performed in the Philippines between March 2018 to June 2019. The tilapia grow-out ponds and hatcheries included in the survey were from tilapia farms identified by NFRDI-FBC as areas where there were reported disease problems, and farms that were willing to participate in the survey.

Each farm was visited once, and the questionnaire completed by the author or NFRDI-FBC staff during face-to-face interviews. Secondary data were provided by Bureau of Fisheries and Aquatic Resources-Batangas Inland

Fisheries Technology Outreach Station (BFAR-BIFTOS) to provide context in the management of tilapia farms in the sampled area as well as to confirm the primary data results.

2.3.2 Tilapia Farm Survey

The survey was done mostly in Luzon with two in Visayas and one in Mindanao from 2018 and 2019. There were a total of 16 farm sites included in the survey, separated into 12 grow-out farms and 4 hatcheries (Table 2.1). The grow-out farms included freshwater floating cages (n=8), freshwater submerged cage (n=1), seawater floating cage (n=1) and brackishwater earthen ponds (n=2), which were distributed throughout the study sites in the 3 major islands.

Table 2.1 The distribution of tilapia farms included in the study

	Provinces	Samplite Sites	Freshwater Floating Cage	Freshwater Submerged Cage	Freshwater Earthen Hatchery	Brackish water Earthen Pond	Seawater Cage	Total
Luzon	3	Taal Lake, Laguna Lake, Laguna, Pampanga	7	1	4	1	0	13
Visayas	2	Batad, Silay	1	0	0	1	0	2
Mindanao	1	Panabo, Davao	0	0	0	0	1	1
Total	6		8	1	4	2	1	16

2.3.3 Questionnaire Design

A questionnaire was designed to enable primary data to be collected regarding the current husbandry and health management practises in tilapia grow-out farms and hatcheries in the Philippines. The questions were grouped under various topics: farm background, background of person interviewed,

production environment and system, husbandry, feed and water management, and disease and health management. The questionnaire included both open and closed questions. The questionnaire remained in English but was translated in Filipino during the interview. Prior to the interview, the assisting staff were briefed and trained regarding the questionnaire. The final English questionnaire is included as Appendix 1.

The questionnaire was pilot tested with three tilapia farms which were not then included in the larger survey. Following the pilot test the questionnaire was edited in consultation with the project supervisors. Answers were written directly onto individual questionnaire sheets during the interviews and data were then uploaded in an Excel spreadsheet to which only the researcher and his supervisors had access.

2.3.4 Fish Sampling

At the same time as the questionnaires were administered biological samples were collected from the sites visited. These contributed to Chapters 3, 4 and 5 of the thesis. Approximately seven moribund and three apparently healthy tilapia were sampled from each of the grow-out ponds or cages while three moribund and two apparently healthy tilapia were taken from each hatchery ponds. The fish were scooped out from the pond using a net and then placed in a bag with its pond water and supplied with oxygen and tightly sealed. The fish were then transported to the laboratory or were processed as soon as possible on sites, for farms in remote locations. The tilapia samples were killed

using Schedule 1 method. The wet body weight (grams) and length (mm) were recorded.

The fish were observed for external and internal signs of diseases. The fish were aseptically dissected, and sterile disposable inoculating loop were inserted to the kidney, spleen and brain and plated out immediately for bacterial recovery. Tissue samples from muscle, gills, liver, intestine, heart, brain, kidney and spleen were taken for histopathology. These were done to identify diseases from these sites, full details are provided in Chapter 3.

2.3.5 Data Collection/Analysis

Each farmer/farm was contacted by the Fisheries Biotechnology Centre-National Fisheries Research and Development Institute (FBC-NFRDI), and of the Batangas Inland Fisheries Technology Outreach Station (BIFTOS) through phone calls prior to site visits to help with compliance. Questionnaires were answered and completed by the farm owner or manager or worker at the time of visit.

All the data gathered were coded and entered in Microsoft Excel 2016 spreadsheets. The data were analysed using SPSS 16, the main statistical tools used to be descriptive and cross tabulation. Apart from the descriptive analyses of the data, the relationship between average yield (MT ha^{-1} $crop^{-1}$) and selected parameters such as stocking density (fish m^{-2}), size of fry (g) were analysed as was the relationship between occurrence of disease outbreaks and other parameters. Microsoft Excel was used to produce charts and tables.

2.3.6 Ethical Approval

Ethical approval was granted from the University of Stirling General University Ethics Panel and all data were treated in compliance with the UK Data protection Act 2018 and the EU General Data Protection Regulations.

2.4 Results

2.4.1 Overview

Survey and Interview. All selected farms participated in the interview during the field survey, giving 100% compliance in the survey. Some questions in the questionnaire were not answered by the respondents since they were not applicable to a specific farming system, for example pond fertilisation and water exchange for floating grow-out cages in lakes and seawater. Additionally, some of the respondents said they did not know, or they did not have a record while others did not answer regarding the use of antibiotics for treatment.

The respondents of the interview identified as 14 males (88%) and 2 females (12%). The females described their role as managers of a grow-out farm and hatchery belonging to the same aquaculture company. Two of the male respondents were past 60 years old and identified as owners of the hatcheries which were in operation for more than 20 years, however, they were cautious in giving responses during the survey compared with the younger respondents. Additionally, during the interview in association with the questionnaire the farmers expressed informal opinions or observations. Four of the respondents in Taal Lake said that production cycle decreased from two to

one production per year due to slow growth and mortality of the stocked tilapia. One farmer told that farm workers in most grow-out cages in Taal Lake would collect the diseased fish and sell them to the local market for additional income due to their very low daily wage. In hatcheries, one hatchery operator revealed that he prepare his own medicated feed by buying and using the antibiotic amoxicillin intended for human use from the local pharmacy during disease outbreaks.

Role of the interviewee. Persons interviewed during the survey were classified as either owner (19%), manager (44%) or worker (37%) (Table 2.3). The participants reported attending seminars to obtain information on tilapia farming which was provided by their own company, government (Bureau of Fisheries and Aquatic Resources) or non-government organisation (NGO) specifically Southeast Asian Fisheries Development Centre-Aquaculture Department (SEAFDEC-AQD). A total of 94% of the survey participants attended at least one seminar on fish farm management (Table 2.2).

Table 2.2 Data on participant role and attendance to training/seminar in fish farm management.

People interviewed	Attendance to training/seminar fish farm management	Training/Seminar provider		
		Own Company	Government	NGO
Owner (n=3)	100	0	100	0
Manager (n=7)	100	71	14	14
Worker (n=6)	83	33	50	0

Location and Farming Systems. The freshwater farms were grouped either as grow-out farms or hatcheries while the brackishwater and seawater

were all grow-out farms. The locations and descriptions of these farms is shown in Figure 2.3 and Table 2.3.

Figure 2.3 Map of farm location in various provinces, Philippines. The red markers indicate the location of the sampled farms (red = freshwater; green = brackishwater; yellow = marine water) in the three main island groups of the Philippines.

Table 2.3 Location and description of the tilapia farms surveyed in Philippines

Farm Number	Farm Location*	Farming System**
1	Talisay, Taal Lake	Floating cage$^{FW, I}$
2	Talisay, Taal Lake	Floating cage$^{FW, I}$
3	Talisay, Taal Lake	Floating cage^{FW,I}
4	Laurel, Taal Lake	Floating cage$^{FW, I}$
5	Laurel, Taal Lake	Floating cage^{FW,I}
6	Agoncillo, Taal Lake	Floating cage$^{FW, I}$
7	Agoncillo, Taal Lake	Floating cage$^{FW, I}$
8	Calauan, Laguna	Earthen Pond Hatchery$^{FW, I}$
9	Calauan, Laguna	Earthen Pond Hatchery$^{FW, I}$
10	Calauan, Laguna	Earthen Pond Hatchery$^{FW, I}$
11	Calauan, Laguna	Earthen Pond Hatchery$^{FW, I}$
12	Pila, Laguna Lake	Submerged cage$^{FW, E}$
13	Lubao, Pampanga	Earthen Pond$^{BW, I}$
14	Batad, Iloilo	Floating cage$^{FW, SI}$
15	Silay, Negros Occidental	Earthen Pond$^{BW, I}$
16	Panabo, Davao	Floating cage$^{SW, I}$

*All the farms were in Luzon except for 14 and 15 which were in the Visayas and 16 in Mindanao. **FW = freshwater; BW = brackishwater; SW = seawater; I = intensive; SI = semi-intensive; E = extensive.

The freshwater grow-out farms visited were those on Taal Lake, Batangas and Laguna Lake, all located in the island of Luzond. In Taal Lake, there were seven (7) floating cages from the municipalities of Talisay (n=3), Laurel (n=2), and Agoncillo (n=2) (Figure 2.4). Most of these floating cages were found on the western portion of the craterlake (Figure 2.4).

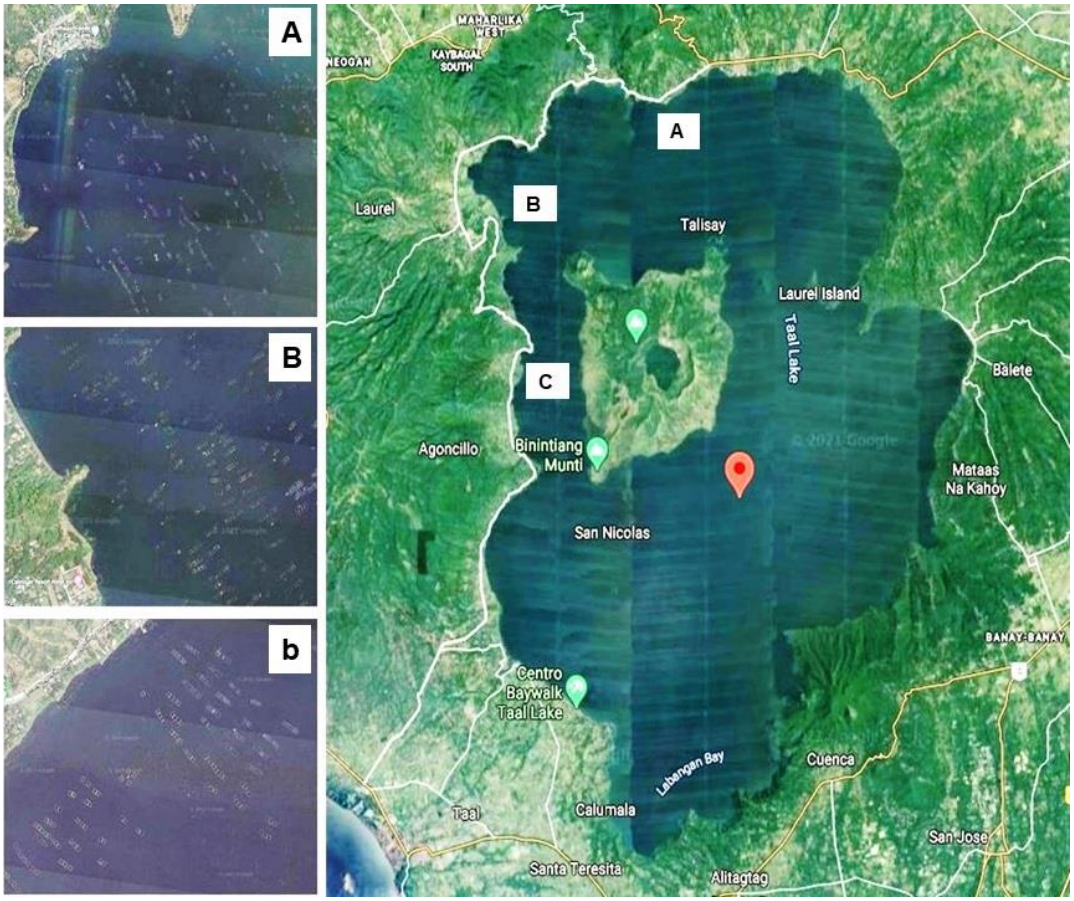

Figure 2.4 Satellite images of sampling sites of freshwater floating cages in: (A) Talisay; (B) Laurel; and (C) Agoncillo all in Taal Lake, Batangas, Philippines. (Google earth).

The cages measured 10x10 m and were floating in the lake. An example of the floating cages is provided in Figure 2.5.

Figure 2.5 Tilapia grow-out floating cages in Taal Lake, Batangas, Philippines.

In Laguna Lake (Figure 2.6 A), a submerged cage was sampled, while in Calauan, Laguna four (4) hatcheries which were earthen ponds were visited (Figure 2.6 B). The depth of the hatchery ponds were at 1.5 m deep.

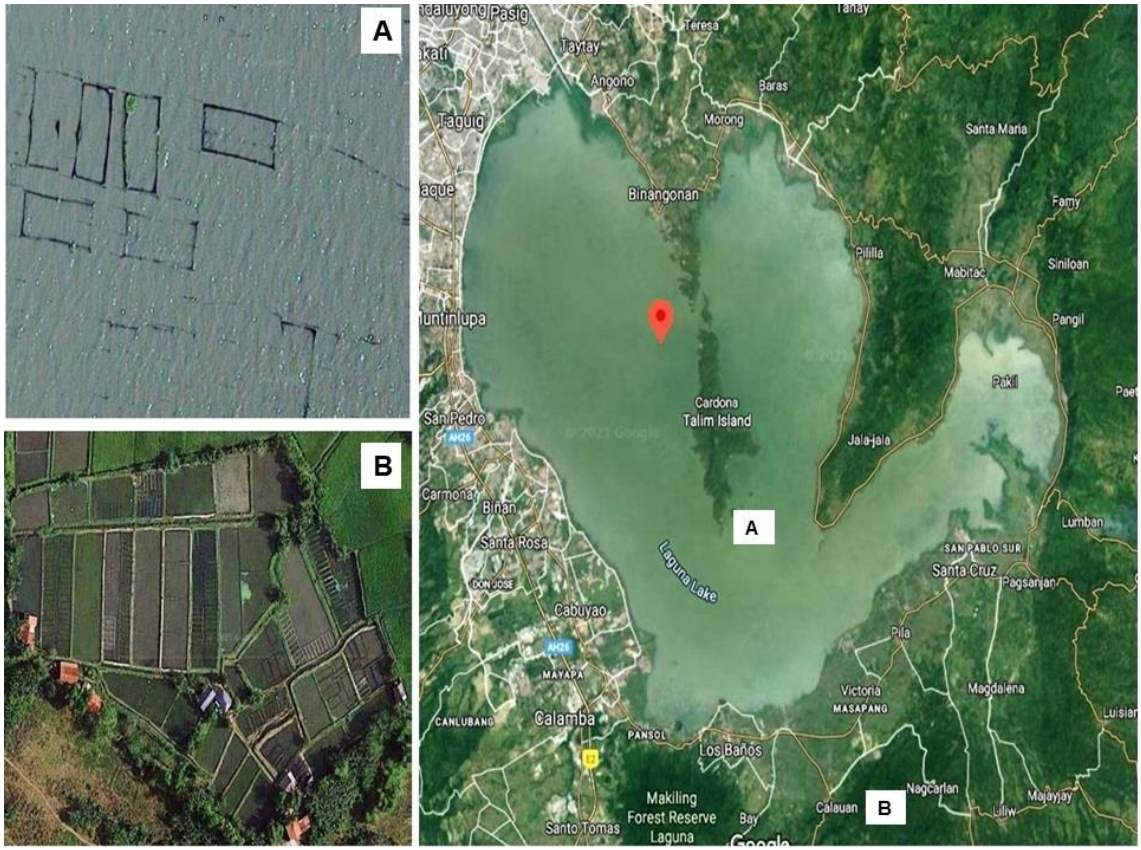

Figure 2.6 Satellite images of sampling sites in: (A) freshwater submerged cage (vague lines since the cages are submerged) in Laguna Lake; and (B) freshwater earthen pond hatcheries in Calauan, Laguna, Philippines. (C) Laguna Lake and adjacent municipality of Calauan, Laguna. (Google earth).

A brackish water earthen pond was visited, and fish sampled in Lubao, Pampanga (Figure 2.7 A) which was 2 m in depth. For Visayas, a freshwater floating cage in Batad, Iloilo (Figure 2.7 B), and a brackishwater earthen pond in Silay, Negros Occidental with a depth of 2 m were sampled (Figure 2.7 C). In Mindanao, a seawater floating cage in Panabo, Davao del Norte was included in the survey (Figure 2.7 D).

Figure 2.7 Satellite images of sampling sites in: (A) brackishwater earthen pond, Lubao, Pampanga; (B) freshwater floating cage in Batad, Iloilo; (C) brackishwater earthen pond in Silay, Negros Occidental; and (D) seawater floating cages in Panabo, Davao del Norte. (Google earth).

2.4.2 Description of the tilapia Farm Sector

Duration of farm operation. For grow-out farms, 42% were ≤5 years in operation while 25% were more than 20 years in operation as tilapia farming. Only 8% of the grow-out farms included in the survey were in operation for 6-10 years. For the hatcheries, 50% were > 20 years old already and they were identified as the family owned backyard hatcheries while the other 50% were ≤5 years and were described as owned by commercial aquaculture companies. From the interviews performed, respondents from both Taal and Laguna lakes

reported longer growing period for their fish which were approximately 8-9 months as compared with previous years when their growth period was estimated at 4-6 months when there were still few cages in operation these areas.

Species of tilapia. The species of tilapia grown in both freshwater and brackishwater farms was the Nile tilapia (*O. niloticus*) (Figure 2.8). The strains of Nile tilapia farmed included the Genomar Supreme Tilapia (GST) strain, Genetically Improved Farmed Tilapia (GIFT), and GET-Excel (Genetically Enhanced Tilapia with excellent qualities) strains. From the interview, respondents revealed that they also used mixed breed strain which they called "Tagalog."

Figure 2.8 Nile tilapia (*Oreochromis niloticus*).

For the seawater site, the tilapia that were grown was the red hybrid tilapia (Figure 2.9) which is a crossbred of the mutant male Mozambique tilapia (*Oreochromis mossambicus*), which had a reddish-yellow colour, with the dark-coloured female of the Wami tilapia (*O. hornorum*). For the source of tilapia fry,

grow-out farms in Taal Lake and Laguna Lake get their fish from the hatcheries in Calauan, Laguna. None of the survey participants claimed that their tilapia fry were health certificated or whether they were disease-free or the broodstocks were specific pathogen-free (SPF) fish.

Figure 2.9 Red tilapia hybrid (O. mossambicus x O. hornorum).

Type of Farm Records. All the farms included in the survey claimed to keep some form of farm records. From the data obtained during the survey, the most commonly held records in all farms were production (harvest) and stocking density which were both kept by 88 % (n = 14) of the farms (Figure 2.10). Whereas mortality records was kept by the lowest number of farms: only 25 % (n = 4) recording these data (Figure 2.10). Water quality data were kept by a total of 56% of all the farming sytems included in the study (Figure 2.10). There was only one farm which kept all of the four types namely production, stocking

density, water quality and mortality and the over-all distribution of record keeping between farming system is provided in Figure 2.10.

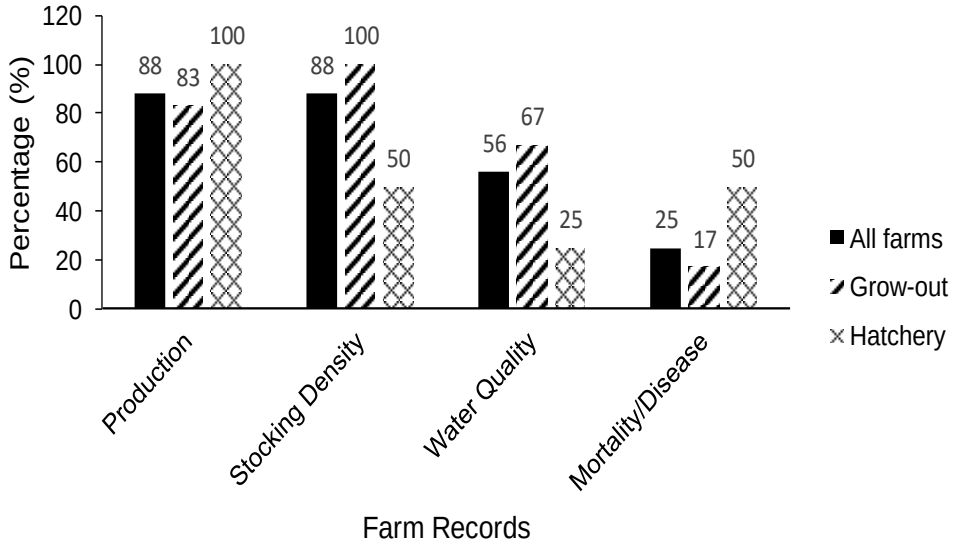

Figure 2.10 Type of farm record kept by all tilapia farms, grow-out farms, and hatchery.

From the growout farms, 100% of the cages and pond farmers included in the study kept records on stocking density (Figure 2.11). However, from the questionnaire data, it can be seen that pond farm sites kept production and mortality/disease more than the cage farm sites (Figure 1.1). Analysis of the questionnaire results showed that it was only in water quality that cages had higher percentage of keeping records than ponds (Figure 2.11.) However, it must be noted that only 2 sites were pond systems compared with a larger number of cage sites included in the study.

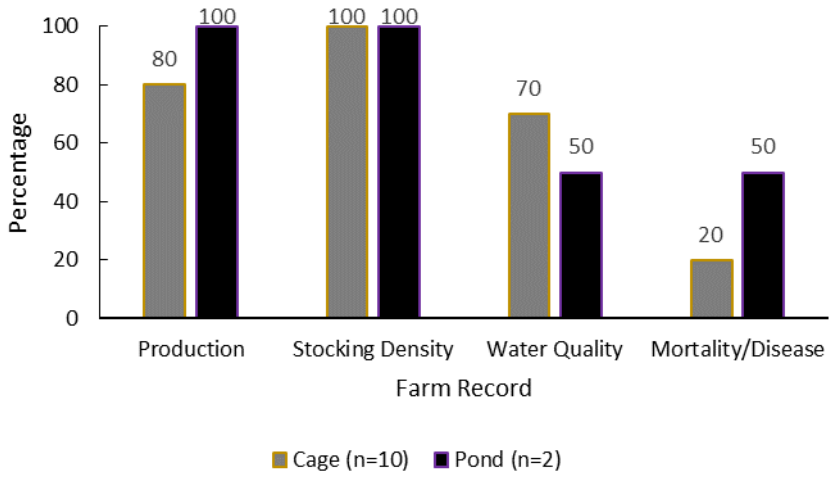

Figure 2.11 Type of farm records kept by growout cages and ponds.

In Figure 2.12, it shows the records held on the various farms, each number and area represents a combination of records held. The overlapping part of the Venn diagram indicate where more than one type of record was held, e.g., 6 farms held production, stocking density and water quality but not mortality. The figure shows that only one (1) grow-out farm kept all types of farm record and that only four farms kept mortality records.

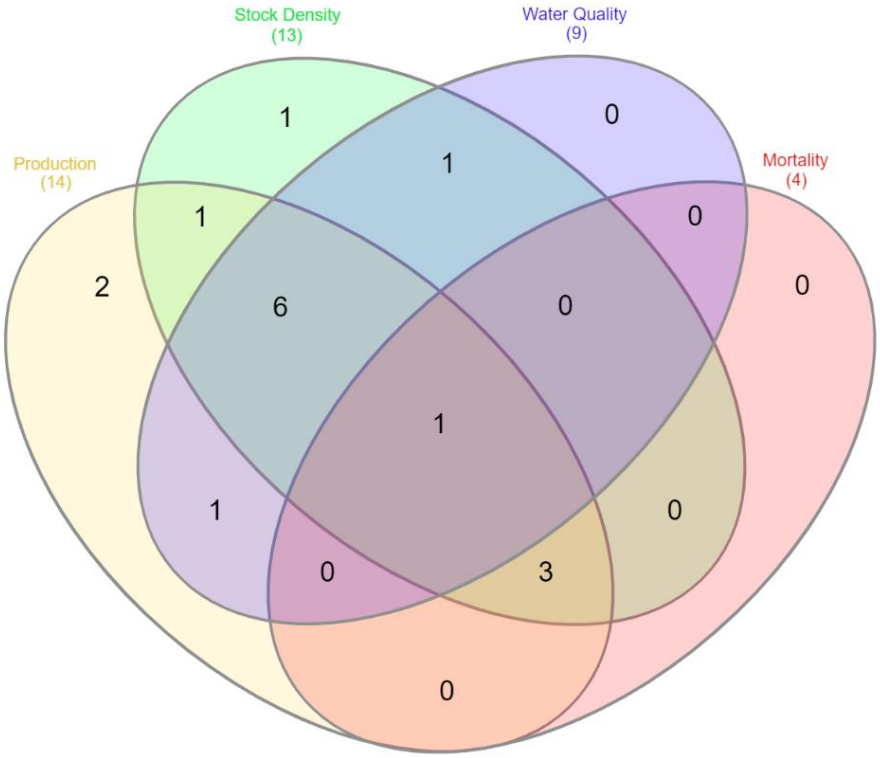

Figure 2.12 A Venn diagram showing the relationship of the different farm records. The coloured oblong represent the specific farm record while the numbers represent the number of farm/s keeping such record. The number of records a farm keeps is shown by the number of overlaps of the coloured oblongs.

Production and Husbandry. From the results of the survey, the grow-out farms were either using floating cages (n=9), submerged cages (n=1) or earthen ponds (n=2). The maximum number of cages was 55 per farm while the minimum was 2. The two brackishwater earthen pond farms had 28 and 4 ponds, respectively. For the hatcheries, the maximum number was 30 ponds while the minimum was 11. The data for number of grow-out cages, ponds and hatcheries are summarised in Figure 2.13.

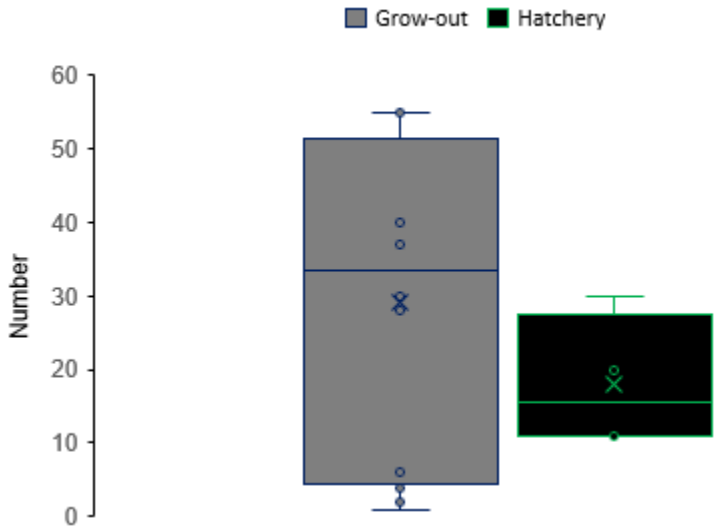

Figure 2.13 Box plots for the number of cages and/or ponds for tilapia grow-out farms and hatcheries. The middle box represents the middle 50% of scores of group. The line that divides the box into two parts is the median. The whiskers represents scores outside the middle 50%. The **x** represents the mean while the **o** are the scores.

Stocking size, number, and density. Stocking size of tilapia varied between farming sites and production systems. From the data gathered this was was maximum 25 g and a minimum of 1g, but the majority of farms (75%) interviewed claimed to stock at 1 g. The higher stocking sizes were from a smaller number of sites e.g. 25 g (17% of farms) or 20 g (8% of farms) (Figure 2.14a). For stocking number, the maximum was at 250 fish/m^3 while the minimum was at 6 fish/m^3 (Figure 2.14b). Stocking densities of 250, 60 and 50 fish/m^3 were reported in freshwater floating cages in Taal Lake while the 6 fish/m^3 was in a submerged cage in Laguna Lake. In seawater floating cages, the stocking density was at 25 fish/m^3. Brackishwater earthen ponds had 30 and 100 fish/m^3. The stocking density (fish/m^3) in grow-out farms are summarised in Figure 2.15. The stocking number of breeders was 1500/pond and the size ranged from 100-500 g. The ratio of breeders in ponds were either 1 male: 5 female or 1 male: 4 female.

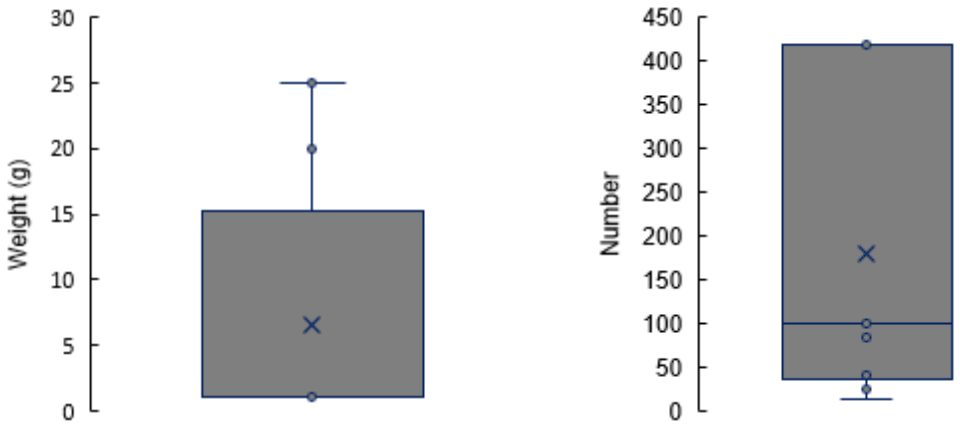

Figure 2.14 Stocking size (a) and number-/m3 (b) of fish in grow-out farms.

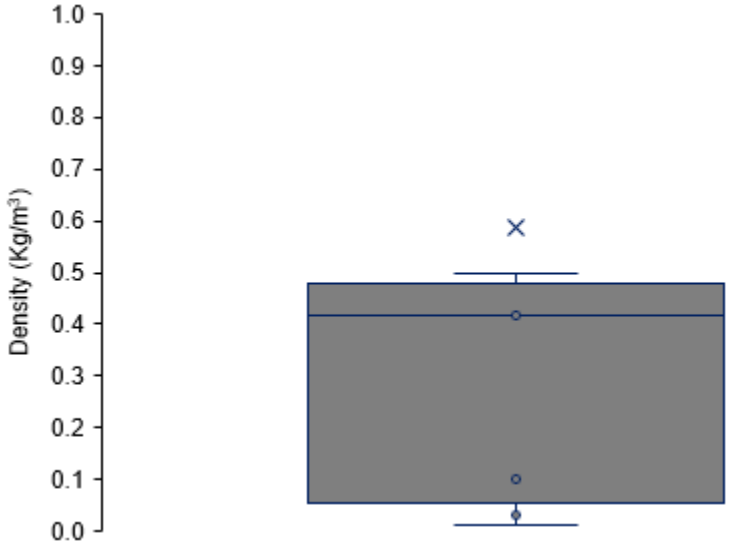

Figure 2.15 Stocking density of tilapia in grow-out farms.

Survival and size of fish at harvest. In grow-out farms, the respondents estimated that the survival of the stocked fry after 1 month was 30% except for the marine floating cage in Panabo, Davao del Norte which was 50%. The percentage of survival in terms of the number of tilapia fry that survived ranged from 900 to 75, 000 fish/ cage or pond (Figure 2.16a.) The

maximum size of the fish at harvest was 0.9 kg while the minimum size was at 0.1 kg. The smallest fish size at harvest was from Laguna Lake. The average size of fish at harvest are summarised in Figure 2.16b.

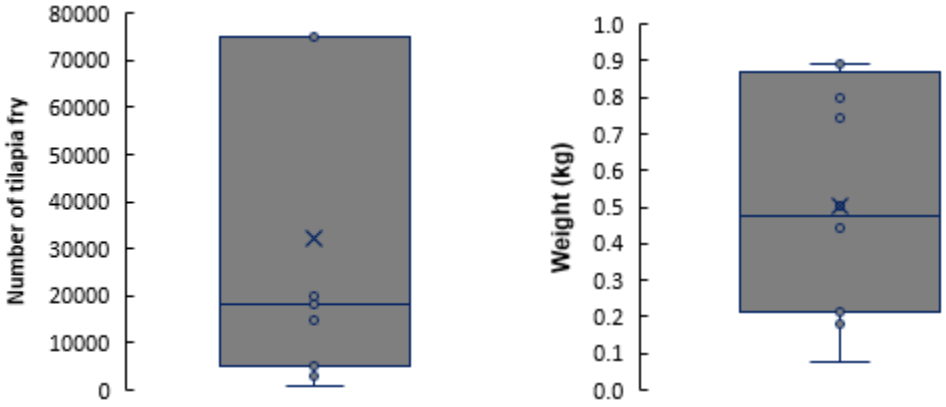

Figure 2.16 Number of stocked tilapia fry (a) and average size of fish at harvest (b) in grow-out farms.

Size of production. The size of production per year of a grow-out cage or pond was 20 kg/m^3 at maximum and 0.2 kg/m^3 at the minimum in the grow-out farms. From all of the interviews performed the highest production volume was in a brackishwater pond in Silay, Negros Occidental, while the lowest was in a submerged cages site, based in Laguna Lake. The pattern can be seen in the annual production size where the highest (1100 kg/m^3) was in floating cage in Taal Lake while the lowest (8 kg/m^3) was in the submerge cage in Laguna Lake. The size of production per cage and per farm in the grow-out are summarised in Figure 2.17.

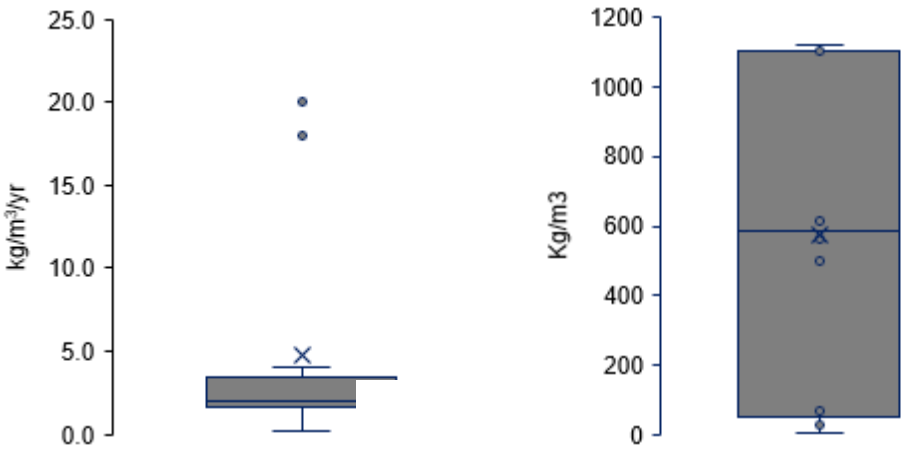

Figure 2.17 Size of production per cage or pond (a) and (b) annual production in grow-out farms.

2.4.3 Pond and Water Management

Water exchange and preparation were not applicable in floating and submerged cages since they are already in the body of freshwater. For brackishwater earthen ponds in Lubao, Pampanga, and in Silay, Negros Occidental, the respondents said that water exchange was done depending on the transparency of the water. If the transparency was low, they exchanged water to increase its transparency. All the hatcheries in Calauan, Laguna claimed to exchange water. Water exchange rates in the brackishwater earthen ponds was reported as being dependent on the tidal force for exchange of water. The earthen ponds farming systems included in the survey were not fertilised. Brackishwater earthen ponds in Silay, Negros Occidental were prepared by liming prior to stocking, and after harvest the ponds were left fallow for 1-2 months before stocking again with fish.

All the grow-out farms included in this study reported that they measured aspects of water quality and nutrient parameters. The most common water quality parameters measured by are provided in Figure 2.18, where the top 3 were salinity (83% farmers), water temperature (75% farmers) and dissolved oxygen (75% farmers). For nutrient parameters (Figure 2.19), ammonia (58%), nitrite (50%) and nitrate (50%) were the most commonly measure while only a single farm (8%) measured phosphates. None of the hatcheries reported that they performed any type of water quality monitoring.

Frequency of water quality management was dependent on the production system with 92% of the grow-out farmers claiming that they performed some type of water quality checks during the production cycle.

From the interview, Taal Lake farmers said that dissolved oxygen (DO) level in the lake is very critical since most of the fish kills that they had experienced were due to low DO. The monitoring of the DO is the work of the BIFTOS-BFAR and they relay the data to fish farmers in order for them to decide for an emergency harvest due to a possible fish kill phenomenon.

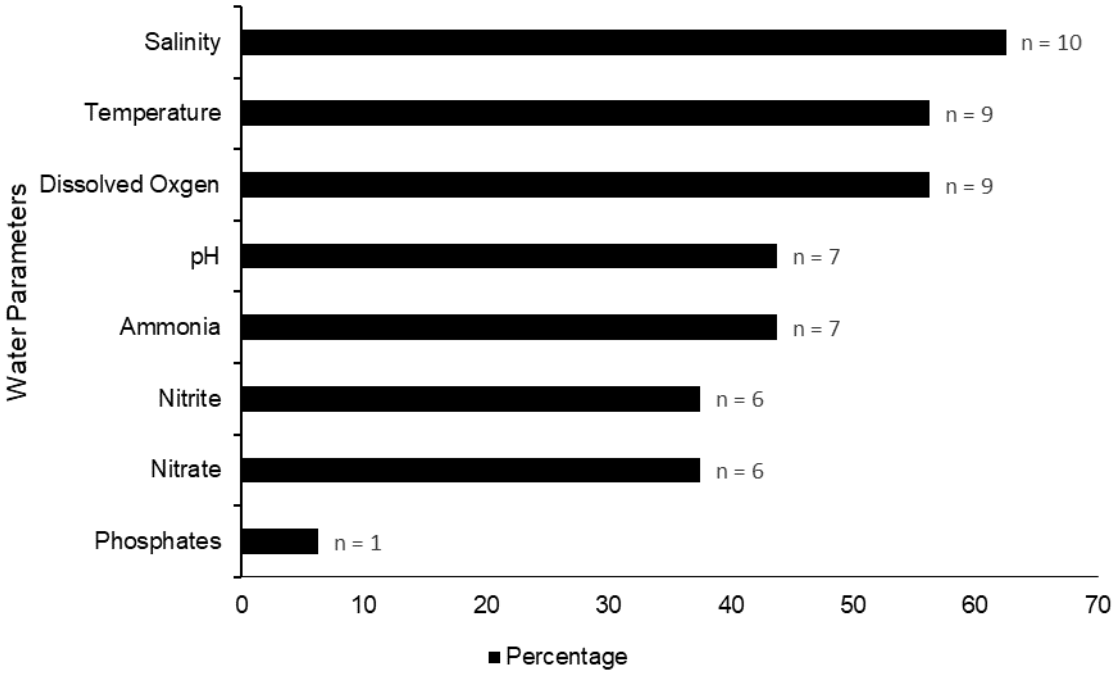

Figure 2.18. Range of water quality and nutrient parameters measured by the tilapia farms (n=16).

2.4.4 Feed Type and feeding regiment

Type of Feed. Commercial feed was used by 88% (n = 14) of tilapia farms while the submerged grow-out cages in Laguna Lake used only natural food and in grow-ot floating cages in Batad, Iloilo used a combination of commercial feed and natural food. The feed used were primarily extruded type. Commercial feeds used were either floating, sinking or a combination of both. A combination of sinking and floating type of feed were used in floating cages. In ponds, sinking type of feed was primarily used.

Feeding Rates. Frequency of feeding in freshwater tilapia grow-out cages was reported as mostly 2x per day (75%) while 17% of the grow-out

brackishwater ponds and marine cages fed 3x per day. All hatcheries fed their fish in a 3x per day frequency. In a 2x/day feeding, the feeding was done in the morning and in the afternoon usually with a duration of 30 minutes to an hour. Other farmers reported that feeding duration depended on the appetite of the fish. Broadcast feeding method using plastic scoop was the most common method to deliver feed to cages and ponds, however 2 earthen pond grow-out farms claimed to use point feeding during the culture cycle in order to make quick estimate of fish population. Point feeding was also used at some parts of the culture of cycle in Taal Lake grow-out cages in to observe fish condition and behaviour.

2.4.5 Health Management Practices

Health interventions. The majority of the farm sites included in the survey (81%) physically cleaned their nets and ponds, whilst a single farm used both physical and chemical (liming) methods of cleaning the pond. Two other grow-out farms did not clean their nets at all. During disease outbreak, farm workers used different approaches in order to treat the disease as observed through clinical signs of the fish, and to reduce mortality (Figure 2.19). The most common of these were treatment (n=8), followed by change of water (n=2) in earthen ponds, and then by changing net (n=2) in grow-out cages. Five of these farms did nothing during disease outbreak while discontinuation of feeding and emergency harvest was done on one farm. Three of the hatcheries (n=3) employed treatment when the fish were sick.

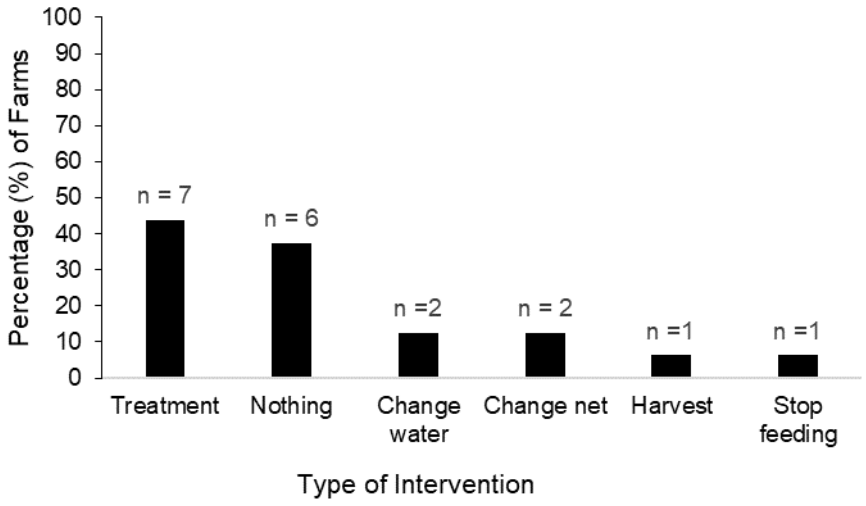

Figure 2.19 Types of interventions during disease outbreak in tilapia farms.

Treatment intervention. A combination of approaches were included in the data gathered under treatment intervention (Figure 2.20). From the data generated, none of the grow-out farms claimed to use antibiotics, and 75% (n=3) of the hatcheries only claimed to use antibiotics (Figure 2.20). The grow-out farms that responed to the question favoured the use of salt (NaC) or lime or claimed to do nothing (Figure 2.20). In terms of understanding where farms obtained information on treatment, approximately 31% of the farms sought advice from BFAR on treatment during outbreaks while 63% used their own knowledge.

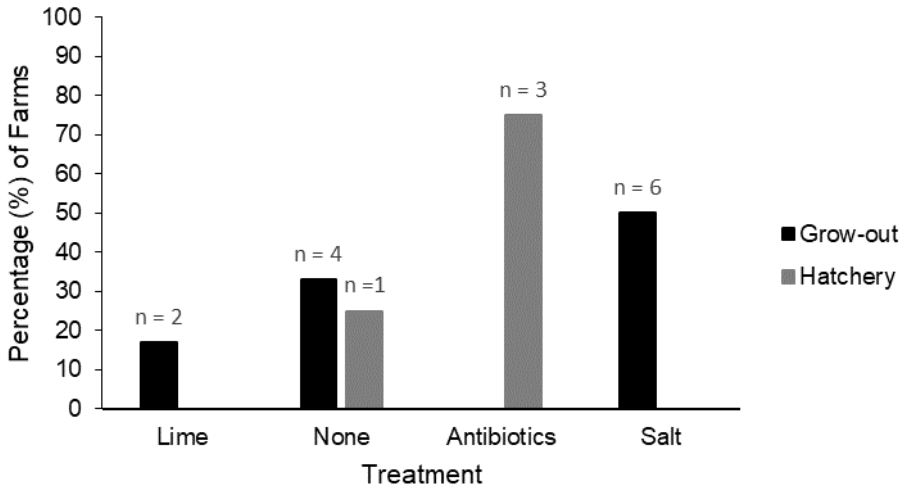

Figure 2.20 Types of treatments during disease outbreak in tilapia farms.

Disposal of diseased or dead fish. When disease outbreaks occurred, moribund fish were primarily discarded and buried (n=10) by farmers in mortality pits with lime. In other farms, the sick fish were collected by people (n=3) but did not disclose the reason or purpose. Others sold them (n=2) in the local town market. It is to be noted that in one farm (n=1), the diseased fish were dried by their farm workers and then the workers eat it. The fate of diseased fish is summarised in the Figure 2.21.

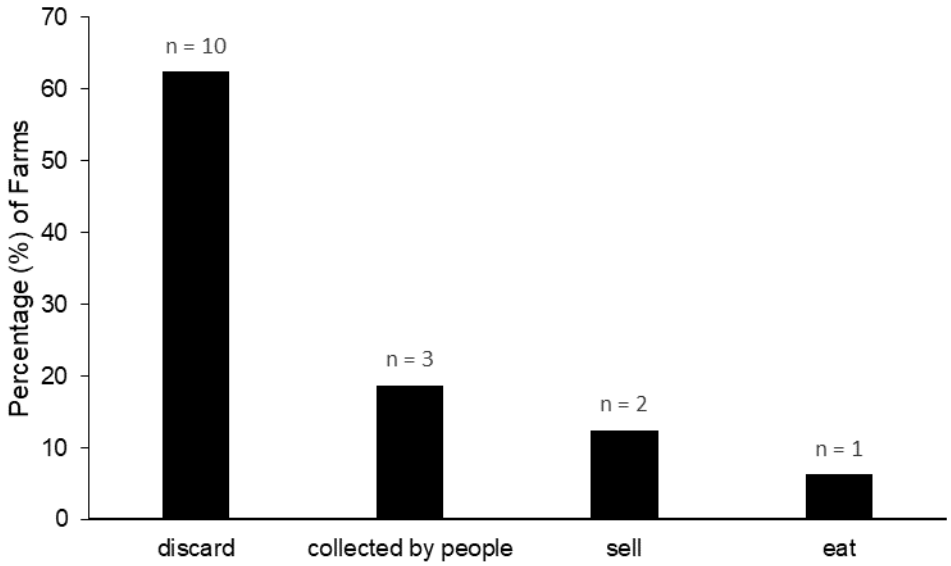

Figure 2.21 The graph shows the percentage of farms for the fate of the diseased/sick fish from the tilapia farms.

2.4.6 Disease outbreaks

Clinical signs of disease. The surveyed tilapia farms and hatcheries all reported that their fish stocks got sick with the majority of the interviewees (88%) claiming that changes in appearance and behaviour were used as indicators of morbidity. These data suggest that the farmers were able to recognise abnormalities in the fish stocks. Only 1 site included in the survey reported taking clinical samples for disease diagnosis, with 94% claiming that they judged ill health of their stocks visually. A range of clinical signs of disease was reported by the farmers during interviews, and the top five included: 1) change of behaviour (n=15); 2) ascites or enlargement of the belly (n=14); 3) corneal opacity/exophthalmia (n=12); 4) dead fish (n=11); and 5) lesions (wounds or ulcers in the body) and marks (n=9). Other observed external signs

were lack of feeding, change of colour, loss of scales, and abnormal shape or size. The different clinical signs of sick fish is summarised in the Figure 2.22.

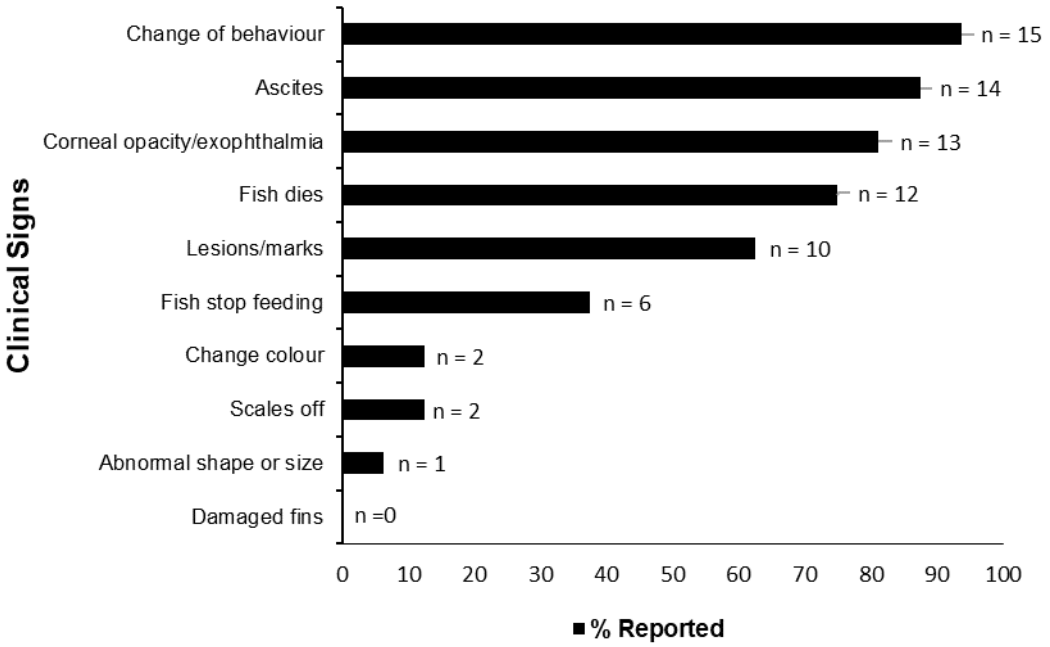

Figure 2.22 Different clinical signs reported by farmers when fish get sick.

The farmers in each of the farms observed one or more clinical signs to identify the diseased fish as seen in Table 2.4. An attempt was made to find a link between clinical signs to the type of farm, but we cannot see any significant trend.

Table 2.4 Clinical sign/s reported by farmers in identifying moribund tilapia per farm.

Range of Clinical Signs	Percentage (%)	Grow-out farms												Hatcheries			
		1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4
Change of behaviour	93.8	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■
Ascites	87.5	■	■	■	■	■		■	■	■		■	■	■	■	■	■
Corneal opacity; exophthalmia	81.2	■	■	■	■	■		■		■		■	■		■	■	■
Fish dies	75	■	■		■	■			■	■		■	■		■	■	■
Lesions/marks	62.5		■	■				■	■		■			■	■	■	■
Fish stop feeding	37.5		■										■	■	■	■	■
Change colour	12.5		■							■							
Scales off	12.5			■				■									
Abnormal shape or size	6.3								■								
Damaged fins	0																

* Shaded box = positive; white = negative.

Time of disease outbreaks. During the survey interview, the respondents of grow-out farms reported that diseased fish occurred year round, however they noted that a higher proportion of sick and dead fish were observed during summer months. This high number of disease and mortality in tilapia stocks were described by them as outbreaks. Reported data from both grow-out farms and hatcheries showed that these peaks of disease/mortality occurred during March (31.3%), April (81.3%), May (87.5%) and June (25.0%). The zero percentage (0%) during the months of September to November does not mean absence of diseased or dead fish but the respondents noted these as insignificant in number. This phenomenon is shown in Figure 2.23. Reports of high level of disease/mortality outbreaks from Taal Lake, where the concentration of grow-out floating cages of tilapia in the country was found, showed the same months of March to June. The same pattern was also

observed in grow-out cages in Taal Lake and in hatcheries in Calauan (Figure 2.24).

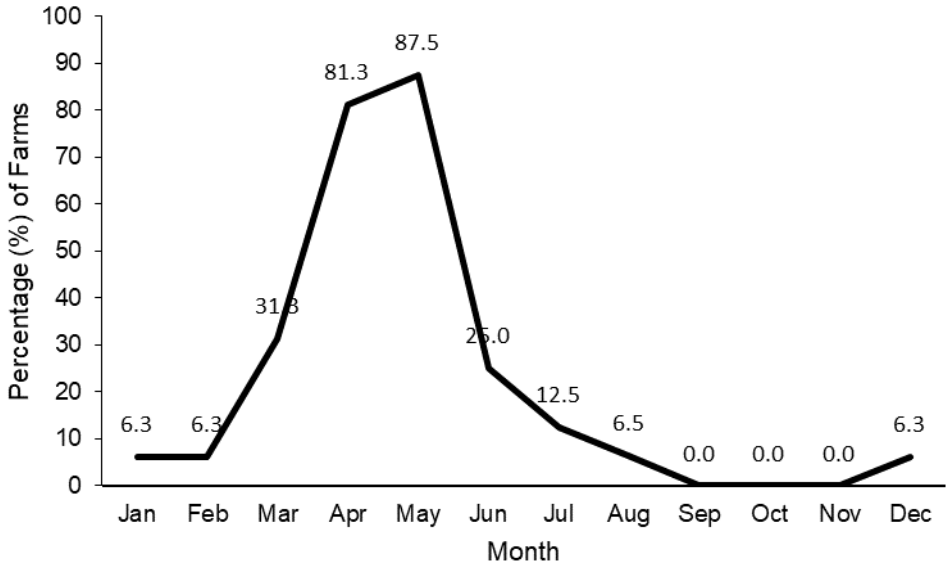

Figure 2.23 Disease/mortality outbreak in tilapia farms and hatcheries in the Philippines.

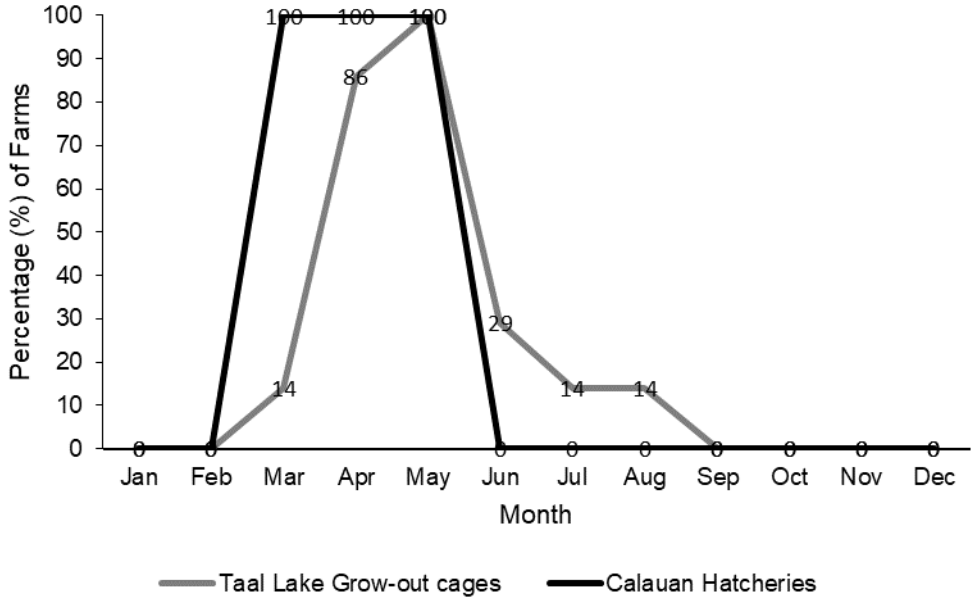

Figure 2.24 Disease/mortality outbreak in tilapia farms in Taal Lake and hatcheries in Calauan.

The seasonal trend in fish morbidity/mortality did not change when the data was compared over 2 years. The data is summarised in Figure 2.25.

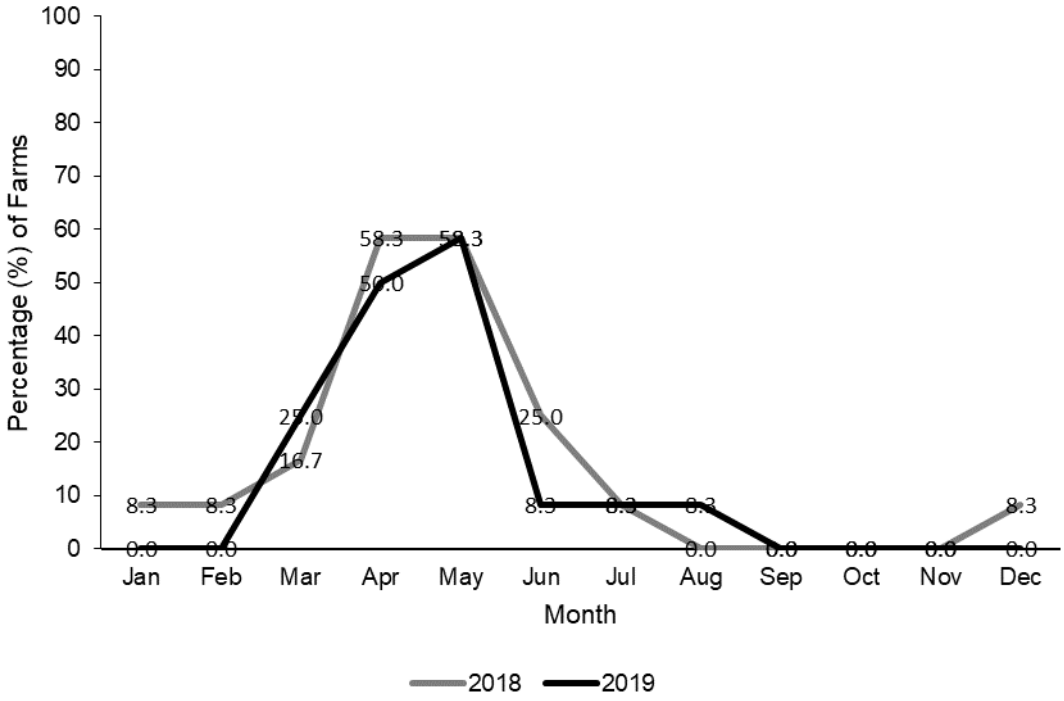

Figure 2.25. Disease/mortality outbreak in tilapia farms in the Philippines in 2018-2019.

The respondents in grow-out farms noted two episodes of mortalities/diseases which were after stocking and the second occurred during the production cycle but most notably during summer months (March-June). The result of the survey showed that 88% of the farms suffered high mortality. In grow-out systems, 67% of the farms experienced high mortality a month after stocking while in the hatcheries it was only during the months of March to May. These mortality outbreaks were not reported to any government agencies by 69% of the farms so that only around 31% notified the Environment and

Management Bureau of the Department of Environment and Natural Resources (EMB-DENR) Region IV-A as required by local regulation.

A 25% or lower disease prevalence was reported by 69% (n = 11) of farms. Two farms reported a 50% disease prevalence while only a single farm noted 75% of it, and the rest (n = 2) did not give data. This is summarised in Figure 2.26. The reported prevalence of disease was noted by farmers after the initial mortality/disease outbreak a month after stocking. The percentage of diseased fish were noted by the respondents for the remaining of the culture cycle.

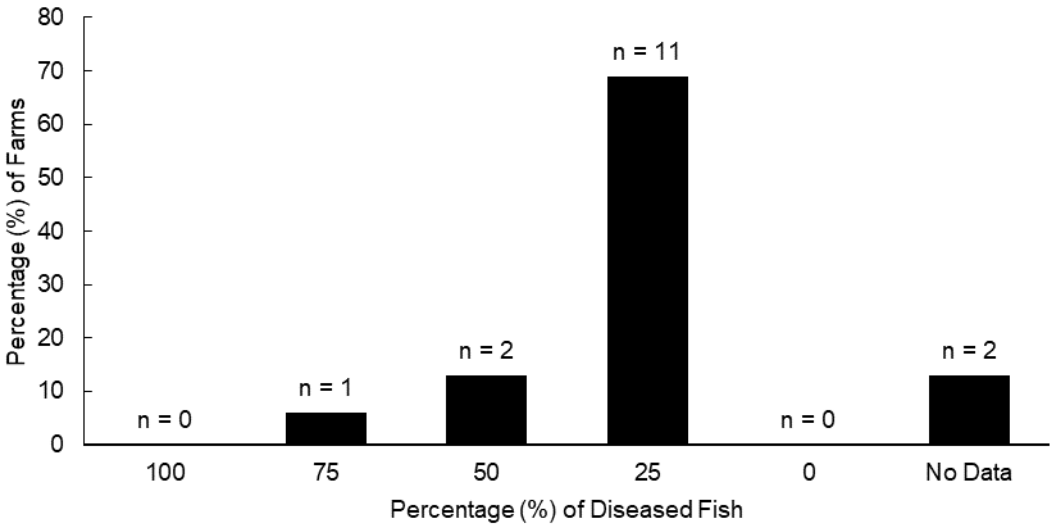

Figure 2.26 Graph showing the percentage of farms showing the percentage of prevalence of disease problems in tilapia stocks in farms and hatcheries after first month stocking.

The percentage of survival and loss of tilapia stocks in a grow-out production cycle after an initial mortality episode one month post stocking was also asked, and result showed that 3 farms reported a maximum of 90%

survival while the lowest was 30% as reported by a single farm (Figure 2.27). There were no data from the hatcheries.

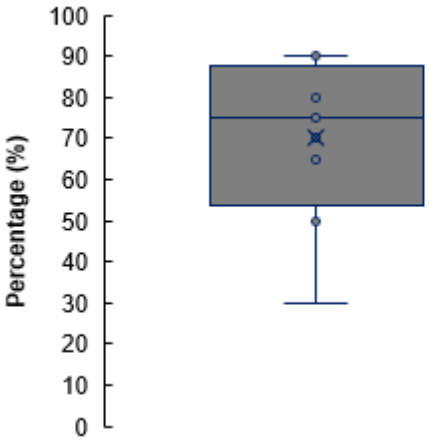

Figure 2.27 Percentage of tilapia survival in a production cycle.

Though it was difficult to ascertain the accuracy, 87% of the respondents said they were able to estimate the fish losses during disease outbreak while only 13% were not. Disease/mortality outbreaks caused significant losses to income in 69% of the surveyed farms while about 31% said that it did not at all affected their income. According to the respondents, the reduction of income and financial losses were due to longer culture period and different sizes.

2.5 Discussion

This study successfully provided current information on the reported disease /mortality outbreaks and mitigation practices applied within tilapia farms and hatcheries, representative of the production sectors in the Philippines. The sampled grow-out farms were mostly freshwater farms as representative of the tilapia farming in the country which primarily is being done in freshwater areas. In the study performed between 2018 and 2019 of the over-all project, all of the

farms contacted agreed to participate and were willing to share information while some were reluctant to share information on specific production figures or use of certain chemicals or treatments. This is expected as a previous study by Aizawa et al. (2014) showed that farmers in the lower Amazon River were also reluctant to provide detailed information about tilapia culture practises which may go against local regulations. In this study the lack of details may have been due to the farmers withholding information due to fear since the survey was done together with BFAR-BIFTOS staff. In Taal Lake, the Batangas Inland Fisheries Technology Outreach Station (BIFTOS) which is an office of BFAR is in coordination with the Taal Volcano Landscape and Management Board (TVPL PAMB) of the Department of Environment and Natural Resources (DENR). The TVPL PAMB together with the Batangas Provincial Government regulates cage farming in Taal Lake and may issue sanctions on the fish farms if found that they are not following the fisheries guidelines or department orders and pertinent laws. Moreover, the lack of some data may be due to the interviewee mood, ulterior motives, or inability to recall information (Tain & Diana, 2007). It is also possible that respondents felt inclined to share positive rather than negative answers with interviewers since during the conduct of the sampling we were accompanied by staff from the BFAR. However, looking at previous published studies (Guerrero, 2019; White et al., 2018; Eguia et al., 2013; White et al., 2007; Vista et al., 2006; Eguia & Romana-Eguia, 2007; ADB, 2005b;) the answers of the respondents were similar, although some people could not recall information or were not allowed to share some data. The present survey was done of reported disease/mortality outbreaks. Previous surveys were done on feed management, husbandry, and water quality. It is

important to compare whether there were changes in these farming practices and that whether these may help to explain the cause/s of problems on diseases or mortality outbreaks.

In this study, the high percentage (94%) of trained tilapia farm workers is in contrast to tilapia farm workers in Bangladesh where 76% of the respondents had not attend any training programs on fish farming (Uddin et al., 2021). In the study of Ituma and Ukah (2017) in Nigeria, they found out that training strengthens fish farmer's ability to efficiently manage farms and to contribute farm's maximum productivity although this is also influenced by the nature of the trainings and abilities of the trainee. These private seed and feed suppliers advise farmers on appropriate practices as well as in improving their products and these have helped contribute to the rapid growth of the tilapia sector (Engle, 2006), however for the past 10 years the volume of production has not seen significant growth due to the limited freshwater areas and other factors that constraint production.

Nile tilapia in freshwater earthen ponds is the most common species and system in the Philippines which reflects the surrounding SEA countries (Yanez et al., 2020; FAO, 2019). However, freshwater earthen pond culture requires higher investment due to the need of land and its development into production ponds (Rojas & Wadsworth, 2007). This high cost of production led to the popularity of cages and pens in lakes and other freshwater bodies. Cage farming of fish has become increasingly popular method of fish culture practised in most developing countries of Southeast Asia (Kashindye et al., 2015). In the Philippines, tilapia cages started using an extensive method of farming, however, the volume of production started to decline in the 1970s (Coche,

1982; Guerrero, 1983) which prompted farmers to begin using supplementary feeds. Moreover, in the extensive method the growing period to produce marketable fish (200 g) increased from 4 months to 6-9 months (Aquino, 1982). These problems led to the uptake of more intensive cage farming of tilapia where grow-out production relies on the use of commercial feed which impacted the water quality of the lakes. Currently under intensive farming which uses exclusively commercial feeds, the average size of the fish at harvest is 500 g and the growing period is similar which is 7 to 8 months. Studies have shown a negative feedback of intensive farming on the nutrient concentration, oxygen level and toxic metabolites in the water leading to eutrophic condition (Eley et al., 1972; Penczak et al., 1982; Phillips et al., 1983). Some disease organisms thrive in eutrophic conditions (Numann, 1972; Lundborg & Lyndberg, 1977) and changes in water quality have also been shown to affect fish production and fisheries (Liang et al., 1981). Given the negative ecological impact on the water quality and carrying capacity of the lakes, and high cost and longer period of production in freshwater cage farming, there is little scope to expand in this type of production system. Moreover, the reliance on commercial feeds resulted to operation and ownership issues of the cages in which financiers and feed companies have become owners and the registered local farmers have become mere caretakers. The number of grow-out farms in lakes in the Philippines is now regulated by the government based on section 51 of the Fisheries Code of 1998 (Republic Act 8550). This law states that “not over ten percent (10%) of the suitable water surface area of all lakes and rivers shall be allotted for purposes like pens, fish cages and fish traps; and the stocking density and feeding requirement which shall be controlled and determined by its carrying

capacity.” Although this law exists, the enforcement specifically on the stocking density and feeding are difficult to monitor and regulate as demonstrated in the results where feeding was arbitrary and most cages were overstocked with actual densities exceeding the recommendation of BFAR.

The main factor that has limited the expansion of the tilapia aquaculture in the Philippines is the availability of lands for pond conversion and the freshwater areas. Currently, almost all the lakes and freshwater reservoirs in the country are engaged in tilapia farming, however, standing laws and regulations limits the conversion of lands into ponds, and the number of cages or pens. Additionally, nutrient pollution of the water, loss of biodiversity and carrying capacity are ecological issues that are of concern which will affect the sustainability of the sector. The potential for the expansion of tilapia farming lies in its diversification from a purely freshwater to brackishwater and seawater culture. Neighbouring Asian countries (e.g., Indonesia, Thailand, and Vietnam) have moved away from an exclusive freshwater tilapia culture into brackishwater farming due to production and environmental constraints (Hishamunda et al., 2009). Moreover, there was an increase in the availability of brackishwater areas as a result of the decline in the culture of marine warm water shrimp species in the early 80's and 90's due to diseases (Cruz et al., 2008; Kautsky et al., 2000). In 2002, large portion of brackishwater ponds (80%) were dedicated to milkfish (Guerrero & Guerrero, 2004) which grows better than Nile tilapia at higher salinities. A small number of marine tilapia floating cages at the time of the survey are being farmed in Panabo Bay in Mindanao using red hybrid tilapia. The uptake of brackishwater and marine farming of tilapia in

Philippine tilapia sector is low. Reasons for this include limited availability of tilapia strains suitable for saline environment, additional cost for salinity acclimation, availability of suitable feeds, and concerns on escape of tilapia into the marine habitat (Guerrero & Guerrero, 2004; Basiao et al., 2005; Eguia & Eguia, 1999). In the Philippines, *O. mossambicus* and the red tilapia hybrid (*O. mossambicus* x *O. niloticus*) can efficiently thrive in brackishwater ponds (Tayamen et al., 2002), while another red hybrid tilapia (*O. mossambicus* x *O. hornorum*) is currently being farmed in seawater at Panabo, Davao. However, the slow growth of *O. mossambicus* and availability of the red tilapia hybrid limit their use hence Nile tilapia is currently the species primarily farmed in brackishwater. Even so, farming Nile tilapia in brackishwater ponds requires periodic monitoring of salinity and exchange of water. Given these issues on suitable strains of tilapia for brackishwater and seawater culture, there is a need to develop fast growing, saline tolerant tilapia strains. Presently, the BFAR is developing tilapia strains such as molobicus (*O. mossambicus* x *O. niloticus*) which will have good growth characteristics in high salinity environments and preferred black silvery colouration. Additional studies should also look into the refinement of husbandry techniques especially on acclimatisation of fry to higher salinity levels, feed development, and disease susceptibility and resistance of the saline tilapia breeds.

The recurring fish kills and deterioration of the water quality of the Laguna Lake caused the farmers in this area to shift towards extensive farming where the fish stocked at low densities in submerged cages depend on natural food (Palanca-Tan, 2020). Using this system, fish cage operators claimed that

farming periods are taking much longer attributing the slow growth of fish to numerous environmental factors aside from the absence of supplemental feeding which is prohibited in the Laguna Lake (Palanca-Tan, 2020; Tamayo-Zarafalla et al., 2002). This same observation was also noted by the respondent during the interview. This longer culture period, aside from lower stocking density and productivity, might contribute to the stagnating volume of tilapia production in this lake. Although a shift from intensive to extensive production system in Laguna Lake was done, survey respondents from the study performed, said that the occurrence of higher disease/mortality outbreaks during March to June was still observed. Taal Lake has lower natural productivity compared with Laguna Lake which has saline flux from Manila Bay which encourages natural plankton production thus enabling aquaculture activities without heavy reliance on supplemental feeding (Saguin, 2015). This low primary productivity in Taal Lake and other enclosed lakes in the country makes a shift from intensive to extensive tilapia production infeasible. Presently, Taal Lake is heavily reliant on commercial feeding to sustain its tilapia production.

Duration of farm operations in this study was composed of relatively new farms (<5 years) with a much smaller number of sites had been in production for more than 20 years. Most of the traditional tilapia cage farmers in Taal Lake had to give up their farms and became caretakers for big companies due to issues with debt. Others made agreements with non-resident financiers or absentee-investors and serve as permanent cage workers for cage farms that are usually registered under their names (ADB, 2005). This scheme of cage ownership brought about by the high cost of intensive cage farming in Taal Lake defeated

the democratisation of access to the lake in which the registrants or owners of the cages should be the local residents. Aside from registered cages, illegal cages are being operated in the lake putting more pressure to it. In 2014, BFAR multi-agency task force dismantled over 7,000 illegally operated cages (Querijero & Mercurio, 2016). Hatchery operation, which is not as costly as grow-out, continued with most families doing it in their backyard especially in the areas of Laguna with some of them are still in operation for more than 20 years. Backyard hatchery operation tended to last longer since the ponds were owned by the family, and members of the family helped in the operation thus reducing expenses. Moreover, backyard hatcheries have small scale operation thus costs and investments are lower, and potential risks are low so potential losses are minimal. The two new hatcheries were owned by the same new grow-out farm company in the Taal Lake. According to these respondents, these big tilapia grow-out farms usually have their own hatchery operation in order for them to have a constant supply of fingerlings. The duration of operation of farms showed no difference in the occurrence of disease outbreaks since all reported similar problem.

The observed practise of the small-scale hatcheries is similar to the previous study of Basiao et al. (2005) where hatchery farms in the Philippines develop their own tilapia strains due to the higher cost of the improved strains. Aside from the cost of improved strains, hatchery owners believed that these locally developed hybrids have improved qualities especially disease resistance. Moreover, from key informant interview, most of the cages in Taal Lake used the Tagalog strain which they claimed to have higher survival compared with commercial strains. However, at the time of writing there had

been no robust work on these local strains or their higher disease resistance. There is also the risk that inbreeding may result in loss of genetic diversity leading to decline in productivity, for example because of undesirable growth characteristics, and depressed fitness (Nguyen, 2016; Fessehaye et al., 2007). Presently, the BFAR is performing trials on the grow-out culture of its SaltUno or molobicus tilapia fish strains (Guerrero, 2018; Yap, 2001), believed to be better suited than the more traditional red-hybrid tilapia, for production in brackishwater and seawater farming. Moreover, this molobicus strain retains its black silvery hue which is preferred in the domestic market since red tilapia is mistaken for a high-priced fish (Eguia & Eguia, 1999). The red tilapia domestic sale is low compared with the black silvery fish since consumers will not buy it thinking it is expensive thus uptake of its farming is low among fish farmers.

Record keeping, if used for constructive purpose in fish farming, can be an important contributing factor to the long-term sustainability and profitability of the business (Opiyo et al., 2017). From the survey performed in this study, all of tilapia farms kept some form of records although the amount and quantity varied considerably. Although not unexpected, an important finding in this study was the fact that most of the tilapia farms do not keep mortality records and yet they reported experiencing fish mortalities on their farms. The reason given for the lack of mortality data included difficulty in monitoring and quantifying of mortalities due to the depth of the cages, and it presented additional workload for the farm workers especially in the deep-floating and submerged cages. Nevertheless, the identification of unusual mortalities may help fish producers identify and address the problem or act as a warning sign in any surveillance programs performed for the sector (Soares et al., 2012; Jia et al., 2018). As the

majority of the farms included in this study had no mortality records, the reported mortality shared during the questionnaire was described as perceived mortality. This was limited only to the number of dead fish without anylaboratory-based diagnosis to determine the cause of mortality. During the interviews, the reported mortalities were mere estimation of the farmers as they were not sure of the exact number. Moreover, they did not share any record book to confirm if they were indeed keeping it in record. It may be that although the farmers were able to identify and give an estimate of the mortalities, the absence of any data is understandable, since the value of these mortality records in fish disease diagnostics and management did not appear to be well understood by the survey participants. This practice of perceived mortality is also seen in red tilapia farmers in Vietnam (Boerlage et al., 2017) which may indicate the lack of understanding by tilapia farmers of the value of such record keeping in health management.

In terms of sustainable development, mortality records are important since it can alert farmers to a problem and they can develop remedies by operational intervention (Ellis et al., 2012) in their husbandry such as reduction of feeding, change of nets or application of antibiotics through medicated feeds. Although perceived mortality cannot replace actual recorded or confirmed mortality (i.e., laboratory-based diagnosis), it remains a good indicator at the farm-level of potential health related issues with their stocks. Farmer-based data may may be used together with actual harvest data or other recorded production level information for estimating over-all fish stock survival because the experience-based farmers' knowledge is often valuable (Stuiver, 2004). Fish health records from the participants in this study were absent since they did not

establish the cause of mortalities, nor did they seem to check the health quality of the fish prior to stocking their farms.

The lack of accessible fish veterinary diagnostic laboratory and the cost of tests maybe the reasons why fish health recording is not done which is a similar observation in tilapia farms in Brazil (Padua & Cruz, 2004). It is understandable that production is recorded, which is evident from data of the survey, because of its direct economic and financial impact on farmers. Moreover, record of stocking density will also allow the fish farmers to project the number and mass of fish that will be harvested at the end of the production period. The lack of mortality record in this study may be due to their observation that the mortalities do not hugely affect their production since the number of fish that survived to harvest is enough for the farms to produce profit.

In the present study, the stocking density and size of tilapia grow-out cages and ponds varied. There were erratic Nile tilapia stocking density survey results from Taal Lake-based cages, which appeared to result from an existing strict regulation against overstocking in lake cages but with the notion that overstocking of fry or fingerlings has nonetheless become customary amongst Nile tilapia cage farmers in the lake. Higher stocking density was mostly noted in floating cages in Taal Lake which were all intensive while it was lower in marine floating cages which were also intensively farmed. The reason for the absence of mortality records from the survey is that farm workers already expect the mortality in the culture period thus they overstock to mitigate the resulting losses. They did not mind if the harvested tilapia is half or lower of the total stocked population as long as the yield is sufficient to allow the farm owners to earn a profit. In the previous study of White et al. (2018), generally

the stocking density minimums and maximums being followed were 50 and 500 fingerlings/m^2 respectively in Nile tilapia cages: two and 10 fingerlings/m^2 in Nile tilapia ponds. However, this cannot be compared with the current study since the stocking density they measured was based on the surface area but not in volume of the cage or pond. In the study of Bhujel (2013), the tilapia cages in Thailand and Vietnam stocked fingerlings sized between 30-50g at densities of 80 fish/m^3 and 175 fish/m^3. In this study, the stocking density in Taal Lake cages was 83-417 fish/ m^3 which is higher than in Thailand but the size of fish that were stocked were smaller (1-25g). In the seawater floating cages of Panabo, stocking density was at 25 fish/m^3 which is notably lower compared with Taal Lake however the stocking size of the fish was larger (20g) with the common stocking size in Taal Lake since they need to be acclimatised first before their release in the marine cages. Another factor during interview was the limited availability of the red tilapia hybrid fry and the associated mortality during acclimation process.

The recommended stocking density for tilapia in cages is 50 fish/m^3 (Yang et al., 1996). A lower stocking density was noted in the interviews performed with the farmers raising fish in submerged cages in Laguna Lake (12 fish/m^3) which are using extensive type of farming. The low stocking densities (40 fish/m^3) were also seen in a semi-extensive farm in Batad, Iloilo. A higher stocking density is suitable if the water flows fast thus maintaining water quality which is not the case in lakes. In a study on the influence of tilapia stocking density in cages on their growth, Yang et al. (1996) recommended 50 fish/m^3, however, this recommendation is rarely followed in Taal Lake cages. In terms of production in grow-out cages high stocking density of tilapia fry at size of 1 g

produced an average weight of 200 g than farms that have lower stocking density regardless of the stocking size of the tilapia (1 g or 25 g) in which the average weight of the fish is around 800 g. From key informant interview, many operators reported longer periods of both intensive and extensive tilapia culture amounting to an additional one to three months of culture period, compared with the situation in the 1990's. In submerged cages, although stocking density is low, the size of tilapia at harvest is also small (100 g/fish) since it only uses natural food compared with other systems which used commercial feeds. For hatcheries, the stocking density for broodstock in hapas in pond was about 7.5 fish/m^2 which is similar to the stocking density in Thailand which is 6 fish/m^2 (Bhujel, 2013). However, in the same study, the ratio of male to female is 1:1 or 1:2 which is lower compared with the Philippines which is 4:1 or 5:1. These ratios of male and female broodstock of Philippines and Thailand are common in Southeast Asia (FAO, 2021). However, a high stocking density may contribute to stress and increase diseases in fish broodstocks. In the study of Pradeep et al. (2016), they have shown that bacterial diseases such as streptococcosis can be vertically transmitted even by asymptomatic broodstock to the offspring. In this study, *Streptococcus* bacteria were recovered from broodstocks and their presence in tissues was confirmed by histopathology (see Chapter 3). It is of note that these hatcheries are the ones that supplies the grow-out farms in Taal Lake. As mentioned earlier, some of the data in production specifically the stocking density is not reliable since most of the farm have incomplete records or no records at all and uncounted stock. There was also a reluctance by farmers to reveal this information. According to BIFTOS, stocking density was routinely violated, and regulators had no way to count the

fish already in the cages aside from the illegal cages which are not monitored. This overstocking has a direct implication to nutrient pollution since higher number of fish will need higher amount of feeding.

The environment, particularly poor water quality affects the feed conversion ratio (FCR) and the health of the fish negatively which in turn impairs the profitability of fish farms (de Verdal et al., 2018; Mengistu et al., 2020). Studies have shown that DO, temperature and pH are important variables to consider in the productivity of tilapia farms (Mengistu et al., 2020; de Verdal et al., 2018; Mjoun et al., 2010). Next to salinity, DO and temperature were also measured by most of the farms. Dissolved oxygen is one of the main limiting factors that affects fish performance. Low DO negatively affects feed intake (Wang et al., 2009), reduces digestibility (Tran-Duy et al., 2012) and the health of the fish. High or near saturation DO may cause improved blood flow to the gastrointestinal tract (Axelsson et al., 2002), lower cost of feed digestion and absorption of nutrients in fish (Duan et al, 2011) which in turn increases feed assimilation for higher growth. Tran et al. (2016) found that Nile tilapia grow less under low DO level (3 mg/L) than normal level (5 mg/L). In the study of Mengistu et al. (2020), they found out that depending on the temperature it can significantly affect FCR but not survival, however in this study water temperature during a particular time of the year is a strong factor that contributes to disease and mortality outbreaks. Moreover, they found that Nile tilapia performed best at the upper optimal temperature of 27-32° C, which agrees with older reports quoting 29-31° C being the optimal temperature range for tilapia growth (Popma & Lovshin, 1996; Popma & Masser, 1999). The range of water temperature of the sampled farms was 28-29° C which is the expected

temperature during dry season in the Philippines. Water temperature management, although possible, in tilapia ponds and cages is impractical since any increase is within the tolerable range which can improve the FCR thus farmers do not employ temperature mitigation strategies in their farms. The study of Mengistsu et al. (2020) found temperature had no significant effect on survival, and increase of temperature may actually be a confounder for a low DO or plankton bloom which are known to cause disease or mortality outbreaks. Another water quality measured by most farms is pH. In the study of Lemos et al. (2018), they found that circumneutral pH (7.14) is the best for Nile tilapia production system since at alkaline pH un-ionised ammonia is higher which is toxic to fish and hydrogen sulphide is toxic at low pH. Additionally, they found out that low or acidic pH promoted high plasma cortisol in the fish which is indicative of stress. While management of pH level is not a problem in ponds via the application of lime, it poses a problem especially in freshwater lakes most especially in Taal Lake. Liming and aeration are not possible in these lake areas. In the case of Taal Lake, the unpredictability of the volcano and seismic activity with ammonia and sulphide releases from the floor of the lake adversely affects its pH which make the fish stocks at risk (ADB, 2005). Ammonia, nitrate, nitrites and phosphates are important water quality parameters that also contributes to the over-all productivity of farms and health of fish. In this study, farmers have noted that DO is critical in the survival of the fish since most of the fish kills they experienced were due to very low DO. Measurement of DO and other water parameters in Taal Lake are monitored monthly by BIFTOS, and the data are relayed to different farms so that in case of critical levels the farmers can decide to do emergency harvest.

Nutrient pollution of the Taal Lake, which causes significant stress to fish stocks, is due primarily to the fish cages itself where discharges of nitrogen and phosphorus derived from uneaten feed, faeces, and excretion via gills and urine (Beveridge & Philipps, 1993; Kibria et al., 1996; Kibria et al., 1998). Commercial feed was used by almost all tilapia grow-out farms and hatcheries as the majority were intensive production methods. This is similar to tilapia farms in Egypt (Eltholth et al., 2015), Brazil (Roriz et al., 2017), Kenya (Opiyo et al., 2017), Southern China (Liu et al, 2013) Vietnam (Boerlage et al., 2017), Bangladesh (Tran et al., 2019), and Thailand (Bhujel, 2013) showing that worldwide tilapia production uses commercial feed in a semi-intensive and intensive farming system. The types of feeds and feed type used by Nile tilapia farmers were a mixture of floating, sinking or a combination of both feeds. Continued use of sinking feeds in Nile tilapia cage operations in Taal Lake is primarily for economic reasons since floating feed is more expensive. This was despite the requirement in the local ordinance requiring the use of floating pellets since sinking feed that are uneaten pollutes the lake (Muyot et al., 2018). Floating feed reduce feed use because of increased utilisation of the fish and actually lessens feed cost (Muyot et al., 2018). In general, floating feeds offer many advantages over sinking feeds such as farmers can directly observe feeding intensity of their fish and adjust feeding rates, and visually monitor health of fish as the come to surface to feed (Tran et al., 2019). In this study, both the grow-out ponds and hatcheries used sinking feeds since almost all of these farms had deep ponds (1.5- 2 m), where the fish have plenty time to eat the feed before it falls to the bottom. A higher feeding frequency is required when Nile tilapia are reared in tanks with increased salinity level since in these

systems there is improved feed utilisation and growth (Daudpota et al., 2016). Tilapia is primarily a freshwater fish so it needs more energy in order to maintain osmotic balance in a saline environment and this can be achieved by eating more food. In hatcheries, the feeding frequency is also 3x/day since broodstocks need to channel high amount of energy for maturation and reproduction.

It is a common practice in tilapia farming in the Philippines that feed manufacturers offer feed loan terms or delayed payment until harvest (ADB, 2005). This practice encourages uncontrolled or over feeding because the farmers have a steady supply of feeds and that they believe that increasing amount of feed will increase growth in short period of time. However, this negatively impacts the water quality and over-all health of lakes largely contributing to eutrophication thus water quality especially DO fluctuate often (Papa et al., 2011; Mamaril, 2001). The current management options to control contamination of the environment by nutrients from the cages are largely limited to controlling the intensity of fish cage production (Naylor et al., 2003; Marte et al., 2000), which is difficult to implement since the power to give license to setting up of cages in the Taal Lake lies with the local government but not to the BFAR. Government officials tends to be lenient since the livelihood of the municipalities primarily depends on these tilapia cages.

Disease or mortality is a specific outcome that is the result of complex interactions involving the environment, fish hosts and pathogens (Snieszko, 1973). Even though the farmers do not test or submit their fish for disease or pathogen diagnosis, all of the farmers said they were able to visually identify and detect if the fish were sick or not using a wide range of factors commonly

applied in freshwater aquaculture systems. This was evidenced during sampling where they were able to pinpoint and collect diseased fish from the apparently healthy ones. This is similar to the practice of tilapia farmers in Brazil which uses gross examination for disease diagnosis (Roriz et al., 2017) where tilapia farm workers can identify and detect diseased fish but are not able to specifically diagnose the specific disease or infection.

The farmers were able to identify and detect disease in tilapia stocks as evidenced during survey when they knew which fish were diseased or moribund to take for sampling. With this knowledge, they have employed some disease interventions or strategies. During disease outbreaks, around half of the surveyed grow-out farms did treatment primarily by throwing salt into the cages which does not affect the salinity level of the lake since it has an outlet towards the sea and there are freshwater inflows from three rivers namely Balete River, Laurel River and Wawa River (Perez et al., 2008). Salt treatment is a common practice for treatment of a range of pathogens in freshwater aquaculture (Lio-Po & Lim, 2002) especially in tilapia in cages (Chitmanat et al., 2016). Its popularity is due to its efficacy, low cost and easy availability (Schelkle et al., 2011), and it is less harmful to the host compared with other treatments (Schelkle et al., 2009). However, its efficacy is dependent on treatment duration, strength and target pathogens (Schelkle, 2011). Moreover, farmers in this study were not sure of its efficacy since there is continued disease and mortalities among the stocks albeit in lower proportions. Studies have shown that salt is effective against protozoans and helminths (Lio-Po & Lim, 2002), however there are very few empirical studies on its efficacy against bacterial fish pathogens (Schelkle, 2011).

One environmental management strategy to help curb fish diseases in earthen ponds is by application of lime. Liming is applied to the pond bottom prior to filling with water so that it is impractical to use it in floating or submerged cages in lakes and rivers (Lazur et al., 1998). Liming of aquaculture ponds is primarily used to enhance the effect of fertilisation by improving phosphorus availability, prevent wide swings in pH, sterilise, and adds calcium and magnesium which are important in animal function (Wurts & Masser, 2004). Although liming does not directly address control of diseases, it does, reduce stress in the fish and improve health since it rapidly raises the pH to 12 or higher that kills pest and disease agents prior to stocking and subsequently stabilises the pH (Lazur et al., 1998). In this study, the use of lime was limited to the brackishwater ponds in Silay, Negros Occidental but not in brackishwater ponds in Lubao, Pampanga and in hatchery ponds of Calauan, Laguna since they did not employ fallow period after one production cycle. During the key informant interview, respondent said that the application of lime in brackishwater ponds of Silay was done after one production cycle after a short fallow period. However, liming did not prevent disease outbreak in Silay ponds as they experienced it also every warm month of the year.

The use of antibiotics in aquaculture is being regulated since its extensive use may result in development of antibiotic-resistant bacteria, and can disturb the environmental microbiota (Austin, 2017). There is a common understanding that the use of antibiotics for protecting fish from diseases should remain low if not discouraged (Aly & Abutti, 2014). Aquaculture farms are advised to use other prevention approaches or health strategies rather than antibiotics but if its use is necessary in some cases, they should administer only

approved drugs for fish at the right amount and period of time (Rogers & Basurco, 2009).

In this study, antibiotic treatment was used exclusively in the broodstock ponds. The hesitance by the participants of identifying the type of antibiotics they used maybe they were administering prohibited antimicrobials which they find more effective than the allowed drugs. Additionally, it could also have been that the respondents were not sure of what specific type of antibiotics were administered since they were not involved in the preparation of medicated feed. From the interview, the owner of a hatchery told us that antibiotics were primarily mixed with farm made feeds. This practice is risky since a correct dosage may not be administered if the added antibiotics are not evenly distributed and bound to the feed pellets.

When disease or mortality outbreaks occur, most of the farmers claimed to discard the dead fish in mortality pits with lime in order to limit the spread of infection. This is much better practise than the other options that have been reported. In contrast, majority of the Vietnamese tilapia farmers sold the dead fish as food to other fish species of other farmers (Boerlage et al., 2017). As far as we can tell from the questionnaire performed in this study, the practice of selling dead fish as food for other fish or animal species did not occur which is good since infected dead fish can be route of disease transmission.

However, the participants did claim that dead and diseased tilapia were sold in local markets for human consumption. We observed that dead tilapia were floating on the water in and out of the cages and also in ponds. Uncollected dead fish poses biosecurity risk since they become source of pathogens that can be disseminated in water that may water down to other

areas. The rapid identification and collect/removal of moribund/dead fish is a welcome practise but can be missed in some farming systems.

In the study performed, interviewees reported that people nearby collected the diseased fish while some workers accumulated them in a separate cage and sold them at a lower price in the market. This practice of selling diseased fish is an additional income to farm workers who are suffering from very low wages which is approximately Php 250 (£3.69)/8-hour day. A small proportion said they eat the diseased fish, but they dried it first. Those people collecting the diseased fish might be also eating or selling it. Danger of zoonotic infection by eating diseased tilapia does not really pose a significant health hazard since Filipinos do not eat raw tilapia fish as it commonly cooked by frying or grilling, however handling and preparation of the raw product may pose a significant route for infection for *S. agalactiae* or *S. iniae* (Chau et al., 2017; Baiano & Barnes, 2009). Reports of zoonotic infection from farmed tilapia is unknown in the Philippines but it does not mean it is not occurring such that additional research or investigation will help clarify this and prevent a future human outbreak.

Tilapia farmers in this study noted gross clinical signs when fish were sick. However, these clinical signs were not specific to single infectious agent since a lot of these are common to a multitude of bacterial, parasitic, fungal, and viral agents (Austin & Austin, 2016). In Taal Lake floating cages, a low percentage of farmers reported that fish stop feeding when they are sick because the cages were quite deep that they are difficult to be seen. These clinical signs are more difficult to observe in submerged cages in Laguna Lake since the cages were almost 1 meter below the surface of the water. The farm

workers do not check the submerged cages every day since they are of extensive type and the fish feed only on natural food. From the interview, farmers were only able to know if there is mortality outbreak if the submerged cage starts to surface or float in the water with the dead fish inside it. The peak of the disease or mortality outbreaks were during the hot months of March to May. The peak of infectious diseases in tilapia was also observed to occur during warm months in Egypt (Elsheshtawy et al., 2019) Brazil (Roriz et al., 2017), and Taiwan (Liao et al., 2020) that an increase of water temperature regardless of the month of the year may be involved or a factor in these disease outbreaks. However, increase in water temperature may also be a confounder since increase in water temperature does not directly cause the disease in tilapia although physiologically it affects them. The increase in temperature may actually affect the expression of virulence of bacterial pathogens in heavily stressed fish in the farms. Although disease peaked during these months, farmers in the grow-out cages in Taal Lake noted that diseases in the stocks persist whole year round, albeit in lower numbers in other months, which is a similar perennial disease problem in Southern China tilapia farms (Liu et al., 2013). This increased in temperature together with low water quality and highly stocked farming system may be the primary factor contributing to disease outbreaks during hot months of the year.

This disease phenomenon was similarly observed in hatcheries in that the broodstocks got sick and died during hot months. From the interview, it is during this time also that hatcheries encounter problems in reproduction since farmers observed that high water temperature ranging from 28-30°C during summer causes decreased volume of spawn of tilapia. Reproductive

performance at temperatures higher than 35°C, which can occur in the afternoon during the dry season in the tropics, has been found to be very poor due to broodfish being highly stressed (Bevis, 1994). The study of Reputola (2018) recommends an average 1.2-meter depth for freshwater breeding ponds to make the water temperature stable which is a little shallower to the 1.5 m depth of the surveyed hatchery ponds. Coupled with disease/mortalities, farmers said that during the hot months of March to June tilapia fry are scarce and expensive. Aside from the seasonal occurrence of disease outbreaks, farmers in grow-out cages especially in Taal Lake observed very high mortality during first month of stocking. The cause of the high mortality was not clarified during the study by the participants and there was not enough data to determine the probable cause but could be due disease and/or stress during transport and handling prior to stocking since the hatcheries were approximately 35 km away from the grow-out sites. In the study of Ofori et al. (2010), tilapia mortality was very high (70%) after stocking which they have associated with handling and transport during stocking although other factors might have contributed to it such as diseases.

It is to be noted from the survey interview of tilapia farmers that their tilapia fry were supplied from hatcheries from a neighbouring province. Based on the interview, the grow-out farmers do not know or do not even care whether the hatcheries were experiencing disease outbreaks in their broodstocks. After the initial mortality 1-month post-stocking, the prevalence of disease problem in the remaining stocks was noted mostly at 25% but this figure is just an estimate of the farmers as most farms do not actually record mortality or disease.

Significant losses to income (69%) due to disease was reported since peak of disease occurrence coincides also with the peak of production from March to June where most fiesta celebrations occur and during which time in Lent, Filipino abstain from meat instead eats fish (ADB, 2005). Production is lowest in the third quarter (July to September) because it is typhoon months. From the interview, farmers revealed that they suffer reduction of income and financial losses which are due to longer culture period and different sizes of fish because of non-uniform growth. Around a quarter said diseases do not affect their income, however, this statement could be due to the fact that the respondents were not involved in the financial aspect of the production since most of them were hired labourers only. Looking at the stagnation of the tilapia development over time in the Philippines, overstocking, water quality issues and disease outbreaks are likely to be drivers contributing towards the lack of sustainable or enhanced development of the tilapia farming sector in this country.

2.6 Conclusion

This study showed that farmers used gross clinical signs only to recognise morbidities and mortalities in their stocks, but further diagnosis was not performed routinely. Furthermore, the farmers included in the study were aware of fish health and factors influencing this e.g., water temperature, seasonality and although they attended training sessions, they did not readily recognise the importance of confirming the cause of mortality/morbidity. This is unfortunately, not uncommon in many parts of the world practising freshwater

aquaculture. As seen elsewhere in other production sectors, farmers used varied practises and approaches e.g., higher stocking densities in anticipation of high mortality. These types of practises can create serious problems for the environment and increase disease potential and complicates farm management. Further work on confirming the cause of the mortalities would be most beneficial for the sector, however, the data presented in this study showed that fish losses impact tilapia production in the Philippines and that disease outbreaks may have contributed towards the stagnating growth in tilapia as a seafood production in the Philippines. Overall, this study showed the critical need for a comprehensive approach, inclusive of fish health management, to support the development of the tilapia sector in the country.

CHAPTER 3.

Disease Status of Farmed Tilapia in the Philippines

3.1 Abstract

In the Philippines, the role of infectious diseases has not been given much attention because of the long-standing belief that tilapia is a robust fish against diseases. The aims of this study were to identify the cause of clinical infections in farmed tilapia and attempt to determine the role of infectious disease as a cause of the mortalities and morbidities experienced on the tilapia farms. This was performed using a traditional and molecular diagnostic approach which included a description of the gross clinical signs, bacterial recovery and identification and corresponding histopathology. The samples were taken during the farm visits, described in Chapter 2.

A total 105 fish were sampled from 16 individual farm sites which included a minimum of at least 1 apparently healthy fish. The other samples per site were animals with overt clinical signs of disease as observed grossly. Bacteria were recovered from tissue samples and the remaining tissues were processed for histology using standard methods. The bacterial identification methods included both traditional (e.g., Gram stain, oxidase), biochemical and *16S rRNA* gene sequencing for confirmation of identification. The histopathology samples were fixed, processed and 5 µm thick tissue sections cut and stained to determine cellular changes and identification of bacteria, where possible.

Results showed a diverse range of clinical signs including, but not limited to, abnormal swimming pattern, lethargy, eye anomalies, ascites, gill pallor, hepatosplenomegaly, body redness and body lesions. Histologically, cellular changes observed included inflammation, severe hyperplasia, bacteria and inflammatory cell infiltration, and hydropic degeneration. Additionally, a number of gill samples were infected with a myxosporean parasite. Multifocal necrosis with infiltration of inflammatory cells, vacuolar degeneration and lytic necrosis were observed in the hepatic tissue. In the spleen, expansion of the red pulp, histiocytosis, splenitis and necrosis with hemosiderosis were present. The kidneys had varying degrees of nephritis with marked tubular degeneration, hyaline degeneration, glomerular dilatation of the Bowman's capsule and necrosis. Pathological changes in the heart were pericarditis, epicarditis, myocarditis, and necrosis of the endocardium with infiltration of mononuclear cells and lymphocytes. Histopathological examination of brain tissue specimens showed massive cellular meningitis accompanied by haemorrhage, meningoencephalitis, vasculitis and encephalitic vacuolation. Gram-stained sections of these tissues showed bacterial colonisation which indicated septicaemia. The histopathological lesions were correlated with the observed clinical signs indicating involvement of infectious agent. Bacterial recovery and identification results were infections from *Streptococcus agalactiae*, *Streptococcus iniae*, *Aeromonas veronii*, *Lactococcus garvieae*, *Vibrio harveyi* among others in diseased fish. The work presented has established the clinical picture of the observed disease/mortality among tilapia farms in the Philippines and confirmed the role of infectious disease.

3.2 Introduction

Infectious diseases causing production level losses have been reported widely in all tilapia producing countries such as the USA, Israel and Thailand (Eldar, Bejerano & Bercovier 1994; Shoemaker, Klesius & Evans 2001; Suanyuk, Kong, Ko, Gilbert & Supamattaya 2008; Evans, Klesius, Pasnik & Bohnsack 2009), China (Ye, Li, Lu, Deng, Jiang, Tian, Quan & Jian 2011) and Brazil (Netto et al., 2011; Figueiredo et al., 2012; Figueiredo et al., 2005; Barony et al., 2015). These include a wide range of microbial and parasitic pathogens where the most common aetiological agents causing disease in farmed tilapia are presented in Table 3.1.

Table 3.1 Important infectious diseases of farmed Nile tilapia.

Pathogen Type	Disease*	Causative agents	References
Bacteria	**Streptococcosis**	*S. agalactiae. S. dysagalactiae. S. iniae, L. garvieae*	Eldar, 1994; Netto, Leal & Figueiredo, 2011; Perera et al., 1997; Evans, Klesius & Shoemaker, 2009
	Aeromonasis	*A. veronii, A. hydrophila, A. dhakensis, A. schubertii*	Huys, Cnockaert & Swing, 2005; Austin & Austin, 2012; Soto-Rodriguez et al., 2013; Ren et al., 2019
	Columnaris	*F. columnare*	Sebastião et al., 2011
	Francisellosis	*F. orientalis*	Soto et al., 2009
	Edwardsiellosis	*E. ictaluri, E. tarda, E. anguillarum*	Dong et al., 2019; Plumb, 1993; Armwood et al., 2019
Viruses	**SHT or TiLVD**	*Tilapia tilapinevirus* or TiLV	Bacharach et al., 2016
	ISKNVD	*Megalocytivirus* ISKNV	Subramaniam et al., 2016
	Viral nervous necrosis	*Betanodavirus* (NNV)	Keawcharoen et al, 2015
	Tilapia parvovirus disease	*Tilapia parvovirus* (TiPV)	Liu et al., 2020
Fungi and Parasites	**Fungal infections**	*Saproglenia, Achlya, Branchiomyces*	Hussein et al., 2013; El-Sayed, 2016
	Ectoparasitic infections	*Trichodina, Dactylogyrus, Ichthyophthirius, Ambiphrya, Amyloodinium*	Bondad-Reantaso & Arthur, 1988; El-Sayed, 2016; Roberts & Sommerville, 1982

*SHT – Syncytial Hepatitis of Tilapia; TiLVD = Tilapia Lake Virus Disease; ISKNV = Infectious Spleen and Kidney Necrosis; NNV = Nervous Necrosis Virus; TiPV = Tilapia Parvovirus.

It is recognised that tilapia production has stagnated in the Philippines (Guerrero, 2019) and the role of infectious diseases is currently unknown. Data acquired from the field study performed in chapter 2 clearly demonstrated that farmers suffered high number of fish mortalities and morbidities on their farms, and they were able to recognise and describe clinical signs of disease in their fish populations. This would suggest a strong awareness by the farming community of disease within these tilapia production units included in this study, however, in general the actual aetiology of the fish morbidities was rarely confirmed. Given the commonality of bacterial infections reported to occur in other tilapia producing countries (Table 3.1), including the neighbouring SEA countries, it is likely that these diseases may be present within the Philippine sector, but either unrecognised or under reported.

In the Asia Pacific region, there has been a dramatic increase in the numbers of emerging infectious pathogens resulting in disease outbreaks within the tilapia aquaculture industry over the last 10 years. Apart from streptococcosis caused by either *S. agalactiae* or S. *iniae* (Anshary et al., 2014; Kannika et al., 2017), tilapia aquaculture in the region has recently experienced severe production losses associated with several emerging pathogens including tilapia lake virus (Amal et al., 2018; Behera et al., 2018), infectious spleen and kidney necrosis virus/iridovirus (Dong et al., 2015; Suebsing et al., 2016), nervous necrotic virus (Keawcharoen et al., 2015), *Hahella chejuensis* (Senapin et al., 2016), *Francisella noatunensis* subsp. *orientalis* (Jantrakajorn & Wongtavatchai, 2016; Nguyen et al., 2016), *Aeromonas veronii* and *Aeromonas jandaei* (Dong et al., 2015; Dong et al., 2017) as well as concurrent infection of multiple pathogens (Dong et al., 2015b). Edwardsiellosis caused by *E. ictaluri*

has been reported in Nile tilapia (*O. niloticus*) cultured in the Northern Vietnam (Dong et al., 2019). Examination of disease outbreaks in surrounding ASEAN countries producing tilapia, has identified several infectious diseases, however, the current status of these and their role in limiting the tilapia production within the Philippines farming sector is unknown.

The serious socio-economic losses and threats to food availability/security brought about by aquatic animal diseases necessitates the development of effective disease control to stop the spread of infectious pathogens and improve the health and welfare of the farmed fish. Aside from a robust health management and husbandry practices, disease monitoring in the form of surveillance and rapid diagnosis is important to allow appropriate action when pathogens are detected, and diseases are diagnosed before they become serious problem for the farmer (Adams & Thompson, 2011). Diagnostics or diagnostic approach is the determination of the cause of the disease which also involves the detection and identification of the pathogens in subclinical or apparently healthy host (Subasinghe, 2009; Austin & Austin, 2016). It is not uncommon nowadays that both traditional and more rapid kit-based approaches are used in aquatic diagnosis studies. Often this depends on the resources available to the investigation. An initial diagnosis can be made in the farm site by receiving background information from the farm regarding the onset of suspected disease problem and during a site visit a visual examination of gross clinical signs of disease. If a bacterial aetiology is suspected, then more emphasis is placed on recovery of the bacteria as this will enable antibiotic sensitivity testing to be confirmed, should a treatment be required. Recovery of viable cultures from diseased fish remains the gold standard, yet this can be

time consuming and laboratory intensive, hence the uptake of more molecular based pathogen identification methods which do not rely on the growth of the viable bacteria. However, the absolute gold standard for any diagnostic investigation would be histopathology samples, as these remain the only methods which can truly diagnose the condition.

Diagnostics plays an important role in aquatic animal health management and disease control (Raja & Rithendran, 2015). Correct diagnosis can lead to effective or appropriate measures to curb disease and mortality outbreaks, and spread of infection, however, an incorrect diagnosis can aggravate the situation and incur more costs. As mentioned earlier, diagnostic approach in aquaculture is primarily used for an initial disease investigation, however, the same laboratory-based methods can be applied in other circumstances. Examples of this may be in confirmation of specific pathogen free status (SPF) stocks or confirmation of health certification. It is not always necessary to use a comprehensive diagnostic approach, particularly when you are doing repeat visits to farms, or have an on-going disease issue which appears annually, however, failure to apply a comprehensive investigative diagnostic approach can result to misidentification of the actual disease problem. This in turn can produce inappropriate control strategies and health management programs that will not reduce or eradicate diseases leading to economic losses to farmers and eventual breakdown of the sector.

To provide a robust description of the disease status of the animals, pathology samples are invaluable at the onset of investigation. It is important to accurately process the tissues and correctly interpret the changes since it will strengthen the diagnosis of disease (Raja & Jithendran, 2015). This is a learned

skill, which has become undervalued due to the emergence of rapid diagnostic kits, which unfortunately when used without wider context, may facilitate poor diagnosis. Whilst the kidney has been the organ of choice for bacterial recovery in diseased fish species (Austin, 2019), as our understanding of the infectious processes and aetiological agents improve, other organs e.g. spleen and brain can be used depending on the suspected disease. Nevertheless, it is very important to understand that mere isolation and identification of pathogen from any host do not warrant that the disease and mortality are due to its presence in the system. To be effective, diagnostic approach needs to consider all available information and methods including the clinical signs of disease before making firm conclusions since the aim of diagnoses is to help, not hinder the management of fish diseases (Austin, 2019).

3.2.1 Study Aim

The aim of this study was to identify the current diseases found in farmed tilapia presenting with clinical signs of disease and disease status. This was achieved by analysis of gross clinical signs of disease presented in the fish samples from the farms with laboratory-based method to determine cellular changes in the tissues and the recovery/identification of bacteria to species level.

3.3 Materials and Methods

3.3.1 Fish samples

From each grow-out farm, a minimum of 8 fish were included for biological samples: five moribund and three apparently healthy tilapia were sampled for disease diagnosis, however, in farms where disease or mortality outbreaks were absent, we sampled minimum of 8 apparently healthy tilapia. From hatcheries, three moribund and two apparently healthy broodstock tilapia were taken. If the farm location was greater than 1 hour from the diagnostic laboratory facilities then the fish were sampled on site, if the farms was located within 1 hour, the tilapia were placed in oxygenated bags with water and transported live to the laboratory where they were sampled for histopathology and bacterial recovery. On arrival at the laboratory the fish were killed with anaesthetic overdose, then weighed and examined for gross presentation of clinical signs of disease both externally and internally.

3.3.2 Bacterial Recovery

Bacteriological samples were taken aseptically from the brain, kidney, liver, and spleen of each fish sampled. The abdomen of the fish was aseptically opened, and the internal organs gently moved to one side being careful not to rupture the gut. A sterile bacteriology loop was then carefully inserted into the brain liver, kidney or spleen of each fish and inoculated onto a Tryptone Soya Agar plate (TSA, Oxoid, Basingstoke, UK) and Edward's medium (Oxoid) supplemented with colistin sulfate (5mg/ml) (Sigma). The TSA plates were for general bacterial recovery and the Edward's medium was used for the selective

recovery of Streptococcal species. These inoculated plates were sealed using Parafilm (Bemis, USA) and then incubated at 28°C for 48 h. All plates were checked for colony growth after 24 h, purified as required and then primary bacterial identification tests performed.

3.3.3 Conventional phenotypic and biochemical tests

Preliminary identification of the recovered bacteria was done using conventional phenotypic and biochemical tests. These included Gram staining, catalase, oxidase, motility, and oxidation-fermentation following the methods of Cowan and Steel (Barrow & Feltham, 2003). Presumptive identification of the bacteria based on the above tests was accomplished following Buller (2004).

3.3.4 Molecular identification of the isolates

DNA Extraction. DNA was extracted from single purified colonies following a crude boiling method with modification from Seward *et al*. (1997). A single colony from each bacterial sample was inoculated in 5 ml TSB (Oxoid) and was incubated at 28° C for 24 hours and then harvested by centrifugation at 3000 g (Sigma 4K15, Sigma Laboratory Centrifuges) for 15 minutes at 4° C. The sample supernatant was discarded, and the cell pellet resuspended in 1.0 ml Sodium-Chloride-Tris-Ethylenediaminetetraceticacid (STE) buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 100 mM EDTA) and then centrifuged for 1 minute at 13000 g (Sigma 1-14 Microfuge, Sigma, Osterode am Harz, Germany). The supernatant was again discarded prior to cell pellet being resuspended in 100 µl of Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and then boiled at

95° C for 10 minutes. After boiling, the tubes were then placed in ice and was let stand for 4 minutes. A final centrifugation for 1 min at 13, 000 g was performed and the upper aqueous phase containing the DNA was removed by pipetting. The concentration (ng/µl) and the quality (260/280 ratio) of the DNA extracts was measured by NanoDrop (Nanodrop ND1000, Thermo Fisher Scientific Inc, Wilmington, USA) spectrophotometer (Appendix 2.1). The DNA samples at a concentration of 50 ng/ µl were stored in sterile tubes in 20 µl aliquots at -20°C until required.

16S rRNA gene sequencing

The *16S rRNA* gene was PCR amplified using universal primers 20F (5'-AGAGTTTGATCATGGCTCAG-3') and 1500R (5'-CGGTTACCTTGTTACGACTT-3') (Weisburg et al., 1991) which amplifies approximately 1501 bp region of the gene. Each PCR was performed in a 25 µl reaction mixture consisting of 12.5 µl of 2X HS MyTaq mastermix (bioline UK), 1.5 µl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 µl of DNA template, and Milli-Q water to volume. The amplification profile consisted of an initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 94°C for 15s, annealing at 56°C for 20s and extension at 72°C for 1 min, with a final extension of 72°C for 2 min. The PCR products were obtained by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE buffer with ethidium bromide (0.5 µg ml^{-1}), visualized on a UV transilluminator (Appendix 2.2). The amplified PCR products were then purified using QIAquick PCR Purification Kit (Qiagen). Then purified products were sent to Eurofins for gene sequencing. Sequences were aligned with

ClustalW algorithm against phylogenetically related organisms available in GenBank in the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) in order to determine the species of bacteria with the highest homology to the queried bacterial isolate.

3.3.5 Histopathology

The brain, spleen, kidney, heart, liver and gills were removed, fixed in 10% (v/v) neutral buffered formalin for 24 hours and then transferred to 70% (v/v) ethanol before processing as described in Del-Pozo et al. (2010). The tissues were cut and processed using standard protocols at the Fish Health Section of SEAFDEC-AQD and then embedded in paraffin wax blocks which were transported to the UoS. The tissues were re-embedded into paraffin wax blocks at the IoA, University of Stirling (Leica Histoembedder, Leica Microsystems Ltd, Milton Kynes, UK) and duplicate 5 µm thick sections cut (Leica RM 2035 microtome, Leica Microsystems Ltd, Milton Kynes, UK). One section was stained with Haematoxylin and Eosin, and the other tissue section was Gram stained with crystal violet and counterstained with 1% neutral red for detection bacteria. These were conducted following routine laboratory methods (Del Pozo et al., 2010). The sections were cover slipped and then examined under light microscopy at up to 100x magnification and images captured using a digital slide scanner (ZEISS Axio Scan.Z1, ZEISS Germany).

3.4 Results

3.4.1 Fish samples

From the 16 farms included in this study, a total of 105 fish were sampled and presented as 61 apparently normal fish and 44 moribund animals (Table 3.2). Fifty-five percent (55%) of the fish weighed 50-149 g while only 4% were below 50 g. The data on farms and fish sampled together with weight range are summarised in Table 3.2.

Table 3.2 Number of farms visited, and fish sampled.

Sampling Site	No. of Farms	Average weight (g)	Total (n)	Fish Sampled Apparently Healthy (n)	Moribund (n)
Taal 1	3	95.53±53.67	19	4	15
Taal 2	2	253.79±85.78	14	5	9
Taal 3	2	126.69±60.90	13	5	8
Laguna Lake	1	137.60±16.35	5	5	0
Calauan, Laguna	4	164.67±81.78	18	6	12
Lubao, Pampanga	1	190.10±38.08	10	10	0
Batad, Iloilo	1	62.22±17.87	9	9	0
Silay,Negros Occidental	1	368.89±43.72	7	7	0
Panabo, Davao	1	161.9±12.33	10	10	0
Total	16		105	61	44

Clinical signs of disease. As expected, a higher percentage of the moribund fish displayed more external clinical compared with the apparently healthy fish (Table 3.3). However, body redness (25%) and ulcers (11%) were seen in both groups of fish (Table 3.3). Most notable clinical signs observed in the moribund fish group were abnormal/sluggish swimming behaviour (33%) and distended belly (29%). For internal clinical signs, the most prevalent were gill pallor (29%), ascites (26%) and hepatomegaly/pale liver (20%). A single onset clinical signs was not identified and instead multiple clinical signs per fish

was observed (Table 3.3) An attempt was made to collate the most observed clinical signs within the moribund sample group. This gave swimming abnormality as the highest (80%), followed by distended belly and gill pallor (59%), and ascites (57%). Gross external and internal abnormalities in the sampled fish are summarised in Table 3.3 and shown in Figure 3.2-3.6. Figure 3.1 shows an apparently farmed Nile tilapia.

Table 3.3 External and internal clinical signs of the moribund Nile tilapia samples.

	Description	**Frequency in moribund samples (n = 44)**	**Percentage (%)**
External	Abnormal/sluggish swimming	35	80
	Distended belly	26	59
	Body redness/petechiae	10	23
	Corneal opacity	13	27
	Ulcers/lesions	6	14
	Discoloration	5	11
	Exophthalmia	5	11
	Swollen anus	2	5
	Fin rot	4	9
Internal	Gill pallor	26	59
	Ascites	25	57
	Hepatomegaly/pale liver	13	30
	Splenomegaly	11	25
	Haemoperitoneum	6	20
	Enlarged kidney	6	18
	Intracranial oedema	4	9
	Enlarged and deep red heart	2	5

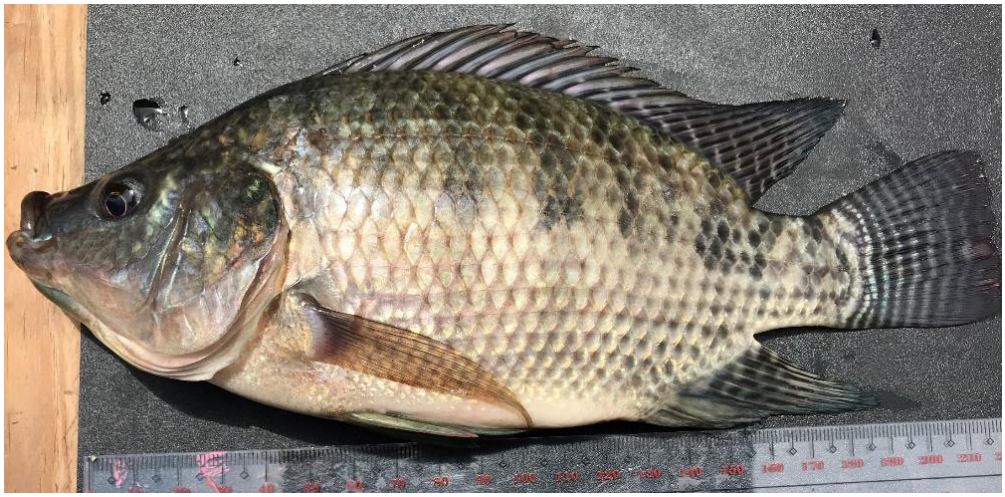

Figure 3.1 An apparently normal farmed Nile tilapia with no gross clinical signs apparent.

Figure 3.2 Figure 3.2. A moribund farmed tilapia, note the presence of body redness (thin arrows) and haemorrhage of the eye (thick arrow).

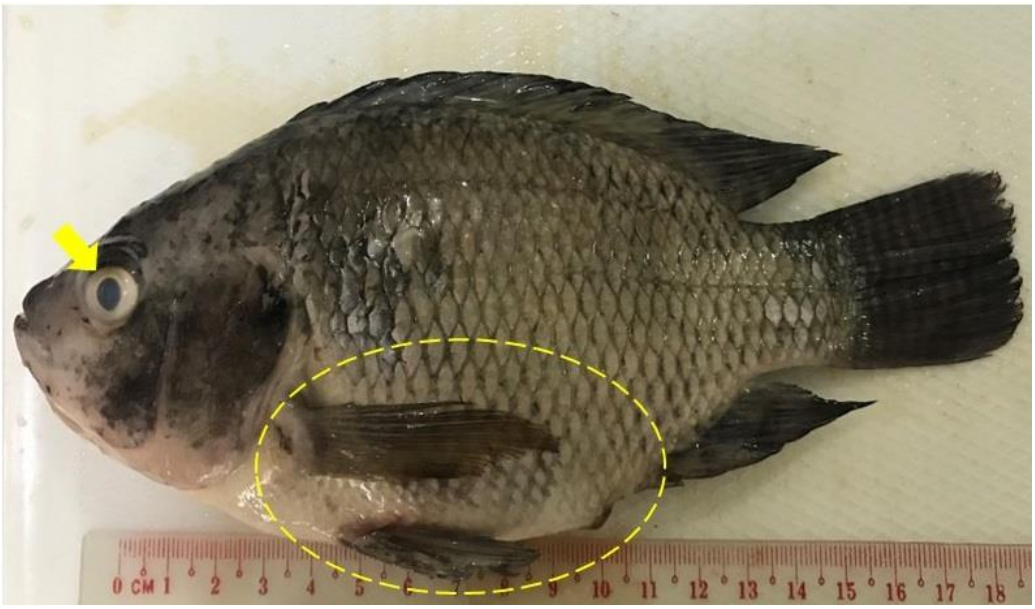

Figure 3.3 Nile tilapia showing distended belly (yellow dashed circle) and cloudy eye (arrow).

Figure 3.4 (a) Bilateral exophthalmia with corneal opacity (thick arrow) and (b) anal swelling (thin arrow).

Figure 3.5 (a) Corneal opacity (arrow) and (b) enlarged kidney (yellow dashed circle).

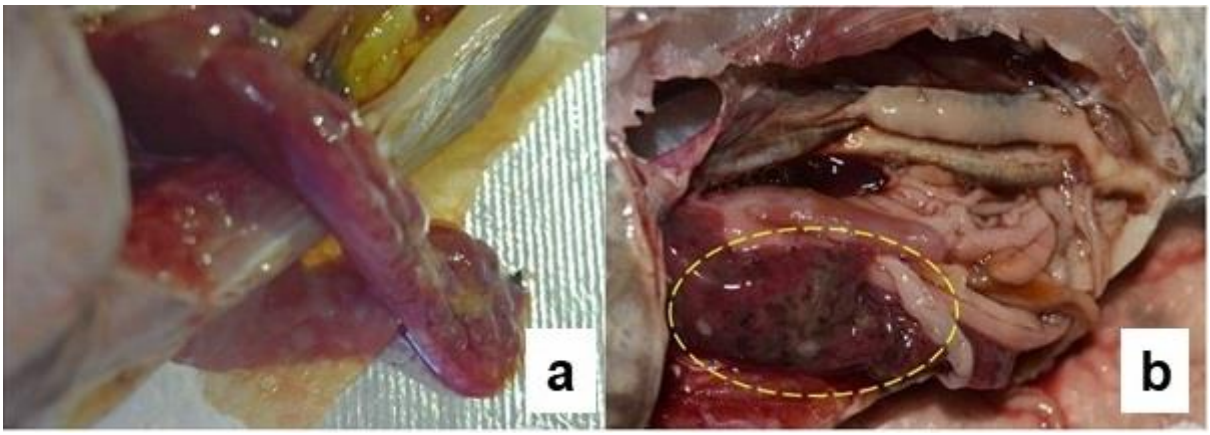

Figure 3.6 (a) Yellowish ascitic fluid and (b) liver lesion (yellow dashed circle).

3.4.2 Bacterial recovery and identification

A total of 102 bacterial strains were recovered from the fish sampled in this study. This was separated into 55 Gram-positive cocci isolates, 45 isolates of Gram-negative rods and 2 Gram-positive rods. Figure 3.7A and B show

typical Gram stain presentation of the Gram-positive and Gram-negative bacteria, respectively. Presumptive identification of the isolates showed that 50% (n=51) of the isolates belongs to the genus *Streptococci* and *Lactococci* while the rest were identified as belonging to *Aeromonas* and *Vibrio* (n=24), *Enterobacteriaceae* (n=21), *Staphylococci* (4), and *Bacillus* and *Lactobacillus* (n=2). The identification profiles and presumptive bacterial identification results are presented in Table 3.4.

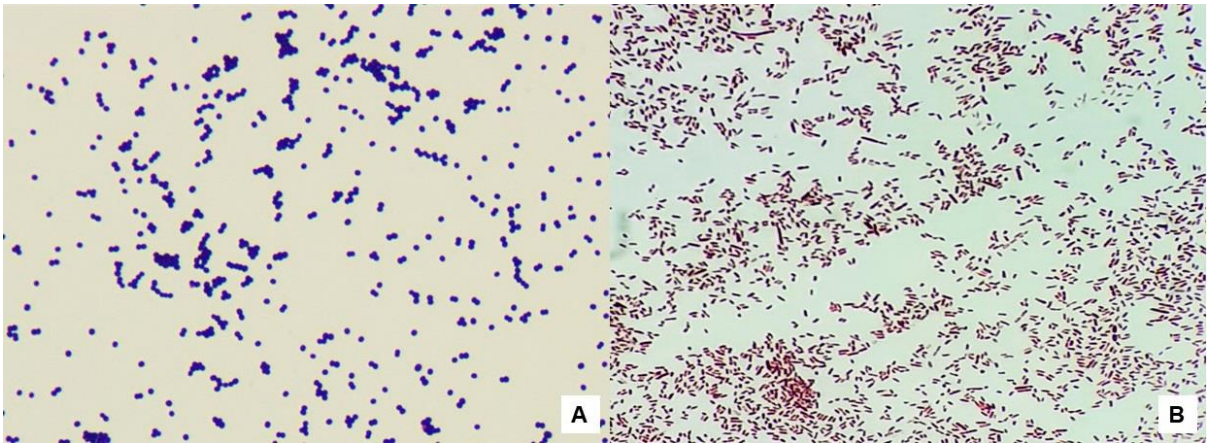

Figure 3.7 Gram-stain showing Gram-positive cocci (A) and Gram-negative rods (B).

Table 3.4 Preliminary identification based on the phenotypic and biochemical tests.

Isolate	Gram reaction	Motility	Catalase	Oxidase	OF test	Preliminary Identification	
	+	-	-	-	+	*Streptococci, Lactococci*	51
cocci	+	-	+	-	+	*Staphylococci*	4
	+	+	+	+	+	*Bacillus, Lactobacillus*	2
bacilli	-	+	+	+	+	*Aeromonas, Vibrio*	24
	-	+	+	-	+	*Enterobacteriaceae*	21

Molecular identification using 16S rRNA gene sequencing. From the *16S rRNA* sequence results, 30 of the Gram-positive cocci were identified as *Streptococcus agalactiae* and 8 were confirmed as *S. iniae*. The remaining 19 Gram positive species were identified as *Bacillus aryabhatthai* (n

=1), *Bacillus kochii* (n = 1), *Enterococcus faecium* (n = 1), *Enterococcus gallinarum* (n = 1), *Enterococcus hirae* (n = 2), *Lactococcus garvieae* (n = 4), *Lactococcus lactis* (n = 2), *Lactococcus taiwanensis* (n =1), *Staphylococcus aureus* (n = 4) and *Streptococcus lutetiensis* (n = 2). For Gram negative bacteria, 14 isolates were identified as *Enterobacter cloacae*, 13 as *Aeromonas veronii*, 7 were *Klebsiella pneumonia,* and 3 each for *Vibrio cholera* and *Vibrio harveyi*, respectively. The remaining 4 Gram negative species were identified as *Vibrio alginolyticus* (n = 2), *Aeromonas caviae* (n = 1), and *Aeromonas dhakensis* (n = 1). The number of bacterial species isolated from tilapia samples is shown in Figure 3.8.

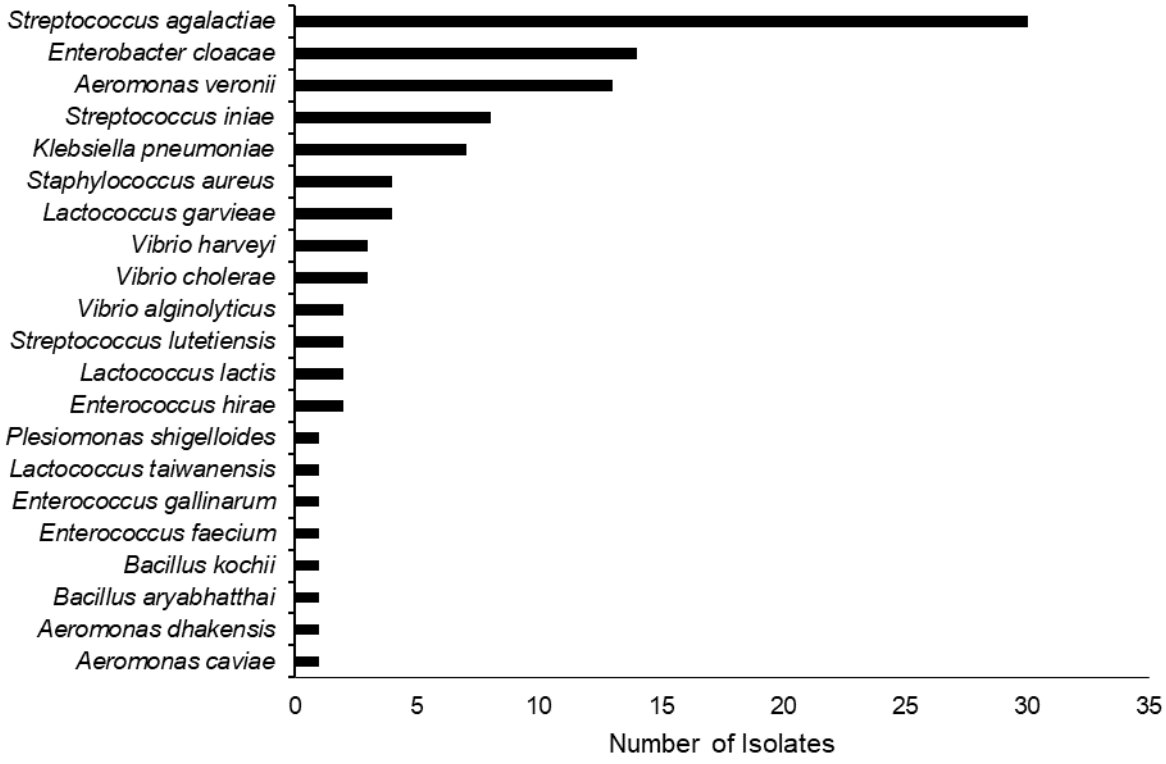

Figure 3.8 Number of bacterial isolates recovered from 16 tilapia farms throughout the Philippines.

The data in Table 3.5 collates the bacterial recovery, identification, histopathology results, and clinical status. This was produced to provide an understanding of the role of the bacterial species in the disease outcome. Table 3.5 shows the recovery of the different fish pathogenic bacteria from 38 Nile tilapia samples showing different clinical signs. Two isolates of *A. veronii* were recovered from two apparently healthy tilapia samples from Laguna Lake. Twelve *S. agalactiae* isolates were recovered from the brain of the fish which accounted for 40% of the total *S. agalactiae* isolates while the rest of the 18 isolates were either from kidney or spleen. In terms of pathology, 49% of the fish showed bacterial septicaemia while the rest have inflamamation in the internal organs including the kidneys, spleen, and liver.

Table 3.5 Summary for the recovered bacterial isolates in relation to location, farm description, clinical signs, and organ the bacteria was recovered, bacterial ID and pathology.

Farm No.	Location	Farm Description	Fish No.*	Clinical Signs	Organ Bacteria Recovered*	Bacteria	Pathology
1	Taal Lake Site 1	Freshwater grow-out floating cage 1	2-M	gill pallor, ascites	K	*A. veronii*	Bacterial septicaemia
			4-M	Gill pallor, ascites	K	*S. iniae*	Bacterial septicaemia
			5-M	One eye absent, ascites	K, S	*S. iniae*	Bacterial septicaemia
		Freshwater grow-out floating cage 2	1-M	Redness, ascites, discoloration, hepatosplenomegaly	K, S & B	*S. agalactiae* Ib	Bacterial septicaemia
			3-M	Gill pallor, hepatosplenomegaly	K, S & B	*S. agalactiae* Ia	Bacterial septicaemia
			4-M	Ascites, hepatosplenomegaly	K, S & B	*S. agalactiae* Ia	Bacterial septicaemia
			5-M	Gill pallor, eyes missing, hepatosplenomegaly	K, S & B	*S. agalactiae* Ia	Bacterial septicaemia
		Freshwater grow-out floating cage 3	1-M	Bilateral exophthalmia, ascites hepatosplenomegaly	K & B	*S. agalactiae* Ia	Nephritis, splenitis, hepatitis
			2-M	Loose scales, discoloration, gill pallor	B	*S. agalactiae* Ia	Nephritis, splenitis, hepatitis
			3-M	Loose scales, gill pallor, ascites	S	*S. agalactiae* Ia	Nephritis, splenitis, hepatitis
			4-M	Corneal opacity, fin rot, ascites	K & B	*S. agalactiae* Ia	Nephritis, splenitis, hepatitis
			5-M	Gill pallor, corneal opacity	S & B	*S. agalactiae* Ia	Nephritis, splenitis, hepatitis
2	Taal Lake Site 2	Freshwater grow-out floating cage 1	2-M	Gill pallor, ascites	K S	*S. iniae* *A. veronii*	Bacterial septicaemia
			3-M	Gill pallor, ascites	K	*S. iniae*	
			6-M	gill pallor, pale and discoloured liver,	S	*A. veronii*	Nephritis, splenitis, hepatitis

				ascites			
		Freshwater grow-out floating cage 2	1-M	Corneal opacity, ascites, hepatosplenomegaly	B	*S. agalactiae* Ib	Bacterial septicaemia
			3-M	Ascites, hepatosplenomegaly	K & S	*S. agalactiae* Ia	Bacterial septicaemia
			4-M	Ascites, redness, hepatosplenomegaly, corneal opacity, fin rot	S B	*S. agalactiae* Ia *S. agalactiae* Ib	Bacterial septicaemia
3	Taal Lake Site 3	Freshwater grow-out floating cage 1	1-M	Discoloration, corneal opacity, ascites	K	*S. iniae*	Bacterial septicaemia
			3-M	Discoloration, corneal opacity, ascites	S S	*A. veronii* *S. iniae*	Bacterial septicaemia
			5-M	Gill pallor, ascites	K	*A. veronii*	Bacterial septicaemia
			8-M	Discoloration, gill pallor, ascites, mild exophthalmos	K	*A. caviae*	Nephritis, splenitis, hepatitis
		Freshwater grow-out floating cage 2	1-M	Anus swollen, petechiae, ascites, gill pallor, ascites	K	*S. iniae*	Bacterial septicaemia
			2-M	Corneal opacity, ascites, gill pallor, reddened heart	S K B	*L. lactis* *L. lactis,* *S. lutetiensis* *S. lutetiensis*	Nephritis, splenitis, hepatitis
			3-M	Gill pallor, redness, ascites, hepatosplenomegaly	K & B	*S. agalactiae* Ia	Bacterial septicaemia
			4-M	Anus swollen, ascites, hepatosplenomegaly	K	*S. agalactiae* Ia	Bacterial septicaemia
			5-M	Anus swollen, ascites, hepatosplenomegaly	S	*S. agalactiae* Ia	Bacterial septicaemia
4	Calauan, Laguna	Freshwater Earthen Pond Hatchery 1	2-M	Redness operculum and body	K	*E. faecium*	Nephritis, splenitis, hepatitis
			3-M	Redness operculum and body	B	*L. garvieae*	Nephritis, splenitis, hepatitis
			4-M	Redness body	K S	*L. garvieae,* *E. hirae* *L. garvieae,* *E. hirae*	Nephritis, splenitis, hepatitis
		Freshwater Earthen Pond Hatchery 3	2-M	Redness of body, petechiae operculum and head	S & B	*S. agalactiae* Ia	Bacterial septicaemia
			3-M	Redness of operculum and body	B	*L. garvieae*	Nephritis, splenitis, hepatitis
			4M	Redness of operculum	B	*L. taiwanenesis,* *E. gallinarum*	Nephritis, splenitis, hepatitis
		Freshwater Earthen Pond Hatchery 4	3-M	gill pallor, redness of body, ascites	K	*A. veronii*	Nephritis, splenitis, hepatitis
			6-M	gill pallor, cloudy eyes, ascites	K S	*A. veronii* *A. veronii*	Nephritis, splenitis, hepatitis
5	Laguna Lake	Freshwater grow-out submerged cage	3-AH	Apparently healthy	S	*A, veronii*	Nephritis, splenitis, hepatitis
			5-AH	Apparently healthy	S	*A. veronii*	Nephritis, splenitis, hepatitis
16	Panabo, Davao	Seawater floating cage	5-M	Red marks in the body, damaged fins ascites	K	*V. harveyi*	Liver necrosis
			6-M	Red marks in the body, damaged fins ascites	K S L	*V. alginolyticus* *V. harveyi* *V. alginolyticus*	Nephritis, splenitis, hepatitis

* M = moribund; AH = apparently healthy

3.4.3 Histopathology

Histopathological examination revealed serous degeneration, necrosis and inflammatory response in multiple organs, which were particularly prominent in the liver, heart, kidney and brain of the fish showing clinical signs.

Liver. Severe vacuolar degeneration, congestion, haemorrhage, and foci necrosis with infiltration of inflammatory cells were observed in the liver (Figure 3.9). Vacuolation with mild hepatitis was observed in tilapia samples from seawater cages, while most of the diseased fish had extensive vacuolar necrotic degeneration of the hepatocytes. All diseased fish showed various level of hepatitis with 14 (25%) exhibiting lytic necrosis, where the normal hepatic architecture was lost, and presence of numerous inflammatory cells were present.

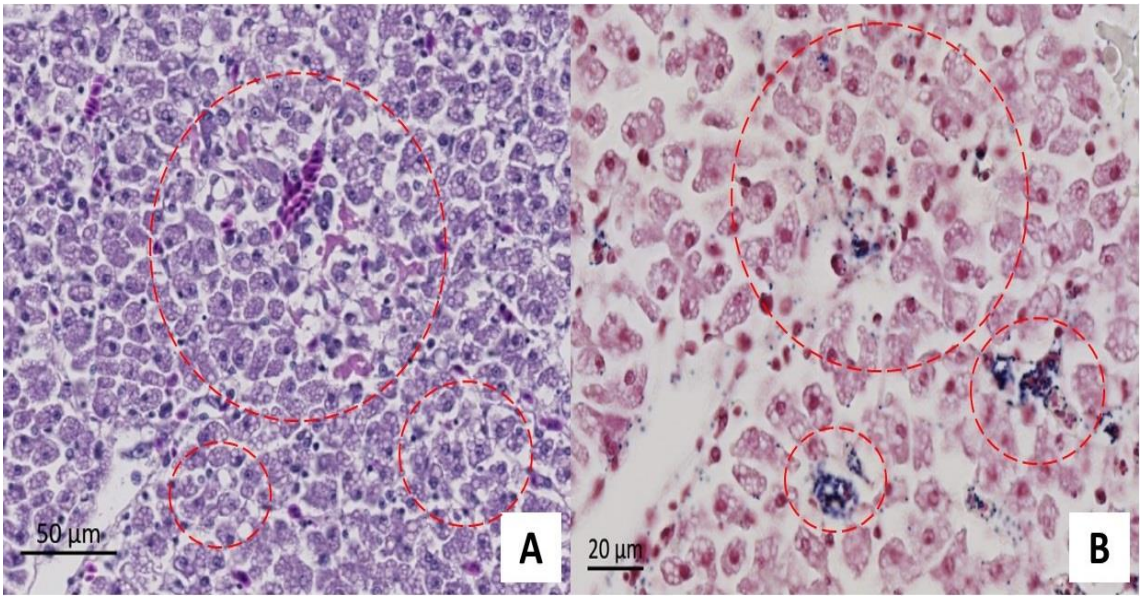

Figure 3.9 Liver showing foci necrosis (dashed circle), infiltration of inflammatory cells, and vacuolation of hepatocytes in H&E stained section (A). Gram stained of the same section (B) showing Gram-positive cocci in the same areas of foci necrosis.

Heart. Pericarditis was present in the heart; the epicardium was thickened by the infiltration of inflammatory cells and oedema (Figure 3.10). Microscopic observation of the heart revealed histological abnormalities in 34% of the diseased samples. Myocardial necrosis with infiltration of inflammatory cells (Figure 3.11) was most notable. Gram-stained of the endocardium showed bacterial and inflammatory cells (Figure 3.12).

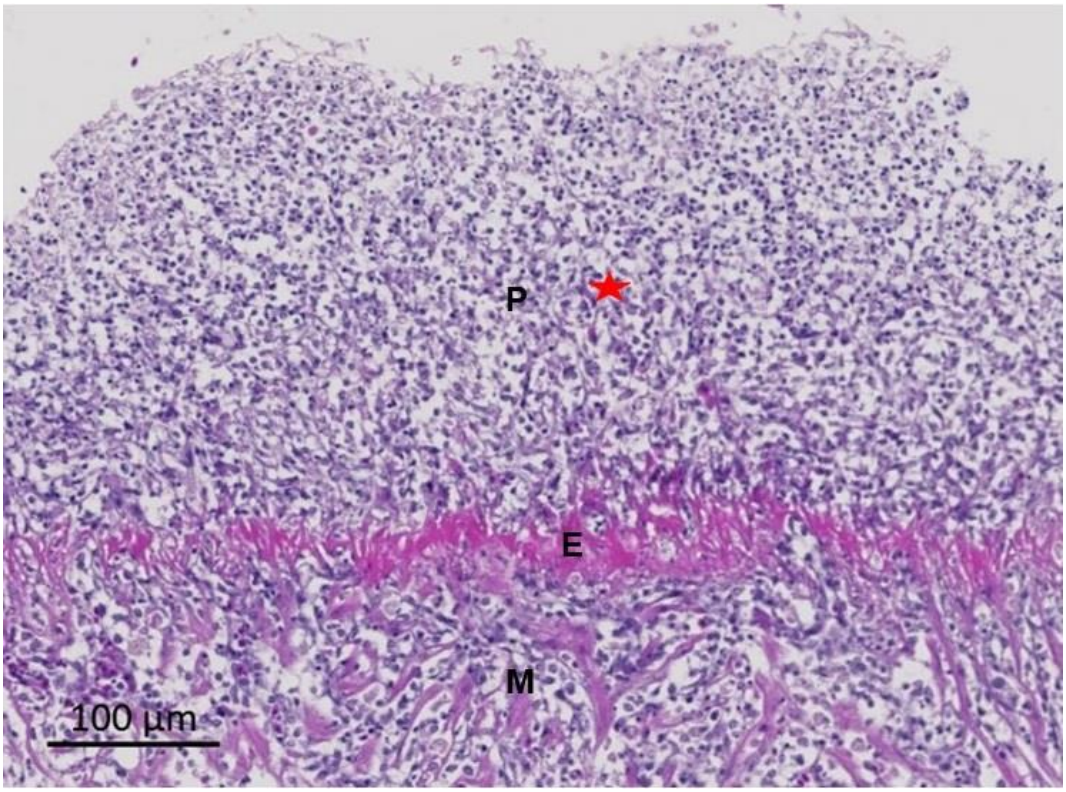

Figure 3.10 An H&E stained section of the heart showing massive pericarditis (star, P), marked thickening of the epicardium (E) and infiltration of inflammatory cells in the myocardium (M).

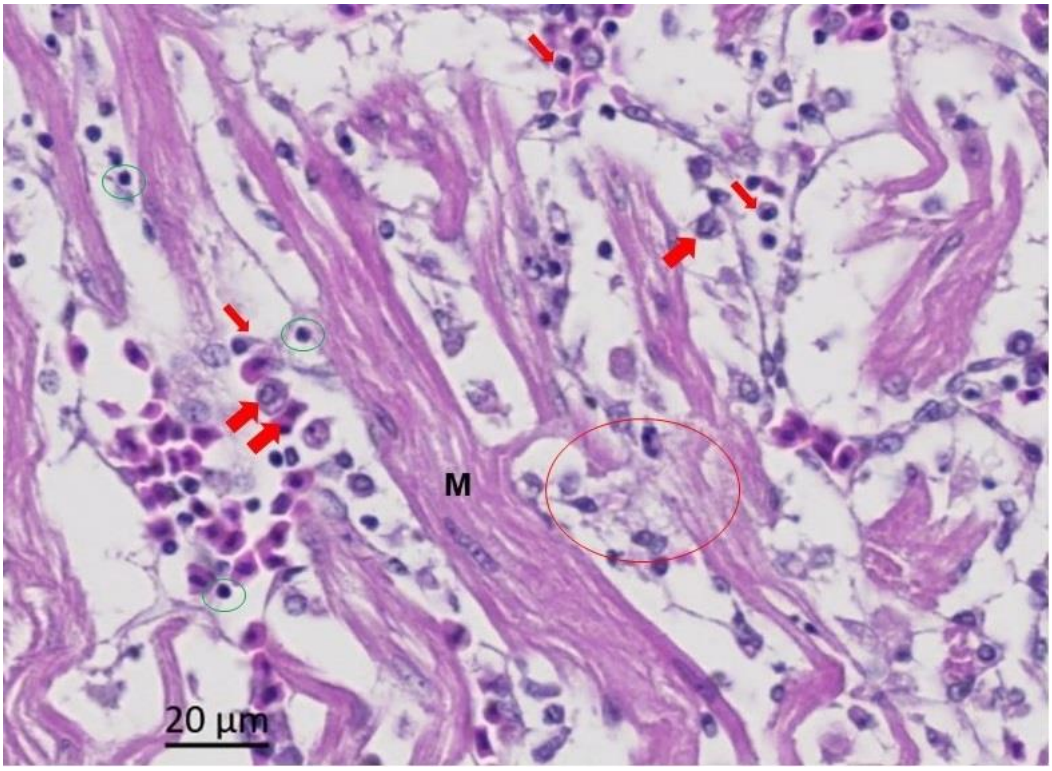

Figure 3.11 An H&E stained section of the myocardium (M) showing necrosis (red circle) with infiltration of lymphocytes (green circles) and macrophages (small & big arrows).

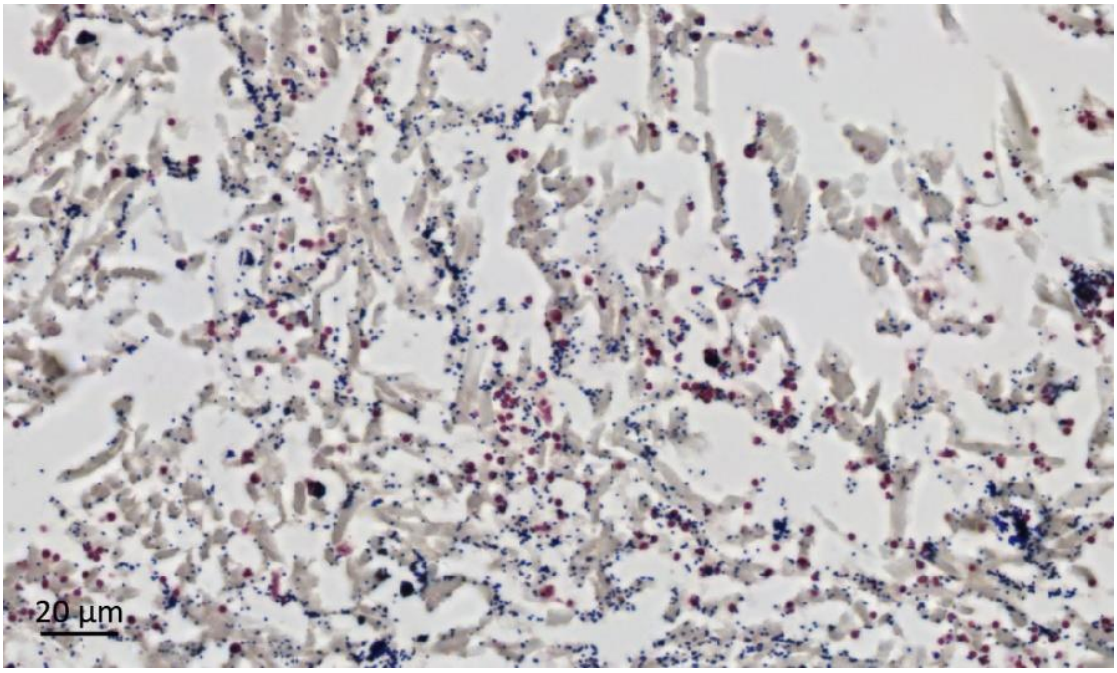

Figure 3.12 Gram stained section of the myocardium showing with numerous Gram-positive cocci bacteria.

Kidney. The kidney showed marked necrosis in haemopoietic tissue and renal tubule showing cells that were pyknotic and karyorrhectic, and with macrophages and lymphocyte infiltration (Figure 3.13). Diffuse interstitial nephritis with diffuse coagulative necrosis of tubular cells and infiltration of inflammatory cells were observed which altered greatly the renal tissue architecture. Most of the tubular epithelium underwent hyaline degeneration and detachment from the basement membrane. The epithelial tissue of the renal tubules and the basement membrane were destroyed by numerous Gram-positive cocci bacteria (Figure 3.14). Aside from tubular damage, renal sections of the diseased fish presented severely damaged glomeruli where they become with infiltration of inflammatory cells and dilatation of the Bowman's capsule.

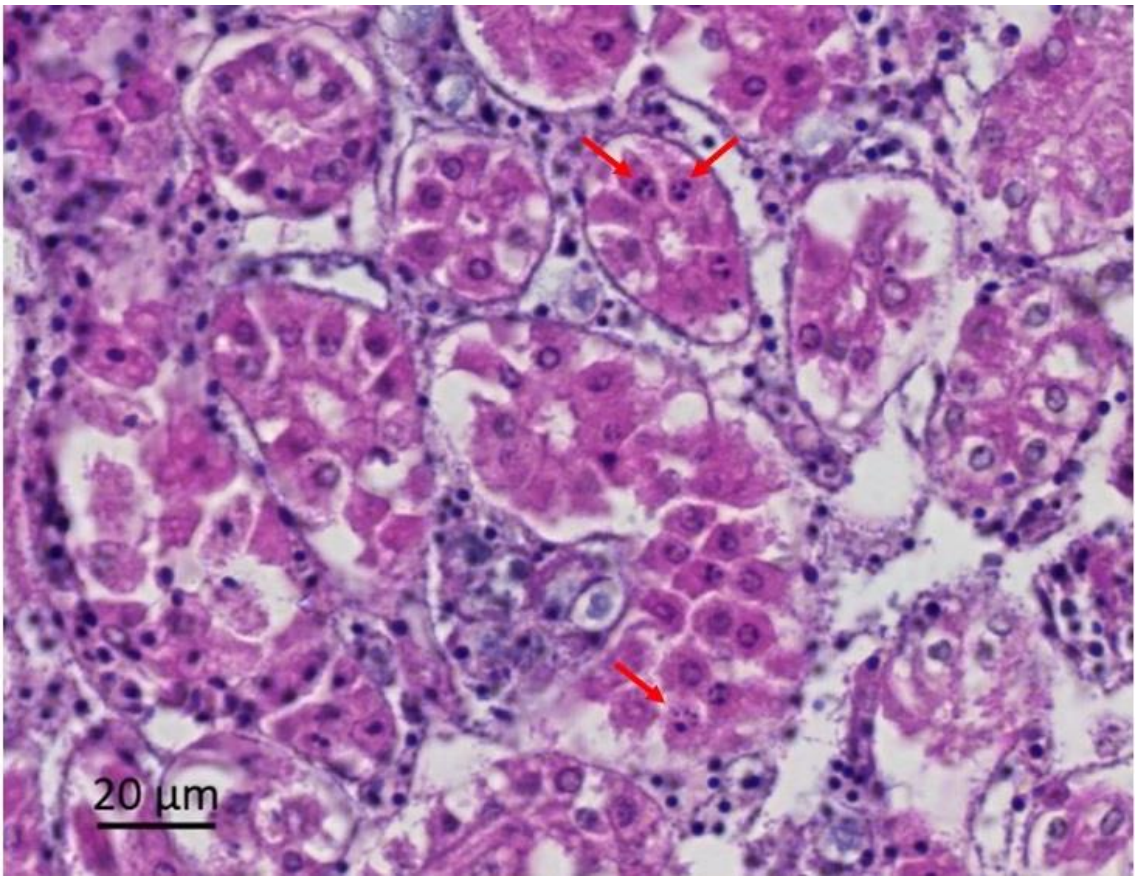

Figure 3.13 An H&E section of the kidney showing necrosis and degeneration of the renal tubules characterised by karyorrhectic cells (arrows).

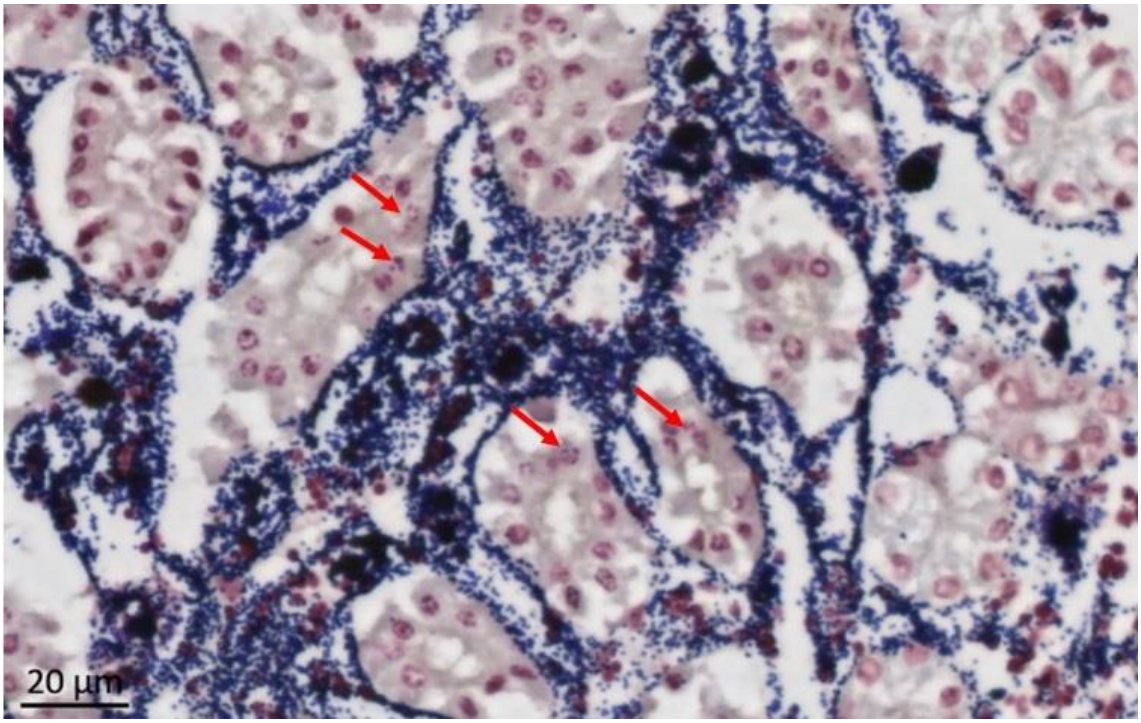

Figure 3.14 A Gram-stained section of the kidney presenting acute tubular necrosis with karyorrhectic cells (small arrows) and massive infiltration of Gram-positive cocci bacteria.

Spleen. The splenic sections of the diseased fish revealed splenomegaly (75%) with most showing acute haemorrhagic and necrotic splenitis. The splenic haematopoietic tissue was necrotic with numerous bacteria, inflammatory cells and melanomacrophages (Figure 3.15). Increased number of melanomacrophage centres and scattered brown pigment granules were seen in most of the samples.

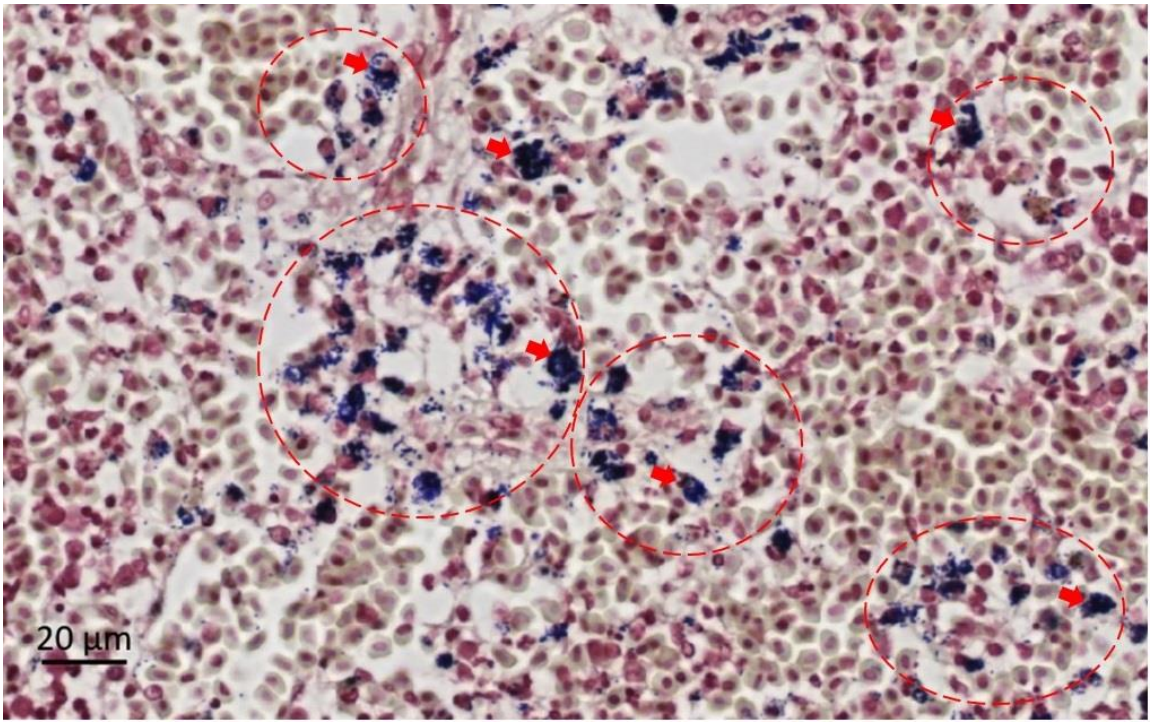

Figure 3.15 A Gram-stained section of the spleen showing foci of necrosis (dashed circle) and macrophages containing bacteria (arrow).

Brain. Changes in the brain included meningitis, necrosis of neuron, oedema and infiltration of inflammatory cells in parenchyma. Meningitis and or encephalitis was observed in 30% of the diseased fish. The meninges were massively thickened (Figure 3.16) and Gram-stained section demonstrated numerous Gram-positive bacteria in the thickened meninges where most of them were also found within macrophages (Figure 3.17). A few samples have mild meningitis, however, there were marked diffuse necrotic encephalitis (Figure 3.18) in the brain where bacteria-filled macrophages were found within the brain cortex (Figure 3.19). It was clearly shown in this study that necrotic neurons or Purkinje cells of the meninx primitive and brain parenchyma, especially in the midbrain and hindbrain, were damaged by numerous Gram-positive bacteria.

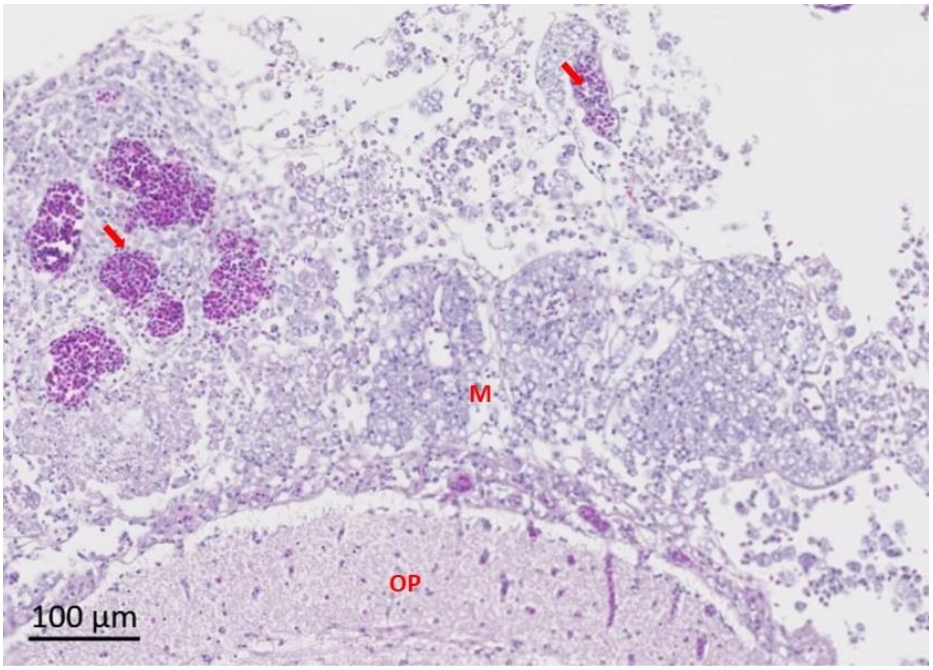

Figure 3.16 Massive cellular meningitis (M) with areas of haemorrhage (arrow).

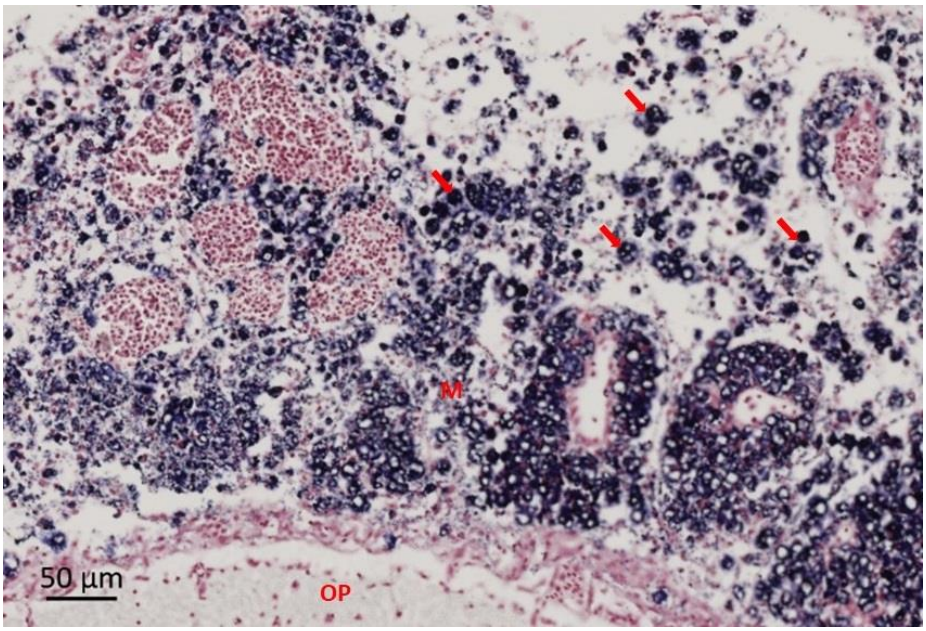

Figure 3.17 Gram-stained section of Figure 3.19 showing numerous bacteria-filled macrophages (small arrows) in the massive cellular meningitis.

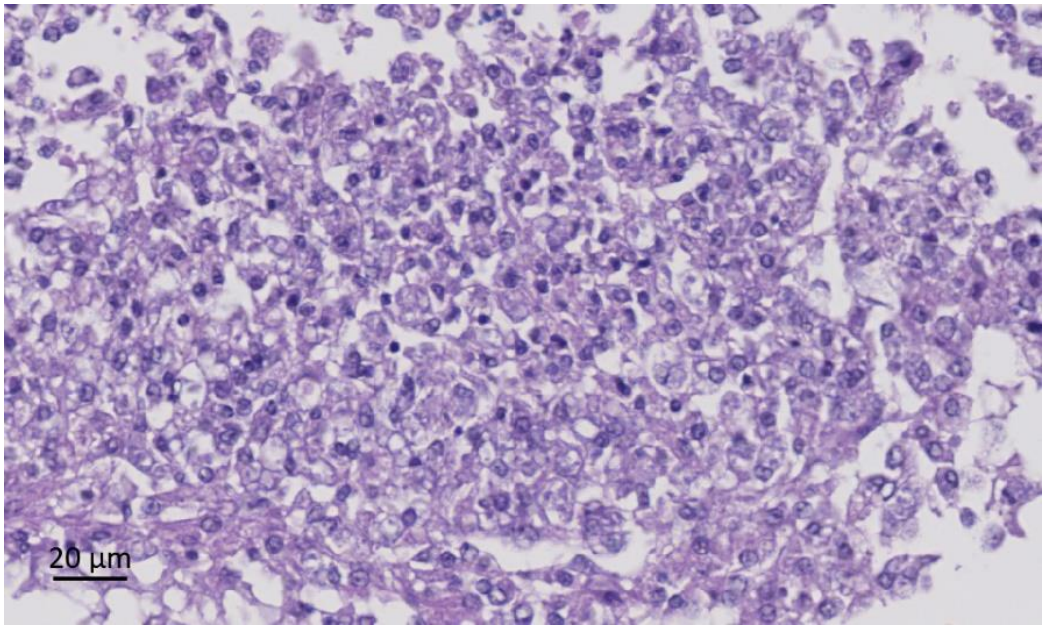

Figure 3.18. Diffuse necrotic encephalitis with infiltration of inflammatory cells in the brain cortex.

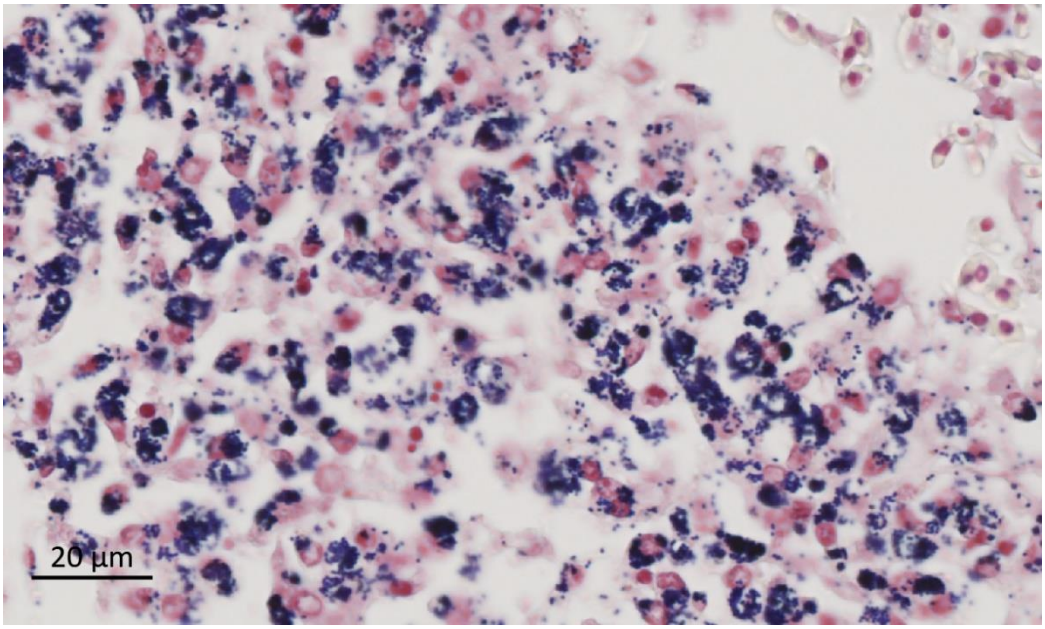

Figure 3.19 A Gram-stained section of the brain in Figure 3.18 showing diffuse necrosis with numerous macrophages-filled with Gram-positive cocci bacteria.

Chains and small clusters of coccus bacteria were found in the liver, heart, kidney, and brain. The main clinical and pathological features were similar to those previously described fish which were infected by

streptococcosis. Additionally, myxosporean parasites were present in the muscles of the gill arches of some diseased fish (Figure 3.20). Gram-negative rods were also seen in in the spleen, kidney, and liver of two diseased fish (Figure 3.21).

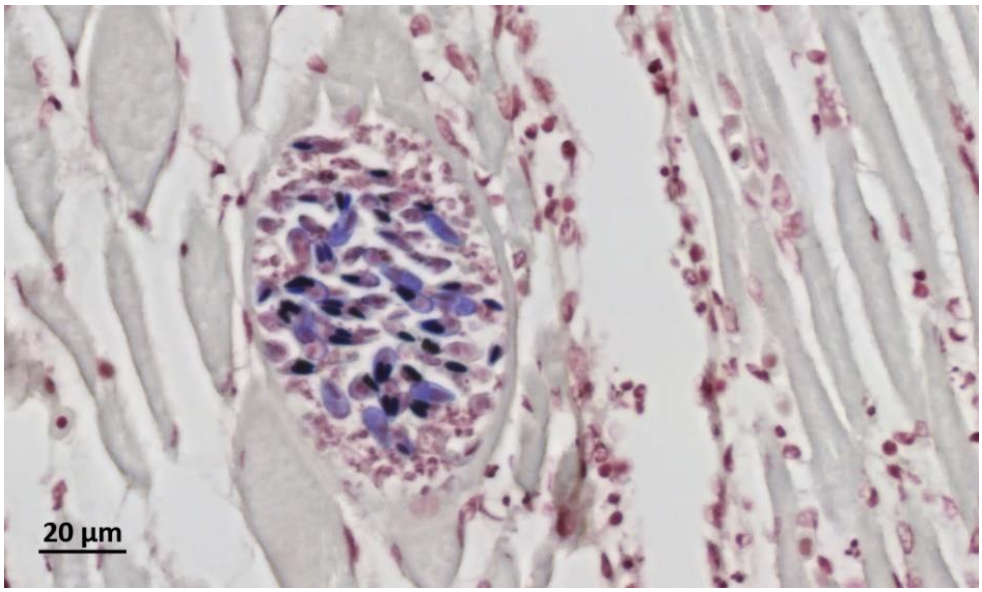

Figure 3.20 Myxosporean parasite (stained purple) encysting in the muscle of the gill arch (Gram stain).

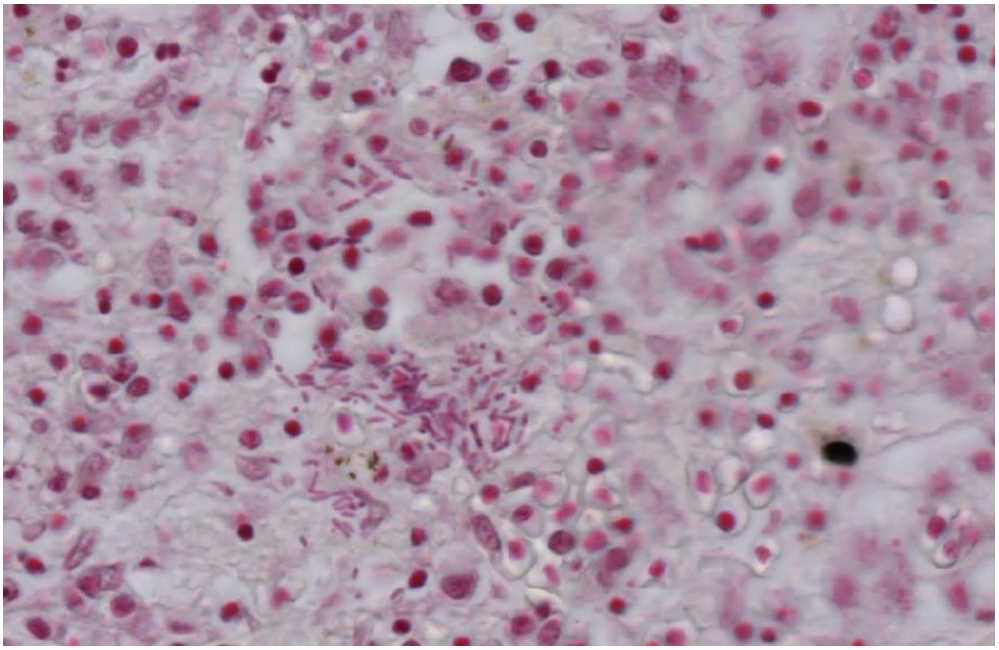

Figure 3.21 Gram-negative rods in the spleen of the diseased tilapia.

3.5 Discussion

Understanding the cause of mortalities and morbidities on tilapia farms is important to support the continued development of this sector. Previously little if any clinical evidence was available on the cause of fish mortalities within the range of production systems in the Philippines. Globally, the intensification of tilapia aquaculture has led to the emergence of diseases which have adversely affected the sector. Although the Philippine fisheries agency already reported that various bacterial, parasitic, and viral disease scourge the tilapia farms (Romana-Eguia et al., 2020), the clinical data was fragmented or not current. The results of this study firmly established the infectious nature of the reported fish losses and identified the bacterial diseases and aetiological agents for fish farmers.

As the purpose of this study was to determine the current disease status in the tilapia farms, a diagnostic approach was adopted. By using such methods this secured the most robust evidence and provided a foundation, from which to build and share, the biological material with others in the future. From the data presented in Chapter 2, farmers reported that diseases affected different life stages in the culture period with highest mortality after 1 month of stocking and progressive presentation of clinical signs up to harvest. Certainly, the biological samples taken and analysed in this chapter would support the farmers' data. It was not expected that a single bacterial species would be recovered, or a single disease would occur at the time of sampling.

Clinical signs observed in the moribund fish farmed in the Philippines were similar to those observed in previous studies in Nile tilapia (Jantrakajorn et al., 2014; Dong et al., 2017; Assis et al., 2016). Moreover, same clinical signs were presented when Nile tilapia were experimentally infected with infectious agents like *Streptococcus agalactiae* (Chen et al., 2007; Pereira et al., 2010; He et al., 2021), *Streptococcus iniae* (Chen et al., 2007; Rahmatullah et al., 2017; Baums et al., 2013), *Aeromonas hydrophila* (Amphan et al., 2019), *Edwardsiella ictaluri* (Soto et al., 2013), *Francisella noatunensis* (Fernandez-Alarcon et al., 2019), and Tilapia Lake virus (TiLV) (Tattiyapong et al., 2017). None of the clinical signs observed in this study were pathognomonic for any specific etiologic agent and instead a diverse range of signs were reported.

None of the clinical signs reported from this study were unique to the fish or the disease conditions. All of these have been reported elsewhere and whilst a complete comparative study was not performed, it is clear that the gross clinical presentation for the Philippine study is similar to that reported for tilapia elsewhere (Jantrakajorn et al., 2014; Dong et al., 2017; Assis et al., 2016). Whilst farmers are able to observe abnormalities in their stocks the relevance of these may not always be apparent. For example, in this study, diseased Nile tilapia showed abnormal swimming behaviour like spiralling or sluggish movement observed surfacing at the water with increased ventilation, which may indicate respiratory problems. Yet the dissolve oxygen level at these sites was acceptable limit (3-5 mg/L). Li et al. (2018) reported that tilapia are able to tolerate low DO levels and so if the perception is that the fish are robust, such behavioural changes in the fish may simply be accepted by the farmer rather that there may be a health issue. In this study, the fish may have reduced

oxygen transportation from haemolysis and anaemia, which was severe enough to induce the observed clinical behaviour and most notably gill pallor in most of the samples. This observation of gill pallor is similar to the findings of various studies where tilapia was infected by megalocytic virus, *F. noatunensis* and *S. iniae* (Subramaniam et al., 2016; Leal et al., 2014; Lahav et al., 2004).

It is critical that farmers understand what is apparently normal and abnormal in their stocks and have the confidence to act rapidly. This will lead to improved health intervention and better welfare of their stocks. However, not all clinical signs noted in this study would be simply from an infection, some may be due to poor water quality or environmental factors. It will be difficult to determine at this point if the type of farms and background environments has an effect to the disease or pathology. Current data from the study will not be able to support this. Therefore, it is important to continue to take farm level data and biological samples and explore the cause of unexpected mortality or morbidity event. However, the first stage in this process is a clear and robust framework from which farmers can recognise abnormal behaviour.

It is clear that further work is required to support the disease diagnosis from gross clinical signs by bacterial recovery, culture, and identification. Bacteria were isolated using a general-purpose medium (i.e., TSA) in order to recover any culturable bacterial pathogens. A streptococci selective Edwards's medium was used together with TSA since the tilapia farms were suspecting streptococcosis as the primary disease in the stocks. Bacterial recovery was performed aseptically from the kidney, and spleen as these organs are where bacteria are usually found during infection (Austin, 2019). Additionally, swabs from the brain were taken as streptococci have a particular tropism for brain

tissue (Russo et al., 2006). In the isolation of streptococci, it was a challenge to discriminately recover *S. iniae* and *S. agalactiae* together in one sample since they are both morphologically identical in TSA and Edward's medium. This problem could have been addressed by using chromogenic agar that differentially show *S. iniae* and *S. agalactiae* in the single agar plate. The advantage of using this chromogenic agar is that co-infection by the two streptococci can be easily determined as their colonies can be isolated and cultured separately. However, because it is expensive it is impractical to use in surveillance studies or routine diagnostics in fish health laboratories. An alternative method for rapid detection of the streptococci or aeromonas pathogens can be done by species-specific PCR using extracted genomic DNA from fish tissue (Rodkum et al., 2012; Cui et al., 2019; Persson et al., 2015). However, the value of this species-specific PCR is mere detection but not for phylogenetic analysis which is important for epidemiological study.

A combination of laboratory-based identification methods were applied in this study, to provide bacterial identification from the strains recovered during the sampling at species level. Initial morphological and enzymatic identification was only able to presumptively identify the recovered bacteria at the genus level. In order to determine the species of the isolates, *16S rRNA* gene sequencing was done. There was good agreement between the traditional and *16S rRNA* sequencing where the most abundant strains recovered from diseased tilapia were *Streptococcus agalactiae*, *Aeromonas veronii* and *Streptococcus iniae*. However, studies has shown that the limitation of *16S rRNA* gene sequencing in speciation of phylogenetically tight *Aeromonas* group (Persson et al., 2015), and also this method cannot differentiate the different

serotypes of *S. agalactiae*. Thus, there is a need for other identification tests. The recovery and identification of *S. agalactiae* and *S. iniae* coupled with clinical signs and histopathology strongly established the involvement of bacteria in the disease/mortality outbreaks investigated. Although it is believed that streptococci caused bacterial disease in Nile tilapia in Taal Lake, this is the first study to recover and identify them from diseased tilapia in the Philippines. Moreover, the recovery and identification of *A. veronii* strains as an emerging motile aeromonad affecting farmed freshwater fishes (Dong et al., 2017) adds to the body of knowledge in the aetiology of motile aeromonad septicaemia especially in farmed Nile tilapia. Further description and characterisation of *S. iniae* and *S. agalactiae* are in Chapter 4 while for motile *Aeromonads* are in Chapter 5.

The recovery of *L. garvieae* from diseased Nile tilapia broodstocks in Calauan, Laguna is the first in the Philippines and this should be a concern since it causes lactococcosis in tilapia (Evans et al., 2009; Vendrell et al., 2006) with clinical signs of the diseased fish similar to streptococcosis (Abu-Elala et al., 2020). *L. garvieae* infected fish suffers from acute haemorrhagic septicaemia with exhibiting similar lesions to *S. agalactiae*-infected fish with high and rapid mortality (Abu-Elala et al., 2020; Vendrell et al., 2006). Moreover, this bacterium is a public health concern due to humans suffering from gastrointestinal infection after eating raw fish (Wang et al., 2007) which synchronised with the aquaculture outbreak of *L. garvieae* (Didinen et al., 2014). Additionally, strains of *L. lactis* which is classified as lactic acid bacteria (LAB) were isolated and they may be leachate from the gastrointestinal tract during dissection. These bacteria are commensal in the gut of the fish, and they

can produce bacteriocin which are antimicrobial proteins which can inhibit similar and/or competitive bacteria species (Kaktcham et al., 2019; Feng et al., 2019). Another LAB identified as *L. taiwanensis* was isolated, and this may be also a commensal in the gut since this bacterium was initially isolated from fermented cummingcordia plant (*Cordia dichotoma*) (Chen et al., 2013). Tilapia, which is a detritivorous fish, harbours gut bacteria that ferments carbohydrates into short-chain fatty acids (Kihara et al., 1997).

Although *Streptococcus* species is recognised as the major causative agent of streptococcosis, *Enterococcus* are also isolated from the diseased fish (Osman et al., 2017). In this study, *E. hirae*, *E. gallinarum* and *E. faecium* were recovered from diseased tilapia showing histological abnormalities such as inflammation and necrosis indicative of bacterial septicaemia. Previous studies have shown that *E. faecalis* as causative agent of streptococcosis in tilapia (El-Sayed & El-Gheit, 2005; Osman et al., 2017), however, a recent study have also shown the involvement of *E. faecium* and *E. hirae* in streptococcosis in tilapia (Akter et al., 2021). Moreover, *E. gallinarum* was also found to be associated with farmed tilapia suffering from septicaemia in Egypt (Osman et al., 2017). *Enterococcus* species in water are the indicator of faecal contamination by the enteric and opportunistic pathogen (Boehm & Sassoubre, 2014; Wen et al., 2020). This is not surprising since data from Chapter 2 (pages) showed that most tilapia farms in the Philippines were close to human communities and the association of these enterococci in streptococcosis of tilapia is alarming for public health and aquaculture in the Philippines. However, the role of pollution, water quality, and *Enterococcus* transmission into farmed tilapia needs further investigation.

Streptococcus lutetiensis, which belong to the *S. bovis* group, was also isolated from diseased tilapia samples in this study. This streptococci species is a commensal or opportunistic pathogens of humans and animals which have been associated with bacteraemia or septicaemia, endocarditis, gastrointestinal and hepatobiliary diseases, and urinary infections (Bayliss *et al.* 1984; Romero *et al.* 2011; Wang et al. 2012; Carrasco et al., 2014; Counihab-Edgar et al., 2012). Presently, there are no studies about the pathogenicity of this bacterium to fish. It is an emerging pathogen, and little is known of its virulence and antibiotic resistance profile (Piva et al., 2019), thus additional research is required to determine is potential as pathogen in cultured fishes.

Some studies have reported the recovery of *S. aureus* from tilapia (Atyah et al., 2010; Soliman et al., 2014; Gaafar et al., 2015). In the study of Soliman et al. (2014), *S. aureus* was implicated in the mortality outbreak in the Nile tilapia, however, there was no histological data to show the pathology in the tissues and the presence of the bacteria. Moreover, these three studies did not perform pathogenicity test in order to confirm if the isolated *S. aureus* strains were truly pathogenic to the fish. Similarly, in this study the recovered tilapia came from apparently healthy fish that did not showed signs of pathology in the tissues or presence of bacteria. Therefore, the recovered *S. aureus* may only represent contamination from the water or leachate from the gut of the fish. However, it will be important to look into the pathogenicity of this bacterium to fish by performing infection bioassay. The recovery of *B. aryabhattai*, which is a rhizobacterium (Lee et al., 2012), and *B. kochii*, an environmental bacterium

isolated from dairy farms and marine environment (Seiler et al., 2012; Chellaram & John, 2015) may also be contamination during sampling.

Gram-negative bacteria were recovered from both apparently healthy and diseased tilapia in the freshwater sites; however, the bacterial strains may represent as normal commensals of the fish. In this study, the recovered bacteria were *E. cloacae*, *K. pneumoniae*, *V. cholerae*, and *P. shigelloides* which were similar to the bacterial microbiota recovered from intestines and gills of tilapia (Pakingking et al., 2015; Sheyin & solon, 2017). The recovery of *V. harveyi* and *V. alginolyticus* from red hybrid tilapia in seawater grow-out cages should be looked further since vibriosis mostly affect grouper and Asian seabass though experimental infection of red hybrid tilapia in seawater causes significant mortality (Abu Nor et al., 2020). Though the culture of red tilapia in seawater is quite new in the Philippines, it is important to conduct research on the pathogenicity of these bacteria for health and management in case of future mortality or disease outbreaks.

Furthermore, result from this diagnostic survey reported for the first time myxosporean infection in farmed Nile tilapia (13%) in the Philippines although *Myxobolus* spp. were detected before from imported fishes like common carp, grass carp and goldfish (Arthur & Lumanlan-Mayo, 1997). The recovery of *L. garvieae* from diseased Nile tilapia is also the first record in the country. Majority of the pathogenic bacteria recovered and identified were from moribund and apparently healthy tilapia from Taal Lake, Laguna Lake and Calauan Laguna which are areas where intensive freshwater farming of tilapia is done. These were also the areas that reported problems on disease/mortality outbreaks.

The changes in the gills observed in this study were consistent with bacterial invasion. It is difficult to determine acute responses in gill histology since changes such as aneurysm or necrosis may actually be associated with tissue fixation (Ferguson, 2006), however this was not the case here. Chronic infections which can provoke the inflammatory response with the migration of neutrophils, thrombocytes, macrophages, lymphocytes, and eosinophilic granulocytes along the gill filaments and in the central filament vessel (Ferguson, 2006; Del Rio- Zaragoza et al., 2010) may result into epithelial hyperplasia with fusion of adjacent lamellae and obliteration of the interlamellar space, increased mucus production due to proliferation of Goblet cells, and branchitis. Gram staining confirmed bacterial involvement in most of the observed pathology such as hydropic degeneration, hyperplasia and leukocytic infiltration which are similar to a previous study in catfish infected by *A. hydrophila* (Laith & Najiah, 2013). It may be that the gill infections resulted in lethargy in the affected fish leading systemic bacterial infection. Additionally, myxosporean infection was also observed in the gill of the diseased and some apparently healthy fish which may had been the predisposing factor for the later bacterial infection. This in turn will affect the fish behaviour and disease susceptibility.

The liver is an organ primarily made-up of polygonal cells called hepatocytes which are considered the first toxicity target of a substance, characterizing it as a biomarker organ of environmental pollution (Steckert et al., 2018; Zelikoff, 1998). In this study, most of the diseased fish exhibited hepatomegaly and paleness and a number were observed to have some necrotic lesions. Vacuolar degeneration may lead to hepatomegaly and

impaired liver function; however, this should be interpreted carefully as intracellular accumulations maybe a nutritional response (Ferguson, 2006). Numerous studies also report hypertrophy, vacuolar degeneration and increased lipid droplets in fish hepatocytes exposed to pollutants (Strmac & Braunbeck, 2002; Giari et al., 2007). In fish culture, commercial feed is known to cause accumulation of fat, degeneration of the liver cell membrane and vacuolation of hepatocytes, which may cause circulatory disturbances (Coz-Rakovac et al., 2005; Bilen & Bilen, 2013). The lesions, especially the vacuolar degeneration observed in this present study, may be due to numerous factors, and it is difficult to establish a threshold of what is healthy and pathological in the liver of cultured fish. Additionally, liver tissue exhibited multifocal aggregation of inflammatory cells surrounding necrotic hepatocytes which was similar to those reported in Nile tilapia (Asencios et al., 2016), the walking catfish (*Claris batrachus*) (Angka, 1990), and channel catfish (*I. punctatus*) (Ventura & Grizzle, 1988). However, in this study, Gram-stained section of the liver showed that 38% have bacterial involvement which could be the cause of the observed hepatic changes since toxins and extracellular enzymes produced by bacteria may induce different organ damage, which cause hepatic lesions characterised by different degrees of hepatocyte degeneration and cell death (Surai, 2015). Notably, lytic necrosis noted in some areas of the liver are abnormal changes, specifically, syncytial cell formation and massive hepatocellular necrosis with pyknotic and karyolytic nuclei similar to the liver cells of TiLV infected tilapia (Tattiyapong et al., 2017). All diseased tilapia showed generalised hepatitis where there was an infiltration of lymphocytes and neutrophils. This inflammatory response is usually preceded by hepatocellular

necrosis, but the converse can also happen (Ferguson, 2006). The lesions observed in the liver, as necrosis and mononuclear infiltration, described in this study are consistent with those reported by Mohamed et al. (2014) in tilapias infected experimentally with *Streptococcus* spp. It is noteworthy that the infiltration of mononuclear cells, mainly macrophages, observed in this study in all organs were also described by Filho et al. (2009) and Iregui et al. (2014).

The spleen is one the two major filtering organs in the vascular system of fish the other being the kidney (Ferguson, 2006). This is the reason why bacterial recovery is primarily done using the spleen and kidney. One of the major pathological changes in this study included expansion of the red pulp, histiocytosis, splenitis and necrosis with hemosiderosis. Careful consideration should also be taken in interpreting hemosiderin and MMC in the spleen since these are normal in fish spleen and some could be repositories of scavenged material and simply represent a metabolic dump (Ferguson, 2006). Gram-stained section of the spleen showed numerous bacteria and inflammatory cells causing severe necrosis obliterating the spleen altering its normal architecture. Hemosiderosis was reported to be associated with bacterial β-haemolysin that cause haemolysis inside the fish body followed by deposition of hemosiderin (Miyazaki & Kaige, 1985). Other authors observed increased number of MMC in the spleen of fish during bacterial infection (Manrique et al., 2014). Thus, the observed increase in hemosiderin and MMC can be safely attributed to bacteraemia. A similar type of splenic lesion was reported in channel catfish following IP injection with *A. hydrophila* (Bach et al., 1978). In the spleen was found congestion, necrotic foci and increased of MMC. Similar histological lesions in the splenic tissue of red tilapia were described by Alsaid et al. (2013).

The cellular alterations and damage to the spleen could be the reason for the splenomegaly that was observed in most diseased tilapia. In some broodstock tilapia, the spleen showed numerous granulomas surround by MMC. This particular may indicate a chronic condition or that the fish was able to control the infection.

Gross external observation of tilapia kidney was quite difficult although some samples showed size enlargement. Histological sections of the kidney of the diseased fish exhibited varying degrees of nephritis. Hyaline degeneration was notable kidney lesion in majority of the diseased fished which was similar to the findings of Abdelhamed et al. (2017) when channel catfish was experimentally infected with virulent *A. hydrophila* strain. These “droplets” are absorbed haemoglobin proteins which have passed through the glomeruli (Croce & Stagg, 1997). Gram-stained kidney sections from these samples showed diffuse necrosis which was so severe in the tubules of some samples that only the basement membrane remained. This diffuse necrosis was similar previous studies in the kidney of crucian carp (Miyazaki & Kaige, 1985), Nile tilapia (Yardimci & Aydin, 2011), and channel catfish (Ventura & Grizzle, 1988). Degeneration of the distal kidney luminal epithelium caused by bacterial toxin was observed as hyaline droplets, all lesions resulted from bacteraemic sepsis were as previously described by other studies (Jantakajorn et al., 2014; Bromage & Owens 2002; Neely et al. 2002; Zamri-Saad et al. 2010). However, these can also occur in the epithelial cells of renal tubules under normal conditions, where bright eosinophilic droplets within the proximal tubular epithelial cells can be found in normal fish (Takashima & Hibiya, 1995; Reimschuessel & Ferguson, 2006). Therefore, the accumulation of protein in

renal tubule epithelium might not specific clinical sign to diseased fish. Additionally, some samples from broodstock tilapia exhibited nephritis with numerous granulomas that indicates the fish is suffering a chronic infection (Ferguson, 2006) or it may have actually recovered from a previous infection.

The heart of the fish is frequently involved in a wide range of diseases and its examination is important (Ferguson, 2006). Pathological changes commonly found in the heart are inflammation of the pericardial sac and heart muscle, called pericarditis and myocarditis, respectively (Steckert et al., 2018; Ferguson, 2006; Takashima & Hibiya, 1995). In this study, around 34% of the diseased tilapia exhibited these pathological changes. The findings of pericarditis and epicarditis found in this study are consistent with those described by Zamri-Saad et al. (2010) in *O. niloticus* infected naturally with *S. agalactiae*. Alsaid et al. (2013) and Pulido et al. (2004) mentioned that inflammation in the epicardium may vary in different degrees, while other organs could be less affected, corroborating the findings observed in this study with types of acute or chronic epicarditis. In myocarditis, the alteration is characterized by the presence of leukocytes and lymphocytes with excessive accumulation in the spaces between the muscular fibres, corroborating the most frequent alterations of cardiac tissue observed in this study, which were the mononuclear and granular inflammatory infiltrates. As the presence of Gram-positive bacteria were observed, these alterations may be associated with the presence of bacteria present in the analysed fish, since such inflammation of the myocardium is usually preceded by bacterial colonisation (Ferguson, 2006). Necrosis of the endocardium with infiltration of mononuclear cells and

macrophage were also evident which could have resulted into cardiomyopathy in some diseased tilapia.

Histopathological examination of brain tissue specimens show meningeal thickening and inflammatory cell infiltration in the brain tissue, indicating meningitis, accompanied by haemorrhage. Meningitis was the most obvious brain abnormality observed in 36% of diseased tilapia. Gram-stained section of the specimens showed numerous bacteria in the thickened meninges and in the macrophages together with inflammatory cells. Moreover, other specimens showed encephalitis where macrophages filled with Gram-positive bacteria are infiltrating the brain cortex. These findings are similar to reports in various study (Abuseliana et al., 2010; Rodkhum et al., 2011; Hernandez et al., 2009; Noraini et al., 2013; Suwannasang et al., 2014). These results demonstrated that macrophages can possibly act as a vehicle for these Gram-positive cocci, allowing it to cross the blood-brain barrier and gain access to the central nervous system, thereby becoming disseminated throughout the organism's organ systems initiating bacterial septicaemia (Bowater et al., 2012). The medial longitudinal fasciculus and its nucleus, which were damaged by Gram-positive bacteria in this study, have been suggested to be involved in the swimming control of the fish by sending axons to innervate the primary and secondary motor neurons of the spinal cord that control swimming activities (Palang et al., 2020; Uematsu et al., 2007). This pathological manifestation in the brain tissue of naturally infected fish correlates with the clinical behavioural abnormalities of erratic pattern of swimming, orientation abnormalities which were the most prevalent clinical signs in the moribund tilapia samples. These results support the observations of the fish farmers that the disease/mortality

outbreaks in their tilapia farms during the warm months of March to June has an infectious etiologic nature.

Over-all, the description of clinical signs from diseased fish together with bacterial recovery and identification, and microscopic analyses of the cellular changes suggest that the disease/mortality outbreaks in farmed tilapia reported by farmers in the Philippines is caused by *Streptococcus* species and motile *Aeromonas* species.

3.6 Conclusion

In conclusion, it appears that farmed Nile tilapia in the Philippines are suffering from myriad of infectious diseases. The recovery and identification of the bacteria correlates with the observed clinical signs and is further strengthened by the abnormality and changes in the tissues examined. Majority of the diseased fish suffered from bacterial septicaemia and generalised inflammatory changes in the internal organs especially the brain. It would also appear from the histopathology result that a single etiologic is not responsible for the reported diseases or mortalities. Although it is possible that myxosporean parasites and viruses like TiLV contributes to the whole disease scenario, streptococci and aeromonads seem to be the major bacterial pathogens causing infectious diseases. The present work from this study now clarified the clinical picture of the observed disease/mortality among tilapia farms in the Philippines.

CHAPTER 4.

Streptococcosis in Farmed Nile Tilapia in the Philippines

Some of work presented in this chapter has been published in Journal of Fish Diseases. Legario FS, Choresca CH Jr., Turnbull JF, Crumlish M. Isolation and molecular characterization of streptococcal species recovered from clinical infections in farmed Nile tilapia (*Oreochromis niloticus*) in the Philippines. *J Fish Dis*. 2020; 00:1–12. https://doi.org/10.1111/jfd.13247

4.1 Abstract

Streptococcosis cause severe losses for global tilapia farming especially in developing countries. The aim of this study was to understand the pathogenic mechanisms of isolates recovered from natural disease outbreaks in farmed tilapia from the Philippines. A total of 25 bacterial strains identified as *Streptococcal* species, were profiled for antibiotic resistance and virulence patterns. There were 7 *Streptococcus iniae* and 18 *Streptococcus agalactiae* included in the study. A mixture of traditional and molecular probes was applied to determine the antibiotic and virulence profiles. Confirmation of species identification of the *Streptococci* were done using API 20 STREP, starch hydrolysis, species-specific duplex PCR and MALDI-TOF MS. The *S. agalactiae* isolates serotyping was done using the multiplex PCR amplification of the capsular *cps* gene. The viable growth of the isolates in different incubation temperatures and salinities (% NaCl) was also determined. Antibiotic profiling was performed using the antibiotic disc diffusion method and PCR

amplification of antibiotic resistance genes. The virulence pattern was determined by haemolysis assay, capsule staining, and PCR amplification of virulence genes. The results of the confirmatory identification showed the limitation of API 20 STREP in identifying *S. iniae* isolates but was excellent for *S. agalactiae*. However, when the API profile of *S. iniae* was compared to published works on the identification of piscine *S. iniae*, good agreement and identification were possible. Hydrolysis of starch can differentiate the positive *S. iniae* from the negative *S. agalactiae*. High correlation was found between *16S rRNA* gene sequencing and species-specific duplex PCR identifications. Peak analysis of the spectral profiles from MALDI-TOF can discriminate between *S. iniae* from *S. agalactiae*. Moreover, indicative serotype-specific peaks were observed in *S. agalactiae* isolates. The multiplex PCR serotyping positively identified two serotypes among the *S. agalactiae* isolates which were serotype Ia and serotype Ib. All strains were susceptible to the tested antibiotics and resistant to oxolinic acid while they were highly susceptible to β-lactam antibiotics and florfenicol. Only *S. agalactiae* serotype Ib showed resistance to sulphamethoxazole-trimethoprim. No resistance genes were detected among isolates; however, this is limited by the number of antibiotics used and the ARGs that were assayed. The low incidence of antibiotic resistance among streptococci isolates from the Philippines may indicate low use of antibiotics in the faming of tilapia. Identical virulence profiles were found for all strains of *S. iniae*, while *S. agalactiae* serotypes have different set of virulence genes. All the streptococci have capsules and the virulence genes for its expression. The *S. agalactiae* serotype Ib did not contain the *cylE* gene which is the structural gene for haemolysis. The presence of these virulence genes adds to the evidence of

their pathogenic potential which was supported by histopathology. This is the first study from the Philippines to characterize the streptococci involved in disease outbreaks in tilapia aquaculture. Outputs from this study will promote development of efficacious disease control strategies in tilapia farming for the Philippines and in Southeast Asia.

4.2 Introduction

Streptococcosis outbreaks in farmed freshwater tilapia continues to threaten global production, contributing to severe economic losses encountered worldwide (Amal & Saad, 2011; Li et al., 2015; Liu et al., 2018; Mishra et al., 2018). Streptococcosis in fish can be caused by more than one streptococcus species (Austin & Austin, 2007). The main aetiological agents identified from natural disease outbreaks in freshwater and marine water fishes are the Gram positive, *Streptococcus agalactiae* and *S. iniae* (Agnew & Barnes, 2007; Zhou et al, 2008; Mishra et al., 2018). These two streptococci species present identical gross clinical signs, and it is difficult to differentiate them by microscopic examination of tissue. Moreover, conventional morphological and biochemical tests are not able to accurately identify the species and serotypes (Mishra et al., 2018). It is of paramount importance to discriminate the two species since preventive strategies like vaccination does not provide cross-protection to the fish (Evans et al., 2004). Since their first detection as aetiological agents of streptococcosis in fish, the continued mortalities in farmed tilapia infected by these two streptococcal species is making them fish pathogens of global aquatic importance (Perera & Johnson, 1994; Zamri-Saad

et al., 2010). Understanding the pathogenesis of streptococcal infections in aquatic outbreaks remains complex, as similar to other aquatic pathogens, both bacterial species possess an array of virulence genes and express a range of virulence factors in relation to their pathogenicity. Identification of virulence gene patterns can be used to determine the genetic diversity of fish pathogens belonging to streptococcal species and provide improved understanding of the evolutionary relationship between pathogen virulence and host adaptation (Godoy et al., 2013). This relationship is important to identify efficacious solutions to reduce natural disease outbreaks on the farms.

Antibiotics are widely used to prevent bacterial disease in fish. On a global scale, several of the major classes of antimicrobials are being used or have been used in aquaculture. Among these are sulphonamides, penicillins, macrolides, quinolones, phenicols, and tetracyclines (Sapkota et al., 2008), all of which are listed as critically or highly important antimicrobials in human medicine (WHO, 2011). Currently, the tilapia industry, especially in China, remains reliant on antibiotics to treat bacterial infections in their stocks and that the overuse of antibiotics without legislation has contributed towards the production of AMR within aquatic bacterial strains (Zhou et al., 2021). Furthermore, without appropriate infrastructure and better administration processes, aquaculture can inadvertently contribute towards the rapid production of AMR via anthropogenic drivers, which can lead to human health issues as aquatic strains can carry genetic resistance and virulence markers which allows them to infect humans. In the Philippines, around 20 antibiotics are permitted for use in aquaculture including tetracyclines, erythromycin, amoxicillin, sulphonamides-trimethoprim, enrofloxacin, florfenicol, and oxolinic

acid (FAO, 2020). A major concern surrounding the use of antimicrobials in aquaculture is the potential to favour the development of a reservoir of antimicrobial resistance genes (ARGs) that may eventually be transferred to clinically relevant bacteria by horizontal gene transfer (von Wintersdorff et al., 2016). The indiscriminate use of antibiotics by fish farmers allows residues of antibiotics to remain in both cultured organisms and local environments which can lead to the emergence of drug-resistant strains. Some streptococci, i.e. *S. iniae* and *S. agalactiae*, are known to be zoonotic and emergence of their antibiotic-resistant strains can create serious public health problems (Ghittino et al., 2003). According to several studies, presently there is an increased call for the ban of antimicrobial use especially in animal production, however, addressing food security should not prohibit their usage but instead to minimise it (FAO/OIE/WHO, 2006; Smith, 2008; Heuer et al., 2009; WHO, 2011). The judicious use of antimicrobials in global aquaculture is important to effectively treat bacterial fish diseases and maintain fish health and welfare.

Under experimental conditions, a diverse type of virulence factors of *S. iniae* have been identified and expression confirmed in white striped bass (Baiano & Barnes, 2009). The pathogenic strains of *S. iniae* possess a capsule and the M-like protein called SiM (genes: *sim*A and *sim*B) which when expressed protect *S. iniae* against phagocytosis from host immune cells via opsonisation (Locke *et al.*, 2007a, 2008). Phosphoglucomutase is regulated by the *pgm* gene and is crucial for virulence as it is involved in cell wall thickness and rigidity, surface capsule expression and resistance to cationic antimicrobial peptides (Buchanan *et al.*, 2005). Furthermore, pathogenic *S. iniae* expresses a cytolysin called SLS) which is a peptide toxin homologous to streptolysin S from

Streptococcus pyogenes which is responsible for beta-haemolysis. The *S. iniae* SLS is a pore forming cytotoxin, where the work reported by Locke et al (2007b) confirmed SLS as an important promoter of cell cytotoxicity *in vitro* and expression of SLS was required during an *in vivo* fish challenge study to induce infection and mortalities in injected hybrid stripe bass. Experimental results showed *sagA* mutant *S. iniae* have highly decreased virulence compared to the wild-type strains since the *sag* operon is involved in SLS formation (Locke *et al.*, 2007b).

The virulence genes of *S. agalactiae* or GBS are clustered into three broad groups: adhesins, invasins and immune evasins, all of which play interrelated roles in their pathogenicity in the host after infection (Lin *et al*. 2011). The adhesins are cell surface components of bacteria that promote the adhesion of those bacteria to the host cell surface; for example, fibrinogen-binding proteins allow bacteria to bind with fibrinogen to protect them from opsonophagocytosis (Gutekunst *et al*. 2004; Tenenbaum *et al*. 2005; Buscetta *et al*. 2014). Invasins, are a class of proteins that allow penetration into the host cells. Examples include enabling the adhesive bacteria to cross the mucosal and the blood-brain barrier (Landwehr-Kenzel & Henneke, 2014) by the degradation of hyaluronan (Li & Jedrzejas 2001; Haas *et al*. 2015; Kolar *et al*. 2015) and collagen (Surve *et al*. 2016). The immune evasins, facilitate escape from host immunity; for example, the *pbp1A/ponA* gene that promotes resistance to host's cationic antimicrobial peptides (AMPs) (Hamilton et al., 2006). Investigation of the different virulence factors within a specific bacterial pathogen is important since it confers evolutionary advantages whether in transmission or within-host growth which consequently lead to disease and

mortality (Brown et al., 2012). However, some virulence factors are said to be by products or adaptation to other ecological niches and not directly related to disease (Adiba et al., 2010). These virulence factors act as coincidental by-product of adaptation may help us understand some pathogenic strategies employed by fish bacterial pathogens and their strains as observed in acute and chronic infections.

Pathogenic GBS strains are able to induce haemolysis in the infected host during natural disease outbreaks. This has been reported in human GBS (Doran et al., 2004; Liu et al., 2004) as well as many fish GBS strains (Kayansamruaj et al., 2014). The ability of the bacteria to induce haemolysis of the host cells is via possession of the surface-associated pore-forming GBS B-haemolysin/cytolysin toxins which are encoded by the *cylE* gene (Liu et al., 2004). βH/C is responsible for the characteristic zone of clearing around GBS colonies grown on blood agar plates and is capable of forming pores in a variety of eukaryotic cell membranes. βH/C is thought to contribute to GBS pathogenicity by virtue of its cytolytic properties, its ability to promote bacterial invasion of epithelial and endothelial barriers, and its activation of host cytokines and other inflammatory mediators. Studies have shown that GBS βH/C mutant exhibited decreased virulence in animal models of sepsis and meningitis (Ring et al., 2002; Doran et al., 2003). The work by Whidbey et al. (2013) identified that the ornithine rhamnolipid pigment is an important pluripotent toxin for GBS infections in humans as this pigment/lipid toxin complex, which is produced by the *cyl* operon (Rosa-Fraile et al., 2014; Armistead et al., 2019) enables the bacteria to be cytotoxic to a wide range of host cells. Optimum level production of this pigment enables GBS to induce

cytolysis and apoptosis of phagocytes and contributes to enhanced survival within phagocytes by shielding from oxidative damage caused by reactive oxygen species (ROS) (Armistead et al., 2019; Liu et al., 2004).

However, like the human GBS strains some fish GBS strains are also non-beta-haemolytic like the piscine *S. agalactiae* serotype Ib. In the study of Banno et al. (2017), clinical isolates of *S. agalactiae* from humans that are non-beta-haemolytic were observed to have reduced growth, small colony size, and atypical colony morphology which is called small colony variants (SCVs). The SCVs have been described in several bacterial genera and species such as *Staphylococcus* (Baddour et al., 1990) and *Streptococcus* (Allegrucci et al., 2007)) with their most prominent feature is the nearly one-tenth size of colonies associated with the wild-type bacteria (Proctor et al, 2006). In human medicine, the clinical importance of SCVs is their association with persistent, recurrent, and antibiotic-resistant infections (Proctor et al., 2006). Moreover, these SCVs may cause problems in bacterial isolation and identification which may lead to misdiagnosis and treatment failure. (Banno et al., 2014). To date, piscine GBS with this SCV phenotype have not been described or reported in the literature.

Investigation of the genetic and epidemiological relationship of piscine streptococci is important in finding the appropriate health strategies, which may be unique depending on the geographical area. To support the tilapia farming sector, the “holy grail” is to produce highly efficacious prevention and control measures against fish losses due to streptococcal infections. Disease outbreaks from both of these bacterial pathogens, often present with similar clinical signs, and can be confused with other bacterial infections. In addition, concurrent infections with both *S. agalactiae* and *S. iniae* (Conroy, 2009) can be seen on

single farms. These factors have contributed to a slower development of effective prevention and control strategies within tilapia farming systems globally and have resulted in more fragmented approaches. To reduce disease outbreaks, reliable data on the antimicrobial susceptibility and genetic determinants of virulence are required.

4.2.1 Study Aim

The objectives of the present study were to characterize and profile the antibiotic susceptibility and virulence genes of streptococcal species recovered from clinically sick tilapia farmed in the Philippines, with a view to improving targeted disease prevention and control measures.

4.3 Materials and Methods

The origin and preliminary identification of the strains characterised in this chapter was reported in Chapter 3 (pages x-y). Preliminary identification of the isolates included conventional methods like Gram staining, oxidase, catalase, motility, and oxidation-fermentation while the molecular identification test was *16S rRNA* gene sequencing.

In total there were 7 *S. iniae* and 25 *S. agalactiae*. The *S. iniae* isolates were recovered from moribund tilapia from Taal Lake farms while the *S. agalactiae* isolates were from Taal Lake, Calauan and Nueva Ecija.

4.3.1 Identification of Species

4.3.1.1 Biochemical profiles of the isolates

The biochemical profiles of the streptococci isolates were identified using the API 20 STREP kit (BioMérieux, France) following the manufacturer's instructions but modified incubated at 28° C instead of 37° C (Crumlish *et al.,* 2002).

4.3.1.2 Starch hydrolysis for streptococci

The starch hydrolysis test was performed based on the method of Cowan and Steel (Barrow & Feltham, 2003). A single colony subculture from TSA plate was aseptically streaked onto a starch agar plate (10 g starch/L of Nutrient Broth-Oxoid). The inoculated starch agar plates were incubated at 28° C for 24 h, then flooded with iodine's solution and the presence of zone of clearance around the bacterial growth against a deep blue background was a positive result.

4.3.1.3 Duplex PCR for *S. agalactiae* and *S. iniae*

A duplex PCR assay was performed to test species differentiation between *S. iniae* and *S. agalactiae* strains. Prior to adopting the PCR method, a series of optimisation steps were performed as described.

Optimisation. A duplex PCR was developed to differentiate between *S. agalactiae* and *S. iniae* using species-specific primers: LOX-1 (5'-AAGGGGAAATCGCAAGTGCC-3') and LOX-2 (5'-ATATCTGATTGGGCCGTCTAA-3') for target gene *lctO* of *S. iniae* which amplifies approximately 870 bp of the gene (Mata et al., 2004), and STRA-AgI (5'-AAGGAAACCTGCCATTTG-3') and STRA-AgII (5'-

TTAACCTAGTTTCTTTAAAACTAGAA-3') which targets the *16-23S* intergenic spacer region of *S. agalactiae* which is about 270 bp (Phuektes et al., 2003). Optimisation of the duplex PCR was done by doing specificity and sensitivity tests. For sensitivity test, genomic DNA of *S. iniae* ATCC 29178 and *S. agalactiae* NCIMB 701348 were diluted 10-fold with Milli-Q water starting with a 50 ng/ µl concentration. Then, each concentration of the genomic DNA for each streptococci was PCR amplified using the species-specific primers, electrophoresed and visualised. In specificity test, each specific primer pairs were separately used for PCR amplification using genomic DNA at 50 ng/ µl. Then a duplex PCR was designed using both primers in a single reaction. The genomic DNA of *S. iniae* ATCC 29178, *S. agalactiae* NCIMB 701348, and a mixture of both streptococci were used in a 25 ul PCR reaction at 50 ng/ µl and amplified with an initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 25s, with a final extension of 72°C for 10 min. PCR products were then electrophoresed and visualised in UV transilluminator.

Duplex PCR. Each PCR was performed in a 25 µl reaction mixture consisting of 12.5 µl of 2X HS MyTaq mastermix (bioline UK), 1.5 µl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 µl of DNA template, and Milli-Q water to volume. The amplification profile consisted of an initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 25s, with a final extension of 72°C for 10 min. The PCR products were obtained by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE (Tris-acetate-EDTA) buffer with ethidium bromide (0.5 µg ml^{-1}), visualized on a

UV transilluminator. Positive control isolates were used which included type strain of *S. agalactiae* NCIMB 701348 and *S. iniae* ATCC 29178. (Appendix 2.3).

4.3.1.4 Capsular serotyping for *S. agalactiae*

Molecular serotype based on the *cps* gene was determined using a multiplex PCR described by Imperi *et al.* (2010) with minor modifications. Instead of 19 primers, seven primers (Table 4.1) were used to amplify serotypes Ia, Ib, II and III to reflect the most common serotypes infecting fishes (Delannoy et al., 2016). The *S. agalactiae* (STIR-CD-17) serotype Ib and Milli-Q water were used as positive and negative controls respectively. The PCR reaction contained 12.5 µl of 2X HS MyTaq mastermix, 1.0 µl of 10 pmol of each oligonucleotide primer, 5.0 µl of 20 ng gDNA, and 0.5 µl of milliQ water. PCR amplification was performed in Biometra thermal cycler (Analytik Jena) using a cycling protocol of: 1 cycle of 1 min at 95°C; 15 cycles of 60 s at 95°C, 60 s at 54°C and 2 min at 72°C; 25 cycles of 60 s at 95°C, 60 s at 56°C and 2 min at 72°C; final cycle of 10 min at 72°C. The PCR products were then separated by electrophoresis in 1.5 % (w/v) agarose gel and visualized under UV light. (Appendix 2.4).

Table 4.1 Molecular serotyping primers for *S. agalactiae.*

Serotype	Target Gene	Primer	Nucleotide (5' → 3')*	Target Region (bp)
Ia	*cpsL*	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	
	cpsG	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	272
		cpsG-R	ATGCTCTCCAAACTGTTCTTGT	
Ib	*cpsG*	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	272
		cpsG-R	ATGCTCTCCAAACTGTTCTTGT	
	cpsJ	*cpsJ*-Ib-F	GCAATTCTTAACAGAATATTCAGTTG	621
		cpsJ-Ib-R	GCGTTTCTTTATCACATACTCTTG	
	cpsL	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	
III	*cpsG*	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	352
		cpsG-2-3-6-R	TCCATCTACATCTTCAATCCAAGC	
	cpsL	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	

4.3.1.5 Protein spectral profiles of the streptococci isolates

Ethanol-formic acid extraction. The ethanol-formic extraction method used in this study was carried out following the method published by Freiwald and Sauer (2009). In summary, single colonies were taken using a disposable loop and re-suspended in 300 µL of Milli-Q water before 900 µL of absolute ethanol at RT was added. At this point, it was possible to store the ethanol-inactivated bacteria for several weeks at 4° C. If this was not to be the case, samples were centrifuged at 10,000 x g for 2 min at RT and the supernatants were removed and discarded. Samples were then centrifuged again at 10,000 x g at RT for 2 min and any residual ethanol was removed using a pipette before sample were left open to dry for approximately 2 min. Once samples had been given time to dry, 10 µL of 70% formic acid was added to the pellets and mixed thoroughly by pipetting in order to dissolve extracted proteins. Following this, 10 µL of pure acetonitrile was added to the samples and carefully mixed before

being centrifuged once again at RT for 2 min at 10,000 x g. The extracted proteins were in the supernatant and were transferred into a new sterile eppendorf tube. The extracted protein samples were then stored at 4°C until used.

Spotting of MALDI-TOF slide/target plate

The matrix solution used in all analysed samples was α-Cyano-4-hydroxycinnamic acid (CHCA; $C_{10}H_7NO_3$) which was prepared at a concentration of 5 mg/mL in a solution of 70% acetonitrile and subsequently stored at 4°C for one week.

In order to spot the MALDI target plate (FlexiMass-SR48 steel targets; Shimadzu, Japan), 0.5 µL of the extracted protein samples were added to each spot of the target plate. Samples were left to dry in air for 20 min before 0.5 µL of CHCA matrix solution was added to each spot. Again, the plate was given 20 min to dry in air before being inserted into the MALDI-TOF MS instrument. From each original sample six dilutions (1:1, 1:2.5, 1:5, 1:10, 1:20, 1:40) were prepared with 0.1% trifluoroacetic acid (TFA). Each original and dilution sample was spotted in tetraplicates (technical replicates n=4/dilution).

Analysis of bacterial profiles through use of MALDI-TOF spectrometry

MALDI-TOF analysis was performed using a Benchtop Linear MALDI-TOF Mass Spectrometer 8020 (Shimadzu, Japan) equipped with the Shimadzu software package, for spectra 15 recording in the positive linear mode (laser power set at 50, Pulse extraction value 1800; mass range, 300 Da to 15,000 Da). A total of 300 profiles per spot were accumulated for analysis. Mass calibration was performed using the Protein Mix 1 calibrant from Laserbio Labs

(UK) that contains a mixture of proteins within a mass range of 5,700-17,000 Da.

4.3.2 Viable growth in different incubation temperatures and salt concentrations

From a pure culture, colonies of each bacterial isolate were transferred into 2 ml sterile distilled water with the bacterial density adjusted to a McFarland standard of 0.5 (bacterial density ≈ 1.5 x 10^8/ml). A 40 µl sample of each bacterial suspension was then added to 2 ml TSB. To determine the temperature tolerance, each isolate was incubated at 4, 15, 22, 28, 37, 43 and 47 °C alongside the negative controls which consisted of TSB with no bacteria.

Salinity tolerance was investigated by growing the isolates at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 6.5 and 7.0 (w/v) NaCl in TSB at 28 °C. The bacterial suspensions were prepared as described above. Negative control comprising of TSB without bacteria were made for each salinity group. A positive result for growth is indicated by turbidity while negative result showed absence of turbidity. For both assays viable growth was compared with the controls for 5 days.

4.3.3 Histopathology

The details of the histopathology protocol is found in Chapter 3 (pages). H&E and Gram-stained tissue sections were examined using the digital slide scanner (ZEISS Axio Scan.Z1, ZEISS Germany).

4.3.4 Antibiotic susceptibility and antibiotic resistance determinants

4.3.4.1Antibiotic susceptibility assay

All the isolates were tested for antibiotic sensitivity to antibiotics that are globally licensed for aquaculture which are amoxicillin (AML 10 µg), oxytetracycline (OT 30 µg), florfenicol (FFC 30 µg), sulfamethoxazole-trimethoprim (SXT 25 µg), oxolinic acid (OA 2 µg), enrofloxacin (ENR 5 µg), tetracycline (TE 30 µg) and erythromycin (E 15). Additionally other antibiotics of human health significance were also tested which include ampicillin (AMP 25 µg), penicillin G (P 10 units), chloramphenicol (C 30 µg), and vancomycin (VA 30 µg) (Oxoid UK). The antibiotics selected were those routinely screened in the diagnostic laboratory at the Institute of Aquaculture, University of Stirling and representative of antibiotics licensed for use in global aquaculture. The Kirby-Bauer method of antibiotic suscpetiblitiy testing as described was also followed. was applied: spread plates were produced on TSA by aseptically removing 2-3 single colonies per strain from a single colony subculture plate and emulsifying in 5 ml of sterile saline (0.85% NaCl) to give a concentration equal to McFarland Standard of 1. This gave an approximate bacterial concentration of 3.0 x 10^8 CFU/ml. A bacterial lawn was produced, and the antibiotic discs applied using an Oxoid ™ antimicrobial susceptibility disc dispenser and incubated at 28°C for 72 h. Results were interpreted as sensitive (≥ 16 mm), partially sensitive (12-15 mm) or resistant (≤ 11 mm) based on the diameter zone of inhibitions (Crumlish et al., 2002). Multi-resistance of the isolates is based on the definition as resistance to at least three antimicrobial classes (Schwarz et al., 2010).

4.3.4.2 Detection of antibiotic resistance genes

Based on the antibiotic resistance phenotypes, genes encoding resistance to sulphonamides (*sul1*, *sul*2, *sul*3) and trimethoprim (*dfrA1*, *dfrA12*, *drfA17*) were tested by PCR. The presence of integrons (*IntII*, *IntI2,* Class 1 integron) were also screened in all isolates. The list of primers are shown in Table 4.2. The antimicrobial resistance genes amplified PCR products were purified and sent for sequencing to confirm their identity.

Table 4.2 Primers used for PCR detection of different ARGs in *Streptococci* spp. isolates.

Targeted Gene	Primer Pair	Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
		Sulphonamide Resistance		
*sul1***	sul1-F	CTTCGATGAGAGCCGGCGGC	71	417
	sul1-R	GCAAGGCGGAAACCCGCGCC		
*sul2***	sul2-F	AGGGGGCAGATGTGATCGAC	54	249
	sul2-R	GCAGATGATTTCGCCAATTG		
*sul3***	sul3-F	GAGCAAGATTTTTGGAATCG	52	789
	sul3-R	CATCTGCAGCTAACCTAGGGCTTTGGA		
		Trimethoprim Resistance		
*dfra1***	dfra1-F	ACG GAT CCT GGC TGT TGG TTG GAC GC	65	254
	dfra1-R	CGG AAT TCA CCT TCC GGC TCG ATG TC		
*dfra12***	dfra12-F	ACT CGG AAT CAG TAC GCA	50	462
	dfra12-R	GTG TAC GGA ATT ACA GCT		
*dfra17***	dfra17-F	GAT TTC TGC AGT GTC AGA	50	384
	dfra17-R	CTC AGG CAT TAT AGG GAA		
		Integrons		
*IntI1**	InII-F	CTACCTCTCACTAGTGAGGGGCGG	58	847
	InII-R	GGGCAGCAGCGAAGTCGAGGC		
*IntI2**	IntI2-F	GCAAATGAAGTGCAACGC	56	467
	IntI2-R	ACACGCTTGCTAACGATG		
Class 1 integron*	5'CS	GGCATCCAAGCAGCAAG	64	Variable
	3'-CS	AAGCAGACTTGACCTGA		

*Hossain et al. (2018), **Syrova et al. (2018).

4.3.5 Virulence and virulence genes

4.3.5.1 Phenotypic virulence determinants

Presence of capsule

The protocol for Anthony's capsule stain was based on Hughes and Smith (2007). One colony of each bacterial isolate from a pure culture was placed into 5 ml milk broth (0.95% skim powder milk) and incubated at 28° C, 140 rpm for 18 hours. After incubation, a 20 µl smear of the bacterial suspension was made on a microscope and left to air dry. The slides were covered with 1% crystal violet for 1 minute and washed with 20% copper sulphate solution (see Appendix). *Pseudomonas aeruginosa* strain ATCC 27835 was used as positive control and prepared as above except that it was grown at 22° C. Coverslips were mounted onto slides using Pertex and then visualised using Zeiss Axiocam MRc digital camera on an Olympus BX51 microscope under 100 x magnification. Bacterial cells and the proteinaceous background will appear purplish while the capsule will appear transparent (positive result).

Haemolysis on Sheep Blood

A pure colony of the streptococcal isolate was streaked onto 5% (v/v) sheep blood agar. The plates were then incubated at 28° C and checked daily for five days. A clearing around the colonies on the blood agar plate was recorded as a positive result for haemolysis. The *S. iniae* ATCC 29178 was used as the positive control.

4.3.5.2 Detection of virulence genes

Isolates were screened for a total of 6 and 7 virulence genes for the *S. iniae*, and *S. agalactiae*, respectively (Table 4.3) using conventional PCR. The PCR conditions followed those cited in the literature (Table 2) with minor modifications to the time for denaturation (15s), annealing (15s) and extension (30s). Temperatures for each stage were followed as described in the cited publication.

Each PCR was performed in a 25 µl reaction mixture consisting of 12.5 µl of 2X HS MyTaq mastermix (bioline UK), 1.5 µl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 µl of DNA template, and Milli-Q water to volume. The PCR products were obtained by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE buffer with ethidium bromide (0.5 µg ml^{-1}), visualized on a UV transilluminator.

Table 4.3 Primers used for the detection of virulence genes in *S. iniae* and *S. agalactiae*.

Virulence Factor	Target Gene	Primer	Nucleotide (5'-3')	Target Region (bp)	Function
S. iniae					
M-like Protein*	*simA*	simAfornew	AATTCGCTCAGCAGGTCTTG	994	Adhesin
		simArevnew	AACCATAACCGCGATAGCAC		
C5α peptidase*	*scpI*	scpIfor	GCAACGGGTTGTCAAAAATC	822	Immune evasin
		scpirev	GAGCAAAAGGAGTTGCTTGG		
Phosphoglucomutase*	*pgm*	pgmfor	TATTAGCTGCTCACGGCATC	490	Immune evasin
		pgmrev	TTAGGGTCTGCTTTGGCTTG		
Capsule*	*cpsD*	cpsDfor	TGGTGAAGGAAAGTCAACCAC	534	Immune evasin
		cpsDrev	TCTCCGTAGGAACCGTAAGC		
Polysaccharide deacetylase*	pdi	pdifor	TTTCGACGACAGCATGATTG	381	Adhesin
		pdirev	TTAGGGTCTGCTTTGGCTTG		
Cytolysin SLS*	*sagA*	sagAfor	AGGAGGTAAGCGTTATGTTAC	190	Invasin
		sagArev	AAGAAGTGAATTACTTTGG		

S. agalactiae					
CAMP factor**	cfb	cfb-F	GGATTCAACTGAACTCCAAC	600	Invasin
		cfb-R	GACAACTCCACAAGTGGTAA		
Serine protease**	cspA	cspA-F	CTGCTAAAGCACACCTAAAC	971	Immune evasin
		cspA-R	ATCAGTAGTGGTTCCTTTCC		
B-hemolysin/cytolysin***	cylE	cylE-F	CACTGCCAAGAGCAGTTGATT	558	Adhesin
		cylE-R	TTCCCCTTGGCGGATTTGGA		
Fibrinogen-binding protein A**	*fbsA*	fbsA-F	AACCGCAGCGACTTGTTA	278	Adhesin
		fbsA-R	AAACAAGAGCCAAGTAGGTC		
Hyaluronate lyase**	*hylB*	hylB-F	TCTATGCTGACGGTTCTTAC	323	Invasin
		hylB-R	GAGGTCTAAGTTTCGCTCTT		
Pilus-island 2B****	*PI-2b*	PI-2b-F	ACACGACTATGCCTCCTCATG	721	Immune evasin
		PI-2b-R	TCTCCTACTGGAATAATGACAG		
Penicillin-binding protein 1A**	pbp1A/ponA	pbp1A/ponA-F	AGGGGTAGTAGCATTACCAT	939	Immune evasin
		pbp1A/ponA-R	CAACTATATGACTGGGATCG		

*Baum et al. (2013), **Kannika et al. (2017), ***Jiang et al. (2016); ****Chattopadhyay et al. (2011)

4.4 Results

4.4.1 Identification of the species

4.4.1.1 Biochemical profiles of the isolates

The API 20 STREP biochemical profiles produced higher variation between the *S. agalactiae* strains compared with the *S. iniae*. All of the 7 *S. iniae* isolates tested had identical API 20 STREP biochemical profiles and they differed only from the type strain *S. iniae* ATCC 29178 on beta-glucoronidase (β-GUR) and arginine dihydrolase (ADH) (Table 4.4). The *S. agalactiae* isolates exhibited varied biochemical characteristics and primarily differed from the type strain *S. agalactiae* NCIMB 701348 on β-GUR and lactose fermentation (LAC). All results are summarised in Table 4.4and the *S. agalactiae* isolates were grouped into three biochemical profiles.

Table 4.4. Phenotypic and biochemical characteristics of the different *Streptococci* isolates.

	***S. iniae* ATCC 29178**	***SI* 9***	***SI* 25***	***SI* 105***	***S. agalactiae* NCIMB 701348**	**SA Group 1****	**SA Group 2*****	**SA Group 3******
Gram	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Morphology	cocci	cocci	cocci	cocci	cocci	cocci	cocci	Cocci
Motility	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
O/F	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	-	-	-	-
Haemolysis	+	+	+	+	+	+	+	+
VP	-	-	-	-	+	+	+	+
HIP	-	-	-	-	**+**	**+**	**-**	**+**
ESC	+	+	+	+	-	-	-	-
PYRA	+	+	+	+	-	-	-	-
αGAL	-	-	-	-	-	-	-	-
βGUR	+	-	-	-	**+**	**-**	**-**	**-**
βGAL	-	-	-	-	-	-	-	-
PAL	+	+	+	+	+	-	-	+
LAP	+	+	+	+	+	+	-	+
ADH	**+**	**-**	**-**	**-**	+	+	-	+
RIB	+	+	+	+	+	+	-	+
ARA	-	-	-	-	-	-	-	-
MAN	+	+	+	+	-	-	-	-
SOR	-	-	-	-	-	-	-	-
LAC	-	-	-	-	**+**	**-**	**-**	**-**
TRE	+	+	+	+	+	+	+	+
INU	-	-	-	-	-	-	-	-
RAF	-	-	-	-	-	-	-	-
AMD	+	+	+	+	-	-	-	-
GLYG	+	+	+	+	-	-	-	-

*SI = *Streptococcus iniae*; similar biochemical profile with 7, 16, 18, 31
**SA Group 1 = *Streptococcus agalactiae* isolate 63
***SA Group 2 = *Streptococcus agalactiae* isolates 100, 101, 102
****SA Group 3 = *Streptococcus agalactiae*; similar biochemical profile with isolates 97, 106, 108, 109, 113, 117, 122, 126, 131, 132, 135, 138, 139, 141

A single biochemical profile was obtained for the Philippine *S. iniae* strains in this study (Table 4.5, column A). When comparing the API STREP

profiles with published results, only 3 tests gave 100% positive responses for all studies. This was PYRA, PAL and LAP and only more comparative results were found in the strains being negative for VP, HIP, ARA, SOR, LAC, INU, and RAF (Table 4.5). The remaining tests gave variable results and would therefore not be considered reliable for species identification.

Previous authors have confirmed the variability in using API20STREP kits for the identification of aquatic *S. agalactiae* strains and this was confirmed for the Philippines strains tested in this study (Table 4.5, column A). More tests were negative or variable for the *S. agalactiae* strains tested in this study as well as those published previously.

From the API STREP results, *S. agalactiae* could be differentiated from *S. iniae* based on positive VP, HIP only (Table 4.5).

Table 4.5 Table 4.5. Percentage positivity for API 20 STREP for *Streptococcus agalactiae* and *Streptococcus iniae* isolates from farmed Nile tilapia in the Philippines and in other literature reports.

	S. agalactiae										**S. iniae**									
Reference*	A	B	C	D	E	F	G	H	I	J	A	G	K	L	M	N	O	P	Q	R
Number of Isolates	18	8	4	8	20	20	10	20	9	18	7	10	10	1	3	65	26	11	24	31
Country**	PH	CH	CH	KW	KW	BZ	IS, JP, TW	TH	VT	US, TH, EC, HN, IS	PH	IS, JP, TW	TH	SP	BH	SK	CH	IS	AU	AU
VP	100	n/a	0	0	100	100	100	100	100	100	0	0	0	0	0	0	0	0	0	0
HIP	83	100	100	100	100	0	0	0	100	100	0	0	0	0	0	0	0	0	0	0
ESC	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	3	100	100	100	100
PYRA	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	100	100	100	100	100
α-GAL	0	100	0	0	0	0	0	100	0	11	0	0	60	0	0	0	0	0	0	0
β-GUR	0	100	0	0	70	0	0	0	0	11	0	100	100	100	0	2	100	100	100	100
β-GAL	0	0	0	0	0	0	0	0	0	0	0	0	40	0	100	0	0	0	0	0
PAL	78	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
LAP	83	100	100	100	100	100	100	100	100	94	100	100	100	100	100	100	100	100	100	100
ADH	83	100	100	100	100	100	100	100	33	94	0	100	100	100	100	11	100	27	v	100
RIB	83	100	100	100	100	100	100	100	89	94	100	100	100	100	100	77	100	100	100	100
ARA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MAN	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	69	100	100	100	100
SOR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAC	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TRE	100	100	100	100	45	0	0	100	89	28	100	100	100	100	100	95	100	100	100	100
INU	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RAF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AMD	0	n/a	0	0	0	0	0	0	0	0	100	0	100	0	100	74	100	100	100	100
GLYG	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	57	100	100	0	100

*(A) Current study (B) Wang et al., 2013 (C) Geng et al., 2012 (D) Duremdez et al., 2004 (E) Al-Marzouk et al., 2005 (F) Salvador et al., 2005 (G) Eldar et al., 1994 (H) Maisak et al., 2008 (I) Oanh & Phuong, 2012 (J) Soto et al., 2015 (K) Suanyuk et al., 2010 (L) El Aamri et al., 2010 (M) Yuasa et al., 1999 (N) Nho et al., 2009 (O) Zhou et al., 2008 (P) Colorni et al., 2002 (Q) Nawawai et al., 2008 (R) Bromage et al., 1999.

(VP) Voges-Proskauer; (HIP) Hippurate; (ESC) Aesculin; (PYRA) Pyrrolidonylarylamidase; (α-GAL) α galactosidase; (β-GUR) β glucuronidase; (β-GAL) β galactosidase; (PAL) Alkaline phosphatase; (LAP) Leucine arylamidase; (ADH) Arginine dihydrolase; (RIB) Ribose; (ARA) Arabinose; (MAN) Mannitol; (SOR) Sorbitol; (LAC) Lactose; (TRE) Trehalose; (INU) Inulin; (RAF) Raffinose; (AMD) Amygdalin; (GLYG) Glycogen.

(n/a) Data not available; (v) variable but no value provided. Numbers show percentage of positive strains.

**Country PH = Philippines; CH = China; KW = Kuwait; BZ = Brazil; IS = Israel; JP = Japan; TW = Taiwan; TH = Thailand; VT = Vietnam; US = United States of America; EC = Ecuador; HN = Honduras; SP = Spain; BH = Bahrain; SK = South Korea; AU = Australia.

4.4.1.2 Starch hydrolysis

Results from starch hydrolysis showed that all *S. iniae* were positive as evidence by the appearance of the clearance around the colonies against the dark blue background. The *S. agalactiae* isolates were all negative for the hydrolysis of starch. The positive and negative reactions of the streptococci isolates are presented in Figure 4.1A and B.

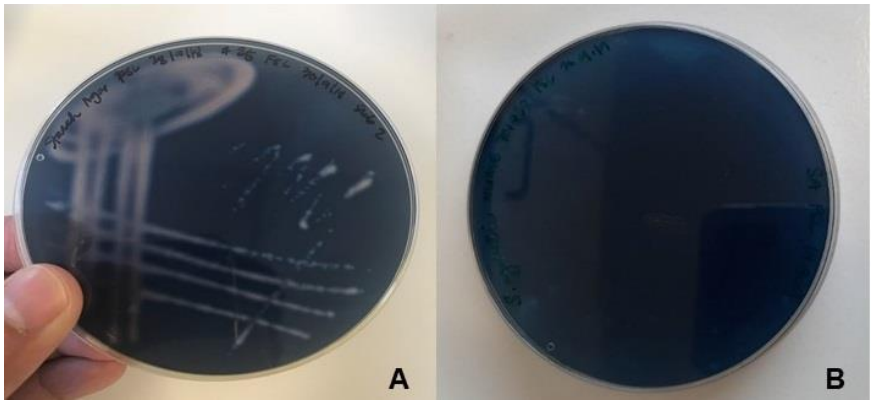

Figure 4.1 Starch agar plates flooded with iodine solution showing *S. iniae* isolate (A) positive reaction and *S. agalactiae* (B) isolate with the negative reaction.

4.4.1.3 Duplex PCR for *S. agalactiae* and *S. iniae*

Optimisation. A species-specific duplex PCR was developed using the primer pairs LOX-1 and LOX-2 (for *lcto* gene of *S. iniae*) and STRA-Ag-I and STRA-Ag-II (for *16-23S* rRNA of *S. agalactiae*). The starting DNA concentration for the sensitivity testing of the PCR is 50 ng/µl. Result on the sensitivity test (Figure 4.2) showed that the LOX primers can amplify the gene up to seven-fold concentration while the STRA-Ag primers can amplify the gene up to the 10-fold concentration.

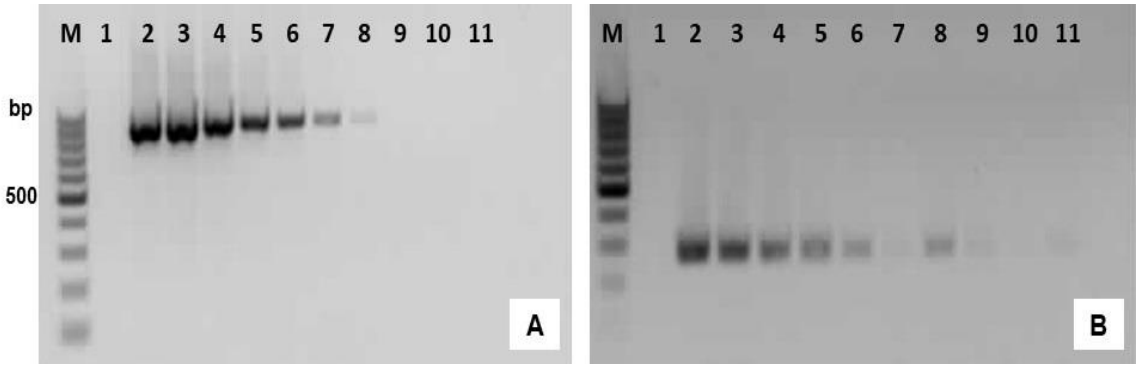

Figure 4.2 Sensitivity PCR for (A) *LOX* gene primers (amplicon size: 870 bp) for *S. iniae ATCC 29178* & (B) *STRA-Ag* gene primers (amplicon size: 270 bp) for *S. agalactiae NCIMB 701348*: Lane M is 100 bp marker; Lane 1 negative control (Milli-Q water); Lanes 2 to 11 are DNA concentrations in a 10-fold dilution (50 ng to 0.5 fg DNA concentration).

For the specificity test, the STRA-Ag primer pair was only able to amplify the target gene (270 bp) of *S. agalactiae* but not the target gene of the *S. iniae* (Figure 4.3A). The LOX primer pair amplified the 870 bp target gene of the *S. iniae* but not the 16-23S rRNA gene of the *S. agalactiae* (Figure 4.3B).

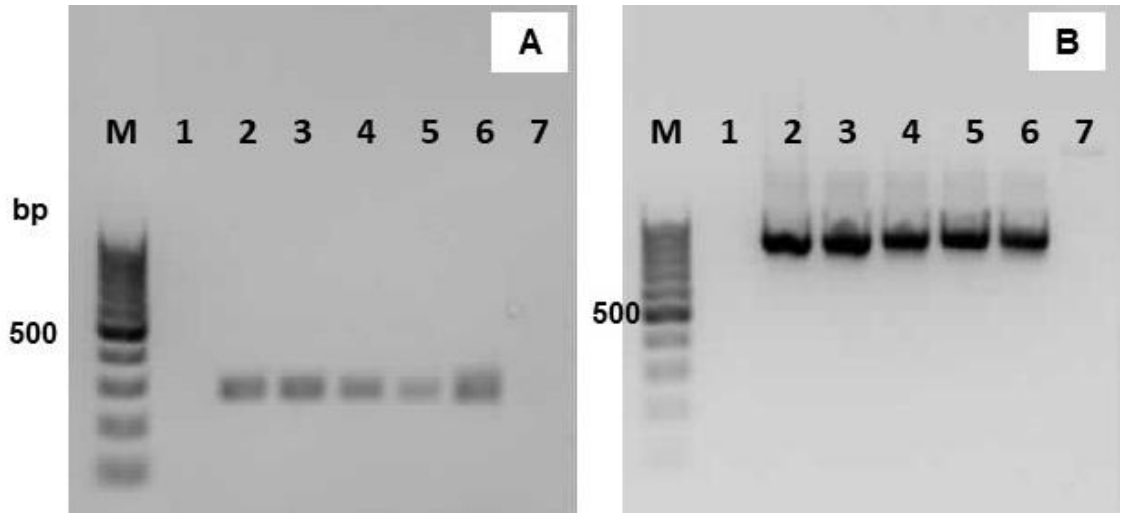

Figure 4.3 Specificity PCR for (A) *STRA-Ag* gene primers (amplicon size: 270 bp) for *S. agalactiae NCIMB 701348*: Lane M is 100 bp marker; 1 negative control (Milli-Q water); Lanes 2 to 6 are *S. agalactiae NCIMB 701348*; Lane 7 is *S. iniae ATCC 29178*; (B) *LOX* gene primers (amplicon size: 870 bp) for *S. iniae ATCC 29178*: Lane M is 100 bp marker; Lane 1 negative control (Milli-Q water); Lanes 2 to 6 are *S. iniae ATCC 29178*; Lane 7 is *S. agalactiae NCIMB 701348.*

The result of the optimised species-specific PCR using primers LOX and STRA-Ag was able to efficiently discriminate *S. iniae* from *S. agalactiae*. In Figure 4.4, samples of the genomic DNA of *S. agalactiae* only amplified a product of only 270 bp which is the *16-23S rRNA* gene target. For the *S. iniae* genomic DNA only samples, gel electrophoresis showed amplicons of 870 bp size which corresponds to the *lctO* gene. A mixture of the genomic DNA of both streptococci species showed positive amplification of both bands at 270 and 870 bp which corresponds to *S. agalactiae* and *S. iniae,* respectively.

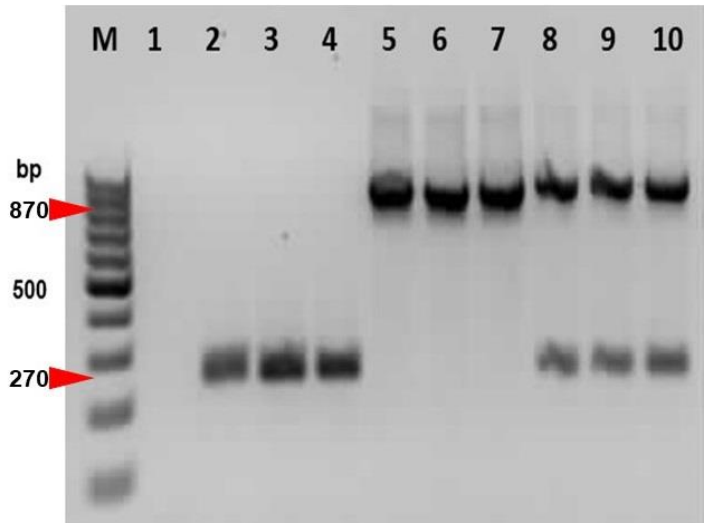

Figure 4.4. Optimised Duplex-PCR amplification of the *LOX* and *STRA-Ag* genes for the identification of *S. iniae* and *Streptococcus agalactiae*. Lane M: 100 bp molecular marker; Lane 1: negative control (Milli-Q water); Lanes 2-4: *S. agalactiae* NCIMB 701348 only (270 bp); Lanes 5-7: *S. iniae* ATCC 29178 only (870 bp); Lanes 8-10 are the band sizes for the mixture of *S. agalactiae* (270 bp) and *S. iniae* (870 bp).

Species-specific Duplex PCR of Streptococci isolates. The species-specific duplex PCR discriminated *S. iniae* from *S. agalactiae*, where a single band was observed at 870 bp for *S. iniae* and at 270 bp for *S. agalactiae* (Figure 4.5 & 4.6). Good correlation was found between the 16S rRNA

sequence results and the duplex-PCR results for *S. iniae* and *S. agalactiae strains*.

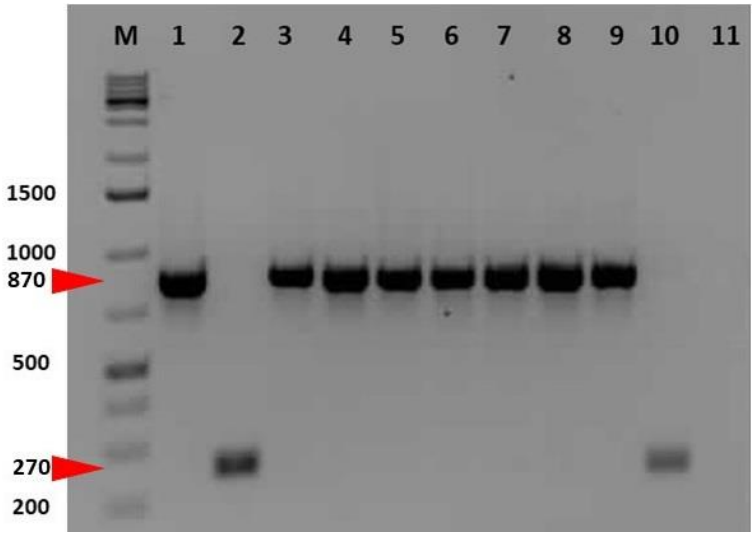

Figure 4.5 Duplex-PCR amplification of the LOX and STRA-Ag genes for the identification of *S. iniae* and *Streptococcus agalactiae* isolates from clinically affected tilapia in the Philippines. Lane M: 1 kb molecular marker; Lane 1: *S. iniae* ATCC 29178; Lane 2: *S. agalactiae* NCIMB 701348; Lanes 3–9 are the band size for *Streptococci iniae* (870 bp); Lane 10 is the band size for *S. agalactiae* (270 bp); and Lane 11: negative control (Milli-Q water)

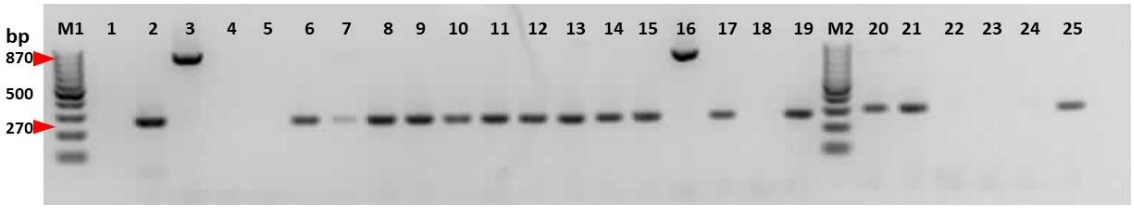

Figure 4.6 Duplex-PCR amplification of the LOX and STRA-Ag genes for the identification of *S. iniae* and *Streptococcus agalactiae* isolates from clinically affected tilapia in the Philippines. Lane M1 & M2: 100 bp molecular marker; Lane 1: negative control (Milli-Q water); Lane 2: *S. agalactiae* NCIMB 701348; Lane 3: *S. iniae* ATCC 29178; Lanes 6–15, 20-21, 25 are the band size for *S. agalactiae* (270 bp); Lane 16 is the band size for *S. iniae* (870 bp).

4.4.1.4 Capsular serotyping of *S. agalactiae*

From the 18 isolates identified as *S. agalactiae*, 15 (83%) isolates were identified as serotype Ia, while the remaining 3 (17%) were identified as

serotype Ib. Serotype Ia was indicated by the presence of *cpsL* (688bp) and *cpsG* (272 bp), while serotype Ib by *cpsL* (688bp), *cpsJ* (621 bp) and *cpsG* (272 bp) (Figure 4.7). Serotype Ia and Ib shares the presence of *cpsL* gene. No other serotype was detected in this study.

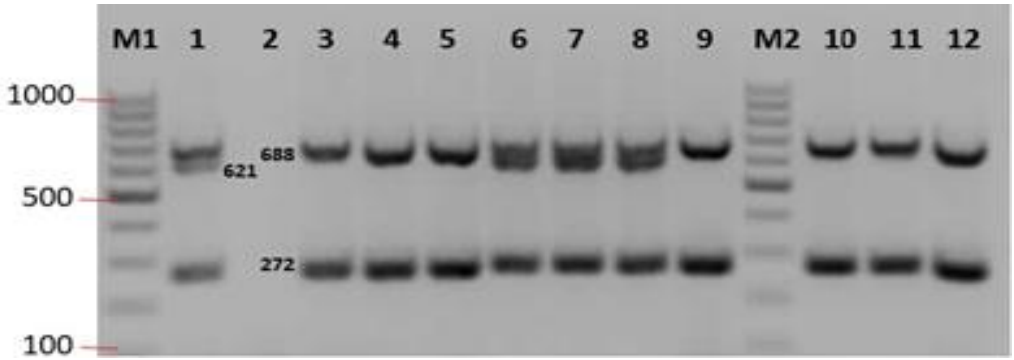

Figure 4.7 Multiplex PCR amplification of *cps* genes of *S. agalactiae*. Lanes 3, 4, 5, 9, 10, 11 and 12 are the band size for serotype Ia (272 bp and 688 bp); Lanes 6, 7 and 8 are the band size for serotype Ib (272 bp, 621 bp and 688 bp).

Taking together the results from API 20STREP, species-specific duplex PCR and *16S rRNA* gene sequencing, most of the streptococci isolates specifically *S. agalactiae* were accurately identified by the three methods except for isolates 100, 101 and 102 which cannot be identified by the API 20 STREP. The *S. iniae* isolates were not positively identified by the API 20 STREP but only by the species-specific duplex PCR and *16S rRNA* gene sequencing. The identification based on the three methods are summarised in Table 4.6.

Table 4.6 Identification results of the streptococci isolates from the three tests.

Isolate	Identification Test			
	API 20 STREP	**ID using Buller and *Streptococci* profile in this study**	**Species-specific Duplex PCR**	***16S rRNA* Gene sequencing**
NCIMB 701348*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*
ATCC 29178**	*S. dysagalactiae* ssp. *equisimilis*	*S. iniae*	*S. iniae*	*S. iniae*
7	Indeterminate	*S. iniae*	*S. iniae*	*S. iniae*
9	Indeterminate	*S. iniae*	*S. iniae*	*S. iniae*
16	Indeterminate	*S. iniae*	*S. iniae*	*S. iniae*
18	Indeterminate	*S. iniae*	*S. iniae*	*S. iniae*
25	Indeterminate	*S. iniae*	*S. iniae*	*S. iniae*
31	Indeterminate	*S. iniae*	*S. iniae*	*S. iniae*
105	Indeterminate	*S. iniae*	*S. iniae*	*S. iniae*
64	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*
97	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*
100	Indeterminate	Indeterminate	*S. agalactiae*	*S. agalactiae*
101	Indeterminate	Indeterminate	*S. agalactiae*	*S. agalactiae*
102	Indeterminate	Indeterminate	*S. agalactiae*	*S. agalactiae*
109	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*
113	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*
122	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*
SA132	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*	*S. agalactiae*

*NCIMB 701348 = *S. agalactiae* type strain; **ATCC 29178 = *S. iniae* type strain

The phylogenetic trees constructed, based on *16S rDNA* sequences of the *S. agalactiae* and *S. iniae* isolates and the reference strains of Streptococci species, strongly supported the identification results from both the conventional and molecular methods (Figure 4.8& 4.9). In *S. agalactiae* isolates, it can be seen that there are two subgroups which are subgroup A (*S. agalactiae* ATCC 13813T, 102, 132, 113, 122 & 109) and subgroup B (63, 97 & 101) while for *S. iniae* isolates there is only one.

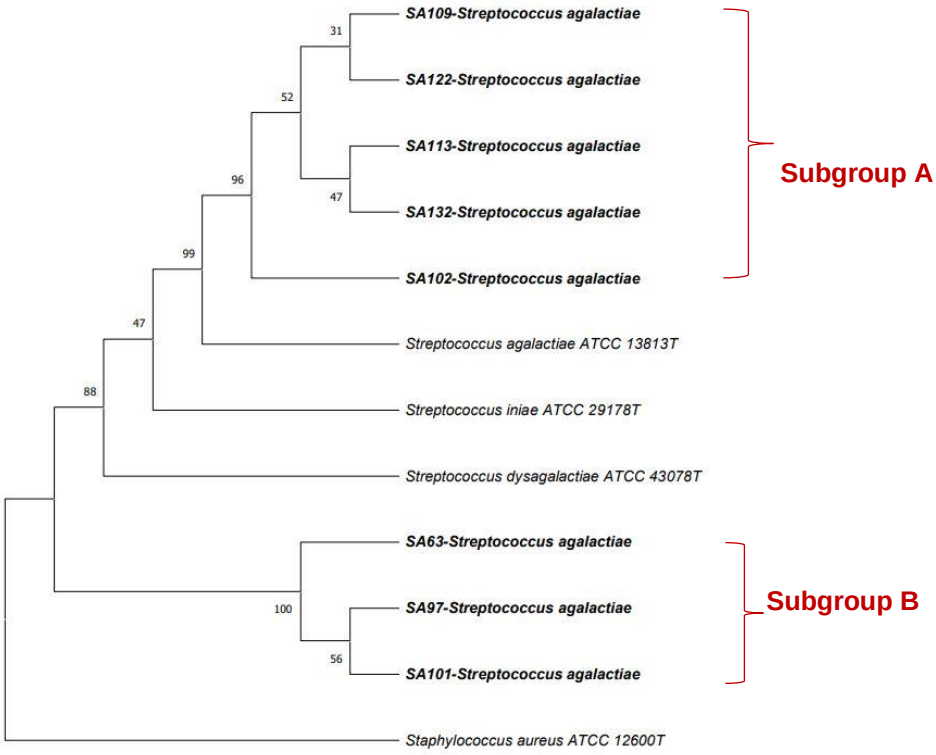

Figure 4.8 Phylogenetic tree was constructed based on the *16S rRNA* of the *S. agalactiae* isolates in this study (bold) and their closely related species. *Staphylococcus aureus* ATCC 12600T was selected as an out-group. Percentage bootstrap values (1000 replicates) are shown at each branch point.

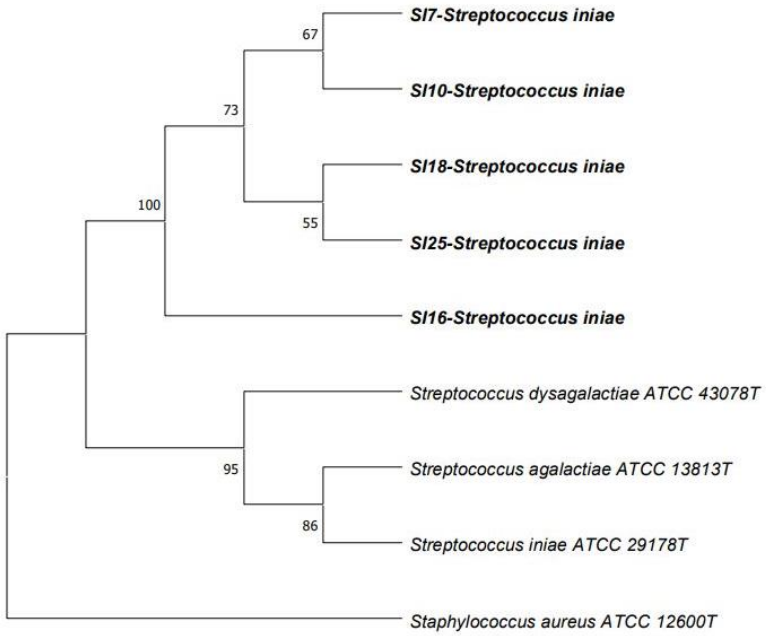

Figure 4.9 Phylogenetic tree was constructed based on the *16S rRNA* of the *S. iniae* isolates in this study (bold) and their closely related species. *Staphylococcus aureus* ATCC 12600T was selected as an out-group. Percentage bootstrap values (1000 replicates) are shown at each branch point.

4.4.1.5 Protein spectral profiles of streptococci isolates

Figures taken directly from the MALDI-TOF MS instrument have been provided in greater scale in the Appendix 3 section of this thesis.

Differences in the peaks from the MALDI-TOF analysis were visible between the *S. iniae* (blue font, Figure 4.10) and *S. agalactiae* strains (purple, green and red font, Figure 4.10). The *S. agalactiae* isolates all possess distinct peaks at approximately 4100, 4278, 6127, and 8195 m/z whereas the *S. iniae* isolate, distinctive peaks were observed at approximately 4737, 5361, 5953, 6276 and 6350 m/z. To make it easier to differentiate, the different peaks and their assignment for the streptococci isolates analysed are presented in Table

4.7. These species-specific peaks allowed for the differentiation between species *S. agalactiae* from *S. iniae*.

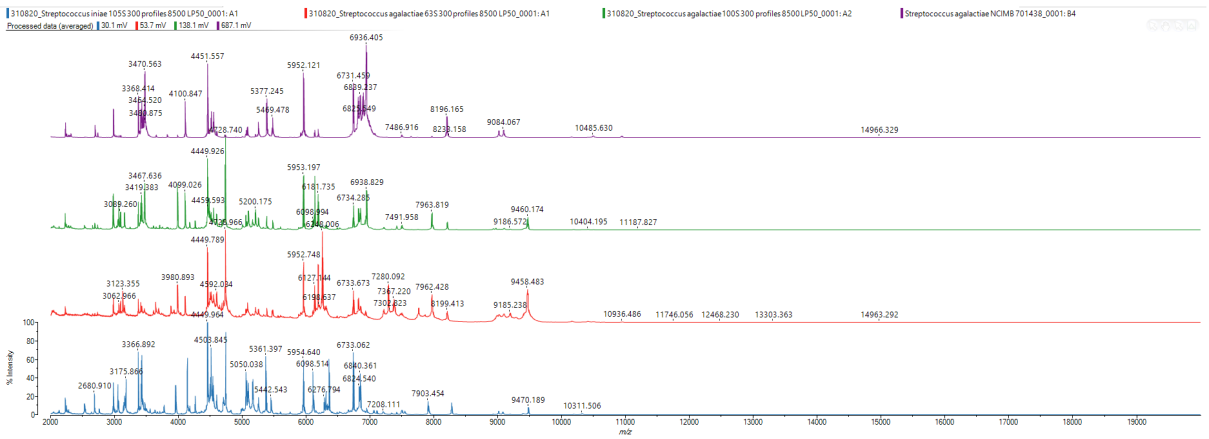

Figure 4.10 Depicts the optimal mass spectrum results achieved with isolates undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The four *Streptococci* isolates were grown for 48 hours before subculture and were procured at subculture 2. The blue spectrum exhibits isolate *S. iniae* 105, the red spectrum displays isolate *S. agalactiae* serotype Ia 63, the spectrum in green shows *S. agalactiae* serotype Ib 100 and the violet spectrum depicts *S. agalactiae* NCIMB 701348. (Appendix 3.1)

Table 4.7 Identification of *Streptococci* isolates by peak analysis by MALDI-TOF MS.

Isolate Number	***16S rRNA* ID**	**Peak analysis (m/z)**											
		4100	**4278**	**4737**	**5361**	**5953**	**6127**	**6181**	**6276**	**6181**	**6350**	**7900**	**8195**
ATCC 29178	*S. iniae*	-	-	+	+	+	-	-	+	-	+	+	-
9	*S. iniae*	-	-	+	+	+	-	-	+	-	+	+	-
25	*S. iniae*	-	-	+	+	+	-	-	+	-	+	+	-
105	*S. iniae*	-	-	+	+	+	-	-	+	-	+	+	-
63	*S. agalactiae*	+	+	-	-	-	+	+	-	-	-	-	+
100	*S. agalactiae*	+	+	-	-	-	+	+	-	-	-	-	+
101	*S. agalactiae*	+	+	-	-	-	+	+	-	-	-	-	+
102	*S. agalactiae*	+	+	-	-	-	+	+	-	-	-	-	+
122	*S. agalactiae*	+	+	-	-	-	+	+	-	-	-	-	+
132	*S. agalactiae*	+	+	-	-	-	+	+	-	-	-	-	+
NCIMB 701348	*S. agalactiae*	+	+	-	-	-	+	+	-	-	-	-	+

An attempt was made to use the protein profiles from the MALDI-TOF MS to differentiate between different serotypes of the *S. agalactiae* strains included in this study (Figure 4.11). For serotype Ia, a peak of approximately 6885 m/z was seen while for serotype Ib there were distinct two distinct peaks at approximately 4257 and 6248 m/z. The serotype II which is *S. agalactiae* NCIMB 701348 did not have these peak masses seen in both serotypes Ia and Ib. The different peaks and their assignment on the different *S. agalactiae* serotypes are presented in Table 4.8.

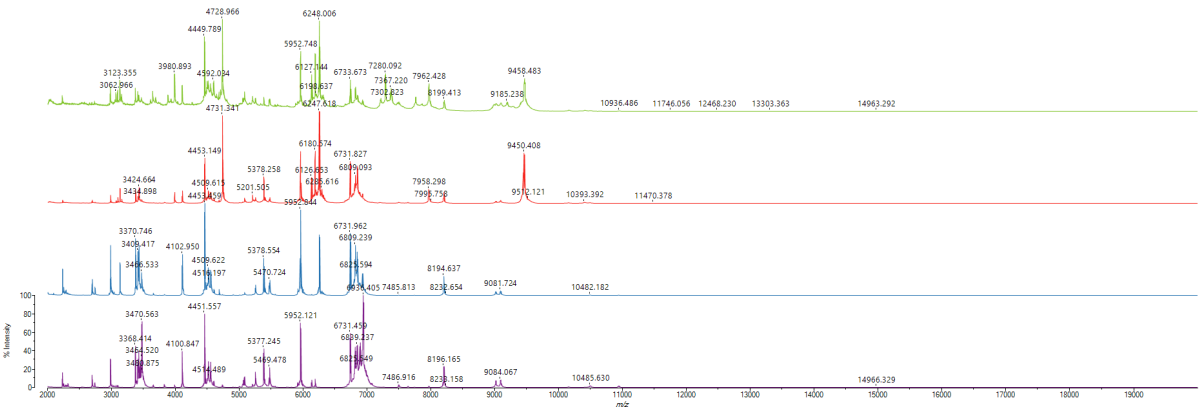

Figure 4.11 Depicts the optimal mass spectrum results achieved with isolates undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The four *Streptococci* isolates were grown for 48 hours before subculture and were procured at subculture 2. The violet spectrum exhibits isolate *S. agalactiae* NCIMB 701348, the blue spectrum displays isolate 122, the spectrum in red shows 132 and the yellow green spectrum depicts isolate 63. (Appendix 3.2)

Table 4.8 Identification of *S. agalactiae* serotypes by peak analysis by MALDI-TOF MS.

Isolate Number	16S rRNA ID	Serotype	Peak analysis (m/z)		
			4257	6248	6885
63	*S. agalactiae*	Ia	-	-	+
122	*S. agalactiae*	Ia	-	-	+
132	*S. agalactiae*	Ia	-	-	+
100	*S. agalactiae*	Ib	+	+	-
101	*S. agalactiae*	Ib	+	+	-
102	*S. agalactiae*	Ib	+	+	-
NCIMB 701438	*S. agalactiae*	II	-	-	-

4.4.2 Viable growth in different incubation temperatures and salt concentrations

All streptococci isolates were able to grow at 15 to 43° C except for *S. iniae* isolate 31 which was only able to grow until 37° C (Figure 4.12). For salinity tolerance, both *S. agalactiae* and *S. iniae* isolates were able to grow 0.5 to 5 % NaCl (Figure 4.13).

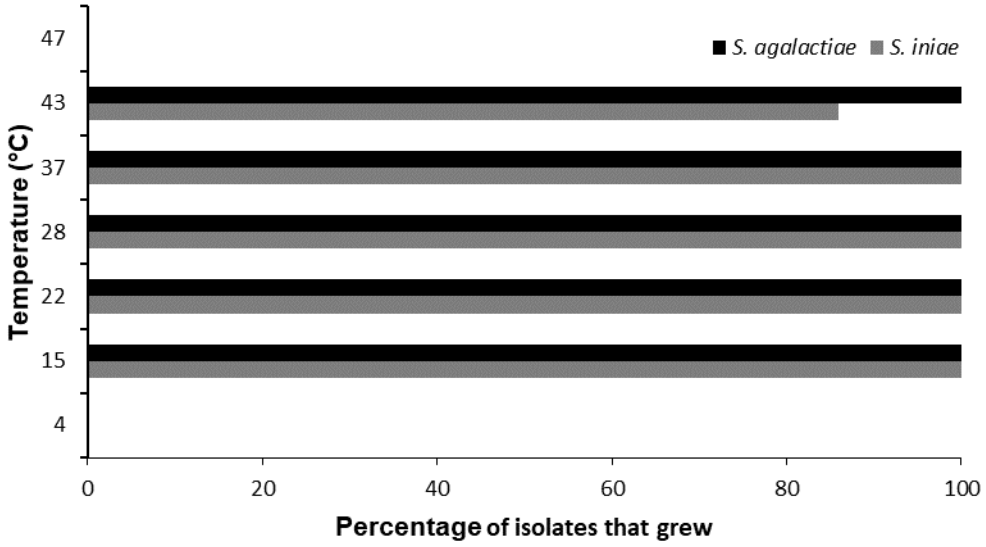

Figure 4.12 The total percentage of *S. agalactiae* (n = 18) and *S. iniae* (n =7) isolates with viable growth in different incubation temperatures.

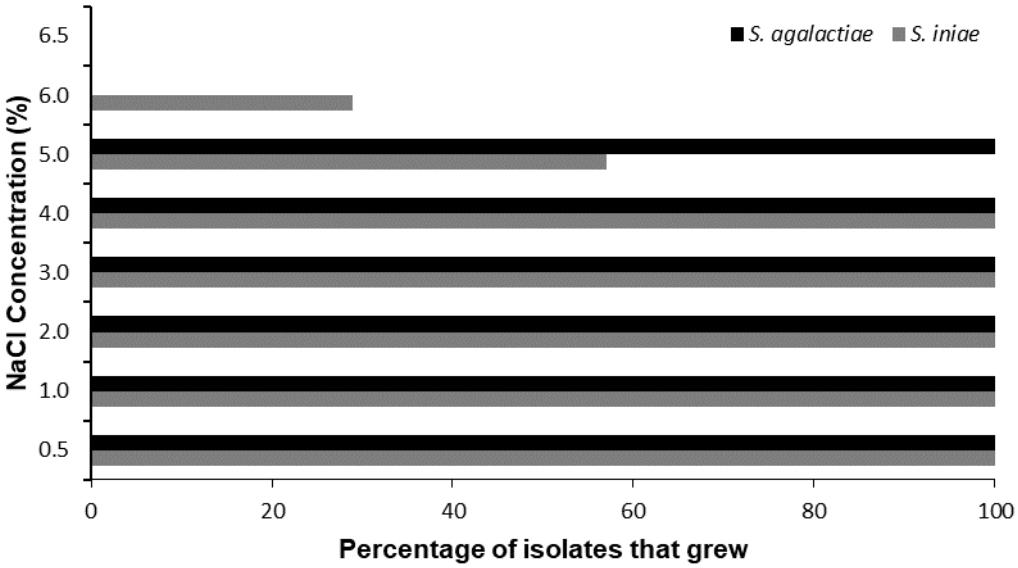

Figure 4.13 The total percentage of *S. agalactiae* (n = 18) and *S. iniae* (n =7) isolates with viable growth in different salt concentrations.

4.4.3 Histopathology

A range of histopathology changes were observed from the moribund tilapia infected with both *S. iniae* and *S. agalactiae*. By far, the greatest histological changes were encephalitis (Figure 4.14) or meningitis (Figure 4.15) which was observed in 20 diseased fish (80%) where the streptococci bacteria were recovered. Other histological changes included myonecrosis, endocarditis, myocarditis, and pericarditis.

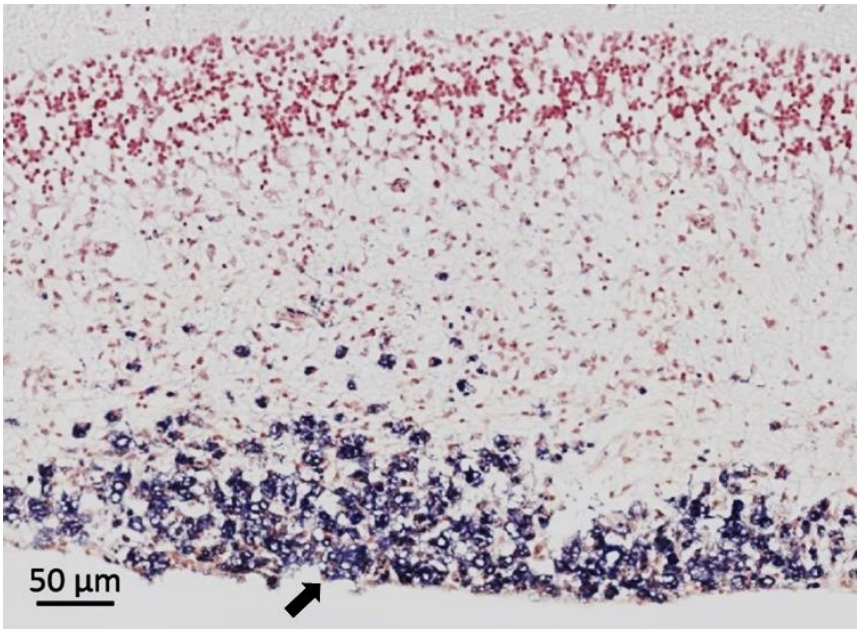

Figure 4.14 Gram-stained section of the basal layer of the optic tectum showing encephalitis (arrow) with infiltration of bacteria-filled macrophages.

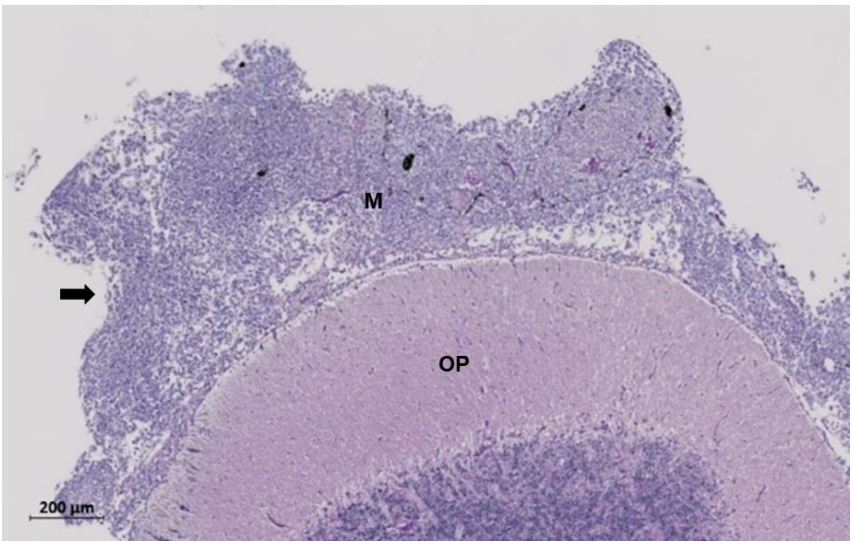

Figure 4.15 H&E stained section of the brain showing massive cellular meningitis (arrow, M) encapsulating the optic tectum (OP) of the brain.

Histiocytosis and increased melanomacrophage centres were noted in the spleen, and glomerulonephritis, interstitial necrosis and tubular degeneration were observed in the kidneys. The liver presented separation of hepatocytes, perivascular inflammation, necrosis and vacuolation. Mononuclear

and bacterial infiltration (Figure 4.16) was seen in most of the sampled organs. These changes were similar to previous descriptions of clinical streptococcosis outbreaks in farmed tilapia (Asencios et al., 2016; Ortega et al., 2017).

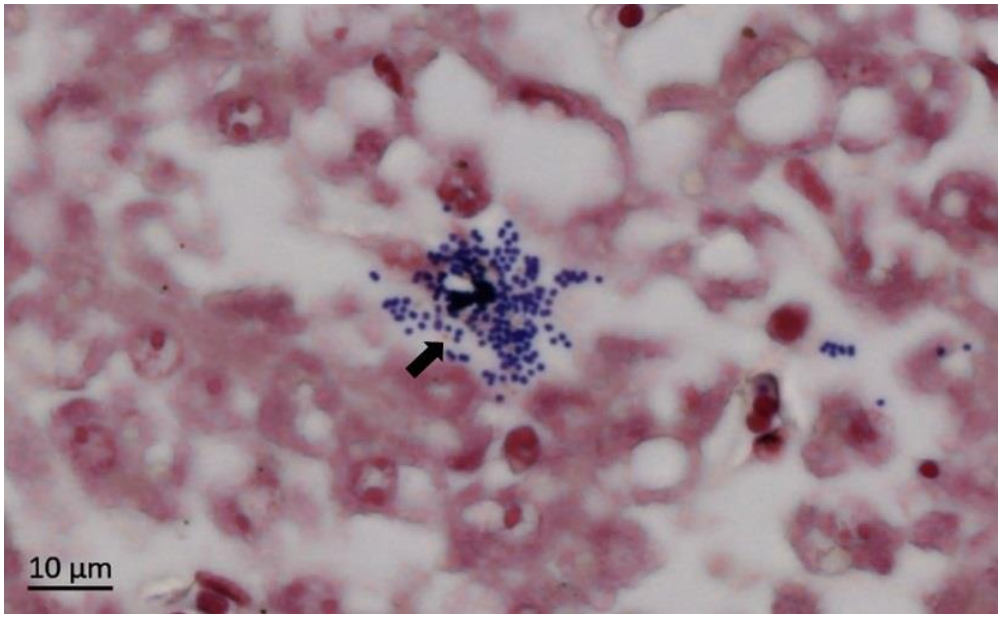

Figure 4.16 Gram-stained section of the liver showing proliferation of streptococci bacteria (arrow).

Histological analysis of the brain tissues of clinically sick tilapia showed subtle differences between *S. iniae*, *S. agalactiae* Ia and *S. agalactiae* Ib. Both *S. iniae* and *S. agalactiae* Ib showed meningoencephalitis with bacteria-filled macrophages in brain cortex (Figure 4.17A). For *S. agalactiae* Ia, although a large number of bacteria was present in the brain capillaries (Figure 4.17B), there was mild meningitis of the brain and absence of meningoencephalitis. However, *S. agalactiae* Ia presented high number of bacteria in the sampled internal organs like the heart (Figure 4.18A) and the kidney (Figure 4.18B).

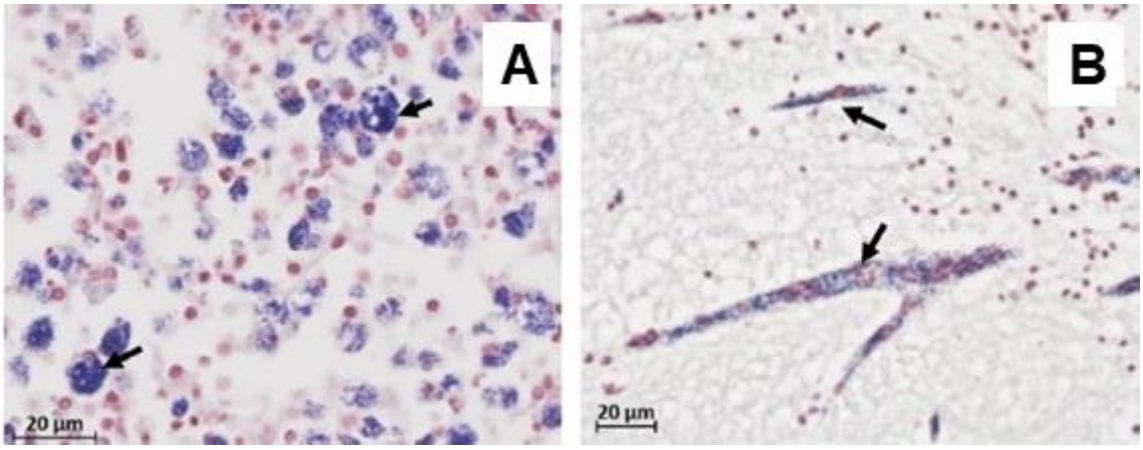

Figure 4.17 Gram-stained sections of (A) brain of tilapia with meningoencephalitis due to *Streptococcus iniae* showing macrophages (arrows) filled with bacteria; (B) meningitis in tilapia infected with *Streptococcus agalactiae* showing bacterial infiltration (arrow) in the brain capillaries.

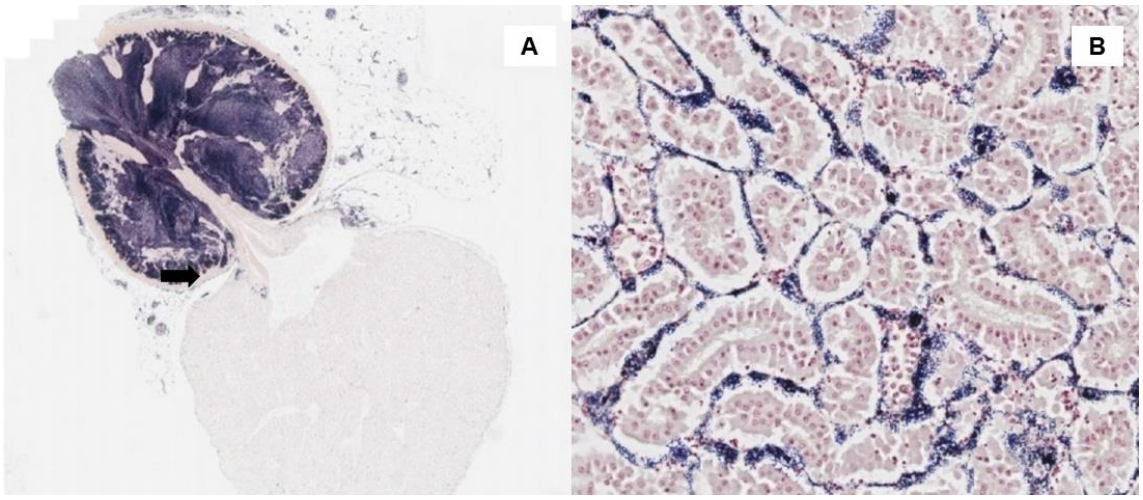

Figure 4.18. Gram-stained section of the (A) heart and (B) kidney showing massive infiltration of Gram-positive cocci bacteria

.

4.4.4 Antibiotic susceptibility and antibiotic resistance determinants

4.4.4.1 Antibiotic susceptibility results and detection of antibiotic resistance genes

The antibiotic susceptibility showed that 100% (*n* = 7) of *S. iniae* isolates were resistant only to oxolinic acid only and susceptible to all other antibiotics tested. For the *S. agalactiae* isolates, 100% (*n* = 18) were resistant to oxolinic acid and 17% (*n* = 3) were resistant to sulphamethoxazole trimethoprim. They were susceptible to all other antibiotics tested. No antibiotic resistance genes were detected in both isolates of *S. iniae* and *S. agalactiae* in this present study. The result of antibiotic response profile and antibiotic resistance genes of the isolates are summarised in Table 4.9.

Table 4.9 Antibiotic response profiles of the different *Streptococci* isolates.

Streptococci Isolate	Multiresistance*	Resistant to**	Susceptible to**	AMR Gene/s Present***
S. iniae 7	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. iniae 9	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. iniae 16	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. iniae 18	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. iniae 25	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. iniae 31	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. iniae 105	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 63	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 97	-	OA	TE, OT, E, ENR, FFC, C, OA	-
S. agalactiae 100	-	OA, SXT	AML, AMP, P, TE, OT, E, SXT, ENR,	-

			FFC, C, VA	
S. agalactiae 101	-	OA, SXT	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 102	-	OA, SXT	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 106	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 108	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 109	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 113	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 117	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 122	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 126	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 131	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 132	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 135	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 138	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 139	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-
S. agalactiae 141	-	OA	AML, AMP, P, TE, OT, E, SXT, ENR, FFC, C, VA	-

* + = resistant to 2 or more antibiotic class; - = resistant to only 1 antibiotic class

**AML = amoxicillin; AMP = ampicillin; P = penicillin; TE = tetracycline; OT = oxytetracycline; E = erythromycin; SXT = sulfamethoxazole-trimethoprim; ENR = enrofloxacin; FFC = florfenicol; C = chloramphenicol; OA = oxolinic acid; VA = vancomycin.

***- = absent

4.4.5 Virulence and virulence genes

4.4.5.1 Phenotypic virulence determinants

Presence of capsule. All *S. iniae* and *S. agalactiae* isolates tested showed the presence of capsule (Figure 4.19B & C) similar to the positive control *Bacillus megaterium* (Figure 4.19A). In the photomicrograph, the bacterial cells and the proteinaceous background appear purplish with the capsule forming a transparent outline surrounding the bacterium.

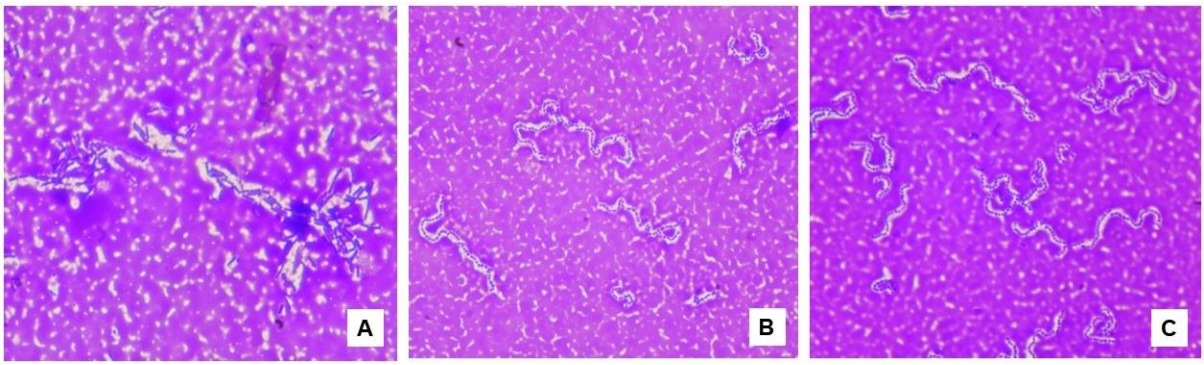

Figure 4.19 The capsules of (A) *Bacillus meagterium* (B) *S. iniae* and (B) *S. agalactiae* isolates under 1000x magnification.

Haemolysis. All *S. iniae* isolates were β-haemolytic. As for *S. agalactiae*, all serotype Ia were β-haemolytic while all serotype Ib were non-haemolytic. As seen in Figure 4.20, the transparent outline around the bacterial colonies are indicative of haemolytic reaction.

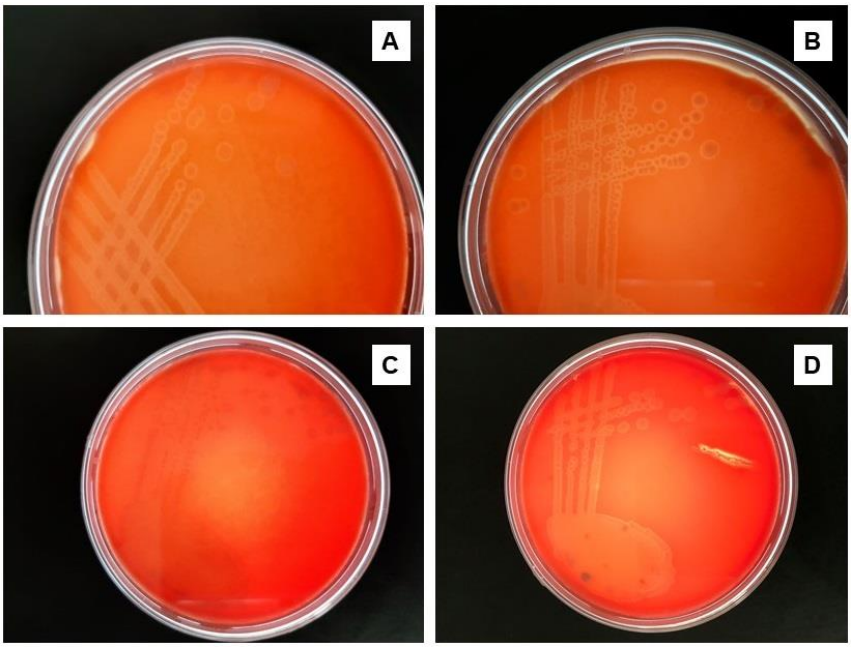

Figure 4.20 Streptococcal isolates on 5% sheep blood agar. Positive haemolytic reaction is indicated by transparent outline around bacterial colonies. A – *S. iniae* isolate; B – *S. agalactiae* serotype Ia isolate; C – *S. agalactiae* serotype Ib isolate; D – *S. iniae* ATCC 29178.

4.4.5.2 Detection of virulence genes

Virulence Genes of S. iniae. All six virulence genes *sagaA* and *pdi*, *simA* and *pgm*, and *cpsD* and *scpI* were detected for all *S. iniae* strains (n=7). The haemolytic activity of the isolates was due to *sagA* gene. The ability to survive in the fish blood and from lysozyme killing is mediated by the *pdi* gene while adherence and resistance to phagocyte killing is contributed by *sim* gene.

The phenotypic (expression) and genotypic (gene detection) virulence profiles for the *S. iniae* strains tested were identical (Table 4.10).

Table 4.10 Phenotypic and genotypic virulence determinants in *S. iniae* isolates.

Streptococcal Isolate	Capsule	Haemolysis	Virulence Gene					
			simA	***scpI***	***pgm***	***cpsD***	***pdi***	***sagA***
7- *S. iniae*	+	+, β	+	+	+	+	+	+
9- *S. iniae*	+	+, β	+	+	+	+	+	+
16- *S. iniae*	+	+, β	+	+	+	+	+	+
18- *S. iniae*	+	+, β	+	+	+	+	+	+
25- *S. iniae*	+	+, β	+	+	+	+	+	+
31- *S. iniae*	+	+, β	+	+	+	+	+	+
105- *S. iniae*	+	+, β	+	+	+	+	+	+

Virulence Genes of *S. agalactiae*. For *S. agalactiae* isolates, all virulence factors *fbsA* and *cylE, cfb*, *cspA*, *hylB, pbp1a/pona*, and *PI-2B* were detected in 15 strains. Three strains of *S. agalactiae* (BSMF1, BSMF-4 & SMF1) were absent for gene *cylE*. From these data 2 virulence profiles for the *S. agalactiae* strains were constructed: profile I (all virulence genes present) and profile II (absent gene *cylE*). This virulence profiles of the *S. agalactiae* strain are summarised in Table 4.11.

Table 4.11 Phenotypic and genotypic virulence determinants in *S. agalactiae* isolates.

Streptococcal Isolate	Capsule	Haemolysis*	Virulence Gene						
			cfb	***cspA***	***cylE***	***fbsA***	***hylB***	***PI-2B***	***pbp1A/ponA***
63- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
97- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
100- *S. agalactiae*	+	-	+	+	-	+	+	+	+
101- *S. agalactiae*	+	-	+	+	-	+	+	+	+
102- *S. agalactiae*	+	-	+	+	-	+	+	+	+
106- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
108- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
109- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
113- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
117- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
122- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
126- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
131- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
132- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
135- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
138- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
139- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+
141- *S. agalactiae*	+	+, β	+	+	+	+	+	+	+

* β = beta hemolytic

4.5 Discussion

Streptococcosis is one of the most significant diseases of tilapia worldwide (Anshary et al., 2014) which is frequently reported to be caused by *S. iniae* and *S. agalactiae* (Perera et al.1994; Eldar et al.1995; Bowser et al.1998; Salvador et al.2005; Suanyuk et al.2005,2010; Yuasa et al.2008; Hernandez et al.2009; Musa et al.2009; Abuseliana et al.2010; Lusiastuti et al.2012; Najiah et al.2012; Figueiredo et al.2012). Although streptococcosis has been reported affecting the tilapia sector in the Philippines, there was no comprehensive study

on the streptococci species especially on their pathogenic mechanisms and antibiotic resistance. This chapter confirmed the diagnostic findings from Chapter 3 where *S. agalactiae* and *S. iniae* were isolated from different tissues of diseased tilapia. Moreover, the histopathology results positively showed the presence of Gram-positive bacteria most especially in the brain, which is often expressed as target organ for streptococcal infections in tilapia (Su et al., 2015).

The evidence presented in this chapter showed a clear diagnosis of Streptococcosis in the diseased tilapia, exhibiting clinical signs of disease and both S*. agalactiae* and *S. iniae* were recovered from these fish. A wide range of identification methods have been applied to differentiate streptococcal species and confirm serotypes in *S. agalactiae* populations occurring in farmed tilapia (Barkham et al., 2019; Mishra et al., 2018).

Prior to the rapid uptake of molecular probes, bacterial identification methods relied on the use of traditional and biochemical assays. Whilst primary identification tests e.g., Gram stain, oxidase, catalase, OF and motility remain the cornerstone of any bacterial identification work, biochemical profiles can be inconsistent particularly for the Gram-positive piscine strains. Whilst Suanyuk et al. (2008) and Sun et al. (2016) demonstrated good level of accuracy with API 20 STREP kit in identifying GBS where all the isolates in their study belong to serotype Ia, a similar finding was not determined in the current study. The results showed that for the Philippine strains a good level of agreement was found using the API 20 STREP kit, but when compared with results from other published studies, comparable profiles could not be obtained. There was more variability in the biochemical profiles from the Philippine strains which correlated well with the clustering of the strains between phenotypic profile and serotype.

However, this was a very small sample size and further work is required to determine how accurate this test may be to differentiate serotypes. Other authors have also found the biochemical test kits to be less reliable in species identification (Deasy et al., 2000; Garcia-Garrote et al., 2000; Jackson et al., 2004). It is difficult to determine where the variability may lie, is it with kit itself or perhaps with the technical performance or even simple strain variation. The observed reduction in test positivity of these *S. agalactiae* strains in the current study may indicate a slow or impaired metabolic activity by the bacterium or an adaptation to help establish infection in fish (Gori et al., 2020; Lannes-Costa et al., 2021).

For the *S. iniae* isolates, the API 20 STREP database identified them as having unacceptable profile since *S. iniae* is not in the database of API 20 STREP and the best possible identification for its profile would be “unable to identify or unacceptable profile” (Facklam et al., 2005). Moreover, the database results provided a “misidentification” match for the type strain of *S. iniae* ATCC 29178. This was similar to the report of Koh et al. (2004) where their streptococcal isolate was also identified by API 20 STREP as *S. dysagalactiae* ssp*. equsimilis* (SDSE) but *16S rRNA* gene sequencing revealed it was *S. iniae* identical to the ATCC 29178 which is the type strain first recovered from the Amazonian dolphin in 1971. The profile number of 4563117 in the API database should not identify it as SDSE since this profile number has a positive test for pyrrolidonyl-arylamidase while SDSE is negative (Takashi et al., 2011). Although the API 20 STREP kit does not have a code for *S. iniae*, the bacteria isolated from the diseased tilapia gave essentially identical profiles and were identifiable by using a traditional identification key from Buller and from

comparison with the API 20 STREP profiles from previous studies of piscine *S. iniae*. This data is valuable since it can be used to create a profile specific for fish-adapted *S. iniae* thus complementing the limitation from API 20 STREP database which can be used in rapid and easy identification of the pathogen especially in devising treatment for affected fish farms. However, not all laboratories will be able to provide their own databases and so the use of rapid API 20 STREP kit or conventional tube biochemical tests in identifying piscine *S. agalactiae* and *S. iniae* still will give an accurate identification as long as analysts take cognizance of the limitation of the API kit.

Whilst these limitations of rapid kits in identification of aquatic bacterial pathogens is not new information, it is very important in disease diagnostics since most fish health laboratories in developing countries where aquaculture activities are intensive do not have the technical and logistical capabilities to perform molecular identification tests. Therefore, there is continued need to use traditional and where possible more advanced methods. It is questionable now, to say if simple 16S PCR sequence is “advanced” as it has become more common place in many aquatic labs, yet it is not considered a core method. Instead, facilities will rely on a mixture of basic and molecular tools, which may be the most cost-effective. For example, the starch hydrolysis test is a simple yet reliable way of differentiating between the two species and in this study, all *S. iniae* isolates gave positive reaction while all *S. agalactiae* were negative. Although *S. agalactiae* are generally negative for starch hydrolysis, there are some strains that can give a positive reaction (Evans et al., 2004) thus additional confirmatory tests are needed to accurately identify streptococci

species. The value of this test is that it is rapid and cost-effective thus increasing the number of isolates that can be tested at once.

In this study, we found excellent agreement between the duplex species-specific PCR and the *16S rRNA* sequencing in identification of both the streptococcal species. The duplex-PCR method is relatively simple and cheap compared with *16S rRNA* gene sequencing and can be performed within most laboratories with limited training. However, this does not replace the need for more advanced molecular epidemiology methods, for example WGS, but it could be a sensible first step in understanding the aetiology and efficacy of any biosecurity or health management programmes.

From the present data, 2 serotypes of *S. agalactiae* appear to be circulating in the Philippine tilapia farming systems with a slightly higher prevalence of serotype Ia. This serotype was more frequently recovered from the clinically sick grow-out and broodstock tilapia, while serotype Ib was only isolated from grow-out fish samples. Serotyping of *S. agalactiae* isolates is important when considering vaccine development as cross-protection between the different bio/serotypes does not occur in fish (Munang'andu, Paul, & Evensen, 2016). The results from the Philippines agree with data from surrounding SEA countries and China which all reported *S. agalactiae* serotype Ia as the most prevalent and widespread cause of streptococcosis in freshwater tilapia aquaculture (Kannika et al., 2017; Kayansamruaj et al., 2019; Lusiastuti, Textor, Seeger, Akineden, & Zschock, 2014; Su et al., 2019; Syuhada et al., 2020). In this study, *cps* gene serotyping was applied which is used widely and successfully by others as a reliable way to serotype *S. agalactiae* in fish (Kannika et al., 2017). While we recognize the limited sample size in this study,

our findings were similar to surrounding SEA countries (Kannika et al., 2017; Kayansamruaj et al., 2019; Lusiastuti, Textor, Seeger, Akineden, & Zschock, 2014; Su et al., 2019; Syuhada et al., 2020). To address this limitation, both active and passive surveillance should be undertaken by the Bureau of Fisheries and Aquatic Resources in order to collect more samples and do a comprehensive study to further characterise these *S. agalactiae* pathogens to determine the sequence types, genetic and proteomic profiles, and exposure/transmission routes of the serotypes which will be vital for future vaccination programmes in the Philippines.

There is always a desire to have accurate and rapid disease diagnosis, particularly in aquaculture where so many variables can influence the disease outcome. Application of the MALDI-TOF MS method has many adbantages over more traditional profiling: rapid time to result, reduced consumable cost, improved quality of result, does not require high technical skills since sample preparation technique is simple, and may aid in diagnosis of hard-to-identify bacteria (Kostrzewa & Nagy, 2016). In this study, protein mass spectra of both *S. agalactiae* and *S. iniae* isolates showed subtle differences by peak analysis. Grouping the streptococci by their specific peaks was found to correlate with the identification by *16S rRNA* gene sequencing. Additionally, different serotypes of *S. agalactiae* possess specific peak/s that can be used to discriminate them visually, and this also agreed with molecular serotyping using the capsular gene. The discrimination of *S. agalactiae* and *S. iniae* isolates in this study by spectral profile analysis is similar to the results of Piamsomboon et al. (2020) where they were also to differentiate between the piscine *S. agalactiae* and *S. iniae*. This study also attempted to use spectral profile to distinguish different

serotypes of *S. agalactiae* since the study of Rothen et al. (2019) showed that MALDI-TOF can predict their probable capsular serotype.

The peak in the spectral profile needs further tests in order to determine their biological significance such as their involvement in pathogenesis of streptococcosis in fish. This include tests such sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) which will visually show different protein fractions by their relative molecular weights. Then these protein bands from SDS-PAGE can be digested and analysed using liquid-chromatography with tandem mass spectrometry (LC-MS/MS) in order to derive the sequences of a specific peptide for identification. Over-all, the result showed indication that spectral profile peak analysis can speciate *S. inia*e and *S. agalactiae* and also shows indication that it can recognise different capsular serotypes of *S. agalactiae*. However, the limitation of this study is the small sample size that can affect its accuracy. Moreover, further studies should be undertaken such as methods of sample preparation and different percentage of acid used since it can result to inconsistent protein spectral profile (Piamsoboon et al., 2020), and to identify species-specific peaks for protein finger printing in order to aid in the identification. Although MALDI-TOF has the advantage in terms of its rapidity, reliability, and low cost (Rosa et al., 2019), the limitation of this method is its dependence on pure bacteria isolates which PCR methods are more advantageous since it can directly extract genomic DNA from clinical samples and perform identification. This limitation of MALDI-TOF in rapid and accurate pathogen identification is more pronounced in difficult-to-isolate-and-culture bacteria, especially those that are of intracellular in nature, however, for common bacterial pathogens the implementation of MALDI-TOF MS plus peak

analysis for the identification of this group of microorganisms may provide precise species-level information that will allow the early implementation of directed antibiotic therapy (Marin et al., 2017).

In the Philippines, the use of antibiotics in tilapia aquaculture is not common because of the prevailing view that tilapia are resistant to diseases. However, recent episodes of disease outbreaks leading to mass mortality, has led to some fish farmers starting to use antibiotics particularly amoxicillin which is administered orally mixed with feed. The antibiotic susceptibility profile of the Philippine strains was similar to previous reports in Thailand (Jantrakajorn et al., 2014; Dangwetngam et al., 2016; Kannika et al., 2017; Klingklib & Suanyuk, 2017) specifically on their susceptibility to florfenicol and amoxicillin, and their resistance to oxolinic acid. However, emerging resistance to oxytetracycline, enrofloxacin, erythromycin, ampicillin, and sulfamethoxazole-trimethoprim were noted for both *S. iniae* and *S. agalactiae* isolates from Nile tilapia and red hybrid tilapia in Thailand (Janktrakajorn et al., 2014) which currently is still absent in the Philippine streptococci isolates. The resistance to oxolinic acid of the streptococci isolates is primarily mediated by chromosomal mutations in topoisomerase genes and mutations that decrease drug accumulation by reducing uptake or increasing efflux (Miranda *et al.*, 2013; Dangwetnam et al., 2016). For sulfamethoxazole-trimethoprim, resistance was observed only in *S. agalactiae* serotype Ib isolates. Resistance to these antibiotics is mediated by the permeability barrier and/or efflux pumps, naturally insensitive target enzymes, regulational, mutational or recombinational changes in the target enzymes genes and acquired drug-resistance genes (Huovinen, 2001). Over-all the Philippine piscine streptococci isolates were susceptible to other antibiotics

tested in this study. This antibiotic susceptibility profile is similar to most strains of *S. iniae* and *S. agalactiae* from mammalian origin that they are susceptible to β-lactams, macrolides, quinolones, and vancomycin, with penicillin being the drug of choice for treating streptococcal infections when antimicrobial therapy is required (Facklam et al., 2005). The use of antimicrobials in fish culture situations does have some limitations and concerns. The first is selection for resistance amongst dense populations. An evidence of selection for an enrofloxacin-resistant isolate was noted by Stoffregen et al. (1996). Development of resistance may also be increased when bacteria are not fully eliminated from fish or their environment. The ability of streptococci to survive in macrophages (Zlotkin et al., 2003) and the failure of sick fish to eat sufficient quantities of medicated feed may contribute to the development of resistant strains or carrier fish (Shoemaker et al., 2001).

Published studies on the presence of antibiotic resistance genes (ARGs) in piscine streptococci are few. However, it is important to know the genetic and molecular mechanisms for the observed phenotypic resistance to antibiotics in order to determine their clinical relevance (i.e., whether they confer low- vs. high-level clinical resistance, their ability to be mobilized by lateral gene transfer, and their frequency of occurrence) (Miranda et al., 2013). In this study, no ARGs such as β-lactam, tetracycline, sulfonamide, and trimethoprim were detected in the streptococci isolates, even though there were observed phenotypic resistance to oxolinic acid and sulfamethoxazole-trimethoprim. This may indicate the low use of antibiotics in the tilapia farming sector in the Philippines. In contrast, macrolide resistance gene (*ermB*) and tetracycline resistance (*tetM*) were detected in *S. agalactiae* isolates from Nile tilapia in

China. Presently, China is the world's largest tilapia producer and exporter (Yuan et al., 2020). The intensified production and use of antibiotics to meet global demand could play a significant role in selecting bacterial strains with antibiotic resistance genes. Pathogens can evolve resistance to treatments—leading to the re-emergence of disease problems e.g. bacteria evolve resistance to antibiotics (Karunasagar et al., 1994). Thus, knowledge of ARGs from aquaculture environments is important to design and prioritize monitoring programs that may generate data that eventually becomes relevant for performing quantitative risk assessments and develop sound treatment strategies to control fish disease.

In this study, all three functional categories of virulence genes namely adhesins, invasins and immune evasins were detected in all isolates revealing their pathogenic and invasive abilities. Identical virulence gene profiles were found for all the *S. iniae* strains recovered from infected fish which may suggest that they have arisen from a single clone as these strains were all recovered from a single site which is Taal Lake. This agrees with the result of Baum et al. (2013). These virulence genes may be universally present in *S. iniae*, however more strains from different areas should confirm this. In the PCR amplification of the phosphoglucomutase (*pgm*) gene, Baum et al. (2013) indicated the amplicon product of the designed primer as 730 bp while upon examination of the primer by primer blast (NCBI), the amplification product should be 513 bp. In the present study, PCR amplification of the *pgm* gene resulted in a band of approximately 500 bp which was similar to the expected size in the NCBI database. To further confirm the identity of the PCR product for the *pgm* gene, it

was submitted for gene sequencing and the result confirmed this to be the *pgm* gene contrary to the expected size of 730 bp in the study of Baum et al. (2013). Detecting the presence of a particular virulence factor by PCR does not provide any evidence of whether this is expressed during the disease process. In this study, capsule and haemolysis expression were confirmed, *cpsD* and *sagA* genes were all present in the *S. iniae* strains, but the expression were determined through Anthony's capsule staining and sheep blood haemolysis, respectively. These kinds of tests should also be extended to other virulence genes to ascertain their expression; however, these may be difficult and limiting since most of the virulence genes are only expressed when the bacteria is in contact with the host tissues or cells. This presence of virulence may help explain the invasiveness of the *S. iniae* isolates as observed in the tissues of diseased Nile tilapia. The bacteria were found in different organs like liver, spleen, kidney, and the brain. Histopathological findings in the brain revealed meningoencephalitis which will only be possible if these virulence factors are present in the strain.

A study of Delannoy et al. (2013) examined the presence of virulence genes of piscine *S. agalacatiae*, which became the basis for the detection of these factors in the *S. agalactiae* isolates from the Philippines. All virulence genes were present in *S. agalactiae* serotype Ia while the *S. agalactiae* serotype Ib lacked the *cylE* gene. This is the structural gene involved in β-haemolysis/cytolysis of the red blood cells (Pritzlaff et al., 2001). The lack of *cylE* gene was confirmed by the lack of haemolysis expressed in the 3 non-haemolytic *S. agalactiae* isolates tested when grown on sheep blood agar. The serotype Ia isolates from Thailand and Vietnam (Kayansamruaj, 2019) share

the same patterns of virulence genes with the Philippine isolates. Studies have shown that β-haemolysin is a virulence factor that influences *S. agalactiae* survival in macrophages (Doran, Liu & Nizet, 2003; Sagar et al., 2013) and promotes infection of the less or non-haemolytic strains by their ability to evade the host immune response and remain dormant inside macrophages until suitable conditions for their reactivation. The presence of *cylE* gene in *S. agalactiae* serotype Ia is believed to promote invasiveness (Chu et al., 2016) supporting rapid spread in the bloodstream and organs of the infected host, while its absence is considered a factor in the development of more chronic infection in fish (Li et al., 2014). For the nonhaemolytic *S. agalactiae* serotype Ib isolates, the observed numerous bacteria-filled macrophage cells in the brain resulting in meningoencephalitis was indicative of a more chronic type of infection although we do know if they are viable or not. The loss of the *cylE* gene in *S. agalactiae* serotype Ib may actually be an evolutionary advantage to the strain. Although the absence of haemolysis decreases the severity of the infection and invasion leading to decrease in host mortality, it increases the ability of the pathogen to become established in a particular species in a particular geographic area. The loss of the *cylE* gene and the metabolic inactivity might actually increase the virulence of serotype Ib, since virulence evolves when there is a non-negligible trade-off between the rate of spread of infection versus host mortality (Bull, 1994, Frank, 1996, Ebert & Bull, 2003). In the study of Rosinski-Chupin et al. (2013), they observed that in fish adapted *S. agalactiae* serotype Ib specifically the ST 260-261, there was massive gene inactivation and broad changes in gene expression due to genome reduction. They detected the inactivation of the *cyl* locus which explained the absence of

haemolytic activity in this piscine strain which is similar to the serotype Ib strains in this study. Moreover, they found profound remodelling of the metabolic activity in the said sequence types reflecting a reduced capacity to utilise different carbon sources which was also seen in this study from the biochemical tests. The reduction in the catabolic capacities observed in these fish adapted *S. agalactiae* strains is in favour of a transition to an obligate pathogen style-of-life (Rosinski-Chupin et al., 2013). Evolution that increases transmission without affecting host mortality, or that reduces host mortality without decreasing transmission, will be beneficial to the pathogen (non-trade off adaptation) (Murray & Peter, 2005). This loss of the *cylE* gene might explain why *S. agalactiae* serotype Ib is implicated in sudden recurrent mortalities and chronic streptococcosis in tilapia in Brazil (Leal & Figueiredo, 2018). In the study of Owatari et al. (2020), they found that the bacteria can remain lodged in different organs like liver and spleen that are not commonly described as pathognomonic, in addition to haematological alterations. These subclinical lesions in the organs compromises physiological functions of the fish without revealing external clinical signs in the animals. This strategy of a chronic and recurrent infection by non-haemolytic *S. agalactiae* serotype Ib causing subclinical infections is indicative of an obligate pathogen-style of life as suggested by Rosinski-Chupin et al. (2013). This data is important to develop methods of efficiently eradicating these pathogenic strains as they have adapted towards an intracellular behaviour.

The results from the present study did show a limited number of SCV with the *S. agalactiae* strains which all belongs to serotype Ib, non-haemolytic, *cylE* gene deficient and SXT resistant. SCV's are typically resistant to antibiotics

like sulphonamides (Garcia et al., 2013) in which the SA-SCVs in the present study were resistant to SXT. These SCV are noteworthy because this is a form of the pathogenic bacteria that produces subacute infections leading to recurrent and chronic infections, reduced antibiotic susceptibility and resistance to oxidative burst (Proctor et al., 2006; Banno et al., 2014; Painter et al., 2017). This change from wild-type phenotype to SCV could be a strategy for *S. agalactiae* serotype Ib for increased intracellular survival within macrophages as similar to strategies used by SCV of *S. aureus* and *Salmonella sp*. Studies have shown that when environmental stress is removed such as using enriched media over standard cultured, the SCV can revert to WT albeit the revertant is not identical to the original WT progenitor or can produce alternative revertant phenotype (Proctor et al., 2006; Johns et al., 2015). However, the reversal from SCV to WT of the SA-SCV was not explored in this study at the moment. So far this is the first report of SA-SCV in piscine *S. agalactiae*. Currently, there is no or little study regarding the SCV form of piscine *S. agalactiae* and their implications in pathogenesis in fish.

In this present study, the farmed Nile tilapia (*O. niloticus*) which were naturally infected by *S. agalactiae* and *S. iniae* revealed several clinical manifestations consistent with streptococcosis including: lethargy, erratic swimming, exophthalmia, corneal opacity, dropsy, haemorrhage of the fin and internal organs. Histopathological abnormalities observed in the brain, kidney, spleen, heart, and liver tissues are suggestive of septicaemia as observed in natural and experimental streptococcal infection in accordance with those described in fish (Austin & Austin, 2007; Musa et al., 2009; Evans et al., 2006; Abdullah et al., 2013; Suanyuk et al., 2005; Shoemaker et al., 2001;

Rahmatullah et al., 2017). Streptococcosis caused by *S. agalactiae* and *S. iniae* in fish is primarily characterised by septicaemia and meningoencephalitis (Netto *et al*. 2011; Mian *et al*. 2009; Agnew & Barnes, 2007). Gram-staining of tissue sections from moribund tilapia samples confirmed the invasion of Gram-positive cocci bacteria in various internal organs including the brain. The lesions of encephalitis and meningitis in this study are classified as lymphohistiocytic since macrophages and not neutrophils were the dominant immune response cells. This contrasts with the observations of previous studies where experimental infection with *S. iniae* (Eldar & Ghittino, 1999; Bromage & Owens, 2002) and natural infection with *S. agalactiae* (Asencios et al., 2016), the lesions have been described as suppurative. The interaction between *Streptococci* and macrophages is crucial in the pathogenesis of infection of *S. iniae* and *S. agalactiae* as they can enter and survive in the macrophages sufficient to cause bacteraemia required in developing meningitis. This mechanism of *Streptococci* entering the central nervous system through migrating macrophages is postulated as Trojan horse theory (Gottschalk & Segura, 2000; Zlotkin et al., 2003). The pathological findings in the brains correlated with the clinical behavioural abnormalities, such as lethargy and loss of orientation. These findings supported that *S. agalactiae* and *S. iniae* were neurotropic as described in previous studies (Eldar *et al*. 1995; Abuseliana *et al*. 2010; Baums et al., 2013). The histopathological findings observed in other organs revealed common features such as infiltration of inflammatory cells, congestion, degeneration, and necrosis of cells as evidenced by pyknosis, karyorrhexis and karyolysis. Moreover, haemorrhages, deposition of hemosiderin and increase of melanomacrophage centres were also noted

especially in the spleen and kidney. These tissue and cell anomalies may be due to the high number of bacteria producing toxins and enzymes that also stimulates the immune response of infected fish. These lesions are similar to those tilapias which were naturally infected by *S. agalactiae* and *S. iniae* (Chen et al., 2007; Asencios et al., 2016; Perera et al., 1998; Rahmatullah et al., 2017). Over-all, in this study the histopathology confirmed the virulence of both Philippine isolates of *S. agalactiae* and *S. iniae* to induce systemic infection in tilapia. This infectious disease can arise as a septicaemia where expression of bacterial virulence factors and its extracellular products facilitates the establishment of disease leading to entry of the bacteria in the circulatory system and disseminates to the different organs disrupting fish physiological functions and induce variety of pathological alterations that may lead to death or subclinical conditions. Thus, the observed disease or mortality outbreaks in tilapia farms can be due to acute systemic infection of natural infection by *S. agalactiae* and S*. iniae*.

4.6 Conclusion

As with other SEA countries, streptococcal infections cause disease and fish losses within the tilapia production systems in the Philippines. The use of molecular methods such as *16S rRNA* gene sequencing, species-specific duplex PCR, multiplex PCR capsular serotyping, and MALDI-TOF MS analysis will help address the limitations of conventional bacteriological methods for rapid and accurate pathogen identification and typing. Similar to previous

reports from neighbouring countries, both *S. iniae* and *S. agalactiae* are present within the tilapia farming systems, able to cause disease with clinical signs similar to those previously reported. The *S. agalactiae* serotype Ia was the most prevalent pathogen. The non-beta-haemolytic *S. agalactiae* serotype Ib was also detected albeit it was more geographically limited in the Taal Lake in the present study. Antibiotic response profile of the streptococci isolates is also similar to neighbouring Southeast Asian countries. Virulence genes namely adhesins, invasion and immune evasins which are indicative of the ability of the streptococci to cause disease in fish were detected. The *S. agalactiae* serotype Ib lacked the *cylE* gene which is responsible to for the β-haemolysin/cytolysin, however, the absence of this virulence may be a mechanism of the pathogen to persist in fish causing chronic or subclinical infections. This study is the first to confirm that a range of streptococcal species is causing disease outbreaks in tilapia farms in the Philippines and that uptake of these data will better inform the disease prevention and control strategies for the Philippine sector and contribute towards a geographically distinct vaccine.

CHAPTER 5.

Aeromonas spp. Associated with Farmed Nile Tilapia in the Philippines

5.1 Abstract

This study aimed to identify and characterise the motile *Aeromonas* strains recovered from the farmed tilapia presenting with clinical signs of disease. Given how heterogeneous these aquatic strains can be particular emphasis was placed on identification of the antibiotic sensitivity and virulence profiles of *Aeromonas* spp. isolated from farmed Nile tilapia in the Philippines. Conventional and molecular identification methods confirmed 16 motile aeromonas isolates recovered from moribund fish as being strains of *Aeromonas veronii* (n=14), *Aeromonas caviae* (n=1), and *Aeromonas dhakensis* (n=1). The antibiotic sensitivity of the isolates was determined by disc diffusion method while the antibiotic resistance genes were detected using conventional PCR. Virulence profile was determined using both phenotypic expression of virulence by agar plates and detection of virulence genes by conventional PCR. Phenotypic resistance to two or more antibiotic classes was found in 7 isolates (44%, n=16). All isolates were susceptible to enrofloxacin, oxolinic acid, florfenicol and chloramphenicol. Tetracycline *tetE* and sulfonamide *sul1*

resistance, and class 1 integron *IntI1* were detected in three *A. veronii* isolates. The isolates showed three or more virulence genes dominated by the cytotoxic enterotoxin *act* and aerolysin *aer* genes. The different virulence genotypes suggest varied mechanisms used by *Aeromonas* to colonise and cause infection or to mutualistically co-exist with the fish. This is the first study from the Philippines to characterise the antibiotic profile and virulence determinants of *Aeromonas* spp. associated with farmed Nile tilapia. Data from this study will help in the development of polyvalent vaccine and improve diagnosis and control strategies in the sector.

5.2 Introduction

Infectious diseases caused by motile aeromonads are one of the most common problems in freshwater aquaculture (Austin & Austin, 2016) and several aeromonads including *A. veronii, A. sobria* and *A. jandaei,* in addition to *A. hydrophila,* have been associated with diseases in Nile tilapia (Li & Cai, 2011; Dong et al., 2017). Given the ubiquitous presence of *Aeromonas* spp. within aquatic environments and animals, they are typically considered as an opportunistic or secondary pathogen but more recent work has shown it can also become a primary pathogen (Shahi et al. 2013), causing mass mortalities of fish and huge economic losses to the aquaculture industry (Fang et al. 2004; Pridgeon & Klesius 2012). Epidemics caused by motile aeromonas in cultured fish farms, resulting in millions of dollars of lost revenue, have been reported worldwide (Silva et al. 2012; Hossain et al. 2014). Additionally, motile aeromonas septicaemia (MAS) has caused the large economic losses in

aquaculture in tilapia farms worldwide (Nielsen et al. 2001;Pridgeon & Klesius 2011).

Aquatic infections due to MAS has been one of the most common problems described in freshwater fish, and most studies associate this to a single bacterial species, *Aeromonas hydrophila*. Doubt is now being raised about how common this bacterial species is during MAS infections primarily being driven its complex phenotypic and genotypic heterogeneity (Lamy et al., 2010) especially in developing countries like the Philippines where laboratories depend heavily on the use of biochemical test kits such as API for bacterial identification. For a treatment or intervention of the disease, it is rarely needed to differentiate bacteria at the species level. However, many countries are limited in their degree of bacterial speciation which in itself, is not always critical with the motile *Aeromonads* for treatments as the same approach is used. However, understanding the speciation and level of diversity for microbial epidemiology studies is critical, particularly for sectors where MAS outbreaks can significantly impair the productivity and sustainability. Moreover, the mis-identification could be also due to contamination from the leaching in the gut compared with the actual pathogenic strains during isolation as *A. hydrophila* is a known commensal in fish gut (Ray et al., 2012). The ability to refine our understanding of the role of varied species within MAS outbreaks is through the uptake of highly sensitive and specific molecular identification methods such as the *16S rRNA*, *gyrB*, *rpoB* and *rpoD* gene sequencing (Beaz-Hidalgo, Alperi, Bujan, Romalde & Figueras, 2010). These molecular probes have higher resolution compared with the more biochemical identification of these MAS complex. The *rpoD* gene, a house keeping gene, is reported as an excellent

tool for identification and for inferring taxonomy of the genus *Aeromonas* (Beaz-Hidalgo et al., 2010). Several motile *Aeromonas* species, aside from *A. hydrophila*, are reported as aetiological agents of MAS in a wide range of fish species. These include *A. veronii* (Dong et al., 2015), *A. dhakensis* (Soto-Rodriguez et al., 2013), *A. caviae* (Peepim et al., 2016), *A. sobria* (Li & Cai, 2011) and *A. jandaei* (Dong et al., 2017). Some strains of these bacteria also cause broad range of human diseases ranging from diarrhoea to life threatening tissue infections and septicaemia in immunocompromised persons (Parker & Shaw, 2011). However, the human strains appear to be genetically distinct from piscine strains (Pablos et al., 2011). This genetic difference among human and piscine strains of *Aeromonas* indicates a complex adaptation strategy in different host via transmission through the water.

Aeromonas, being ubiquitous, may be constantly exposed to micropollutants, such as residual antimicrobial compounds that may be present from agricultural run-offs, human sewage and fish farms doing antibiotic treatment. Depending on their concentration, residual time in the environment and frequency of exposure, contaminating antimicrobials can exert a selective pressure and may thus favour the spread of AMR among aquatic bacterial populations (Stratev et al., 2015). Since the intensification of tilapia aquaculture, numerous diseases have impacted production which consequently resulted in the need of prophylactic and therapeutic administration of antimicrobial substances. Although the use of antibiotics may positively impact aquaculture productivity, there is a rising concern that their extensive application and way of administration selects for antibiotic-resistant bacteria via the presence of antibiotic resistance genes (ARG).

Antibiotic resistance genes in *Aeromonas* are often harboured on mobile genetic elements including class 1 integrons, plasmids, insertion sequence elements, transposons, genomic islands carrying single or multidrug resistance (MDR) genes and gene cassettes (Hossain et al., 2013; Huddleston et al., 2013; Piotrowska & Popowska, 2015). Horizontal gene transfer (HGT) is a mechanism by which bacteria acquire resistance to antibiotics by exchanging their antibiotic resistance genes among diverse species (Le Roux & Blokesch, 2018), greatly fostering collaboration among bacterial population in MDR development (Sun et al., 2019). HGT is the most important factor in the current pandemic of AMR (von Wintersdorff et al, 2016). Horizontal gene transfer of these elements can significantly expand the ability of bacteria to spread and cause disease leading to the potential transfer of mobile genetic elements across microbiomes of common ecological niches. Many of these mobile elements harbour multiple antimicrobial resistance determinants resulting in the propagation of antibiotic resistance. The ability of the MAS species within the aquatic MAS disease complex can rapidly acquire AMR via HGT and this combined with the diverse range of virulence factors possessed by the members of this MAS complex makes the disease outbreaks difficult to control. This in turn, can have a high economic threat to the sustainable development of freshwater aquaculture species (Yang et al., 2020) and with human health for the MAS strains which able to cause disease in people (WHO, 2011). The global rise in AMR detection from motile *Aeromonas* isolates from different sources may pose a serious threat to public health due to their zoonotic potential (Patil et al., 2016; Lijon et al., 2016; Gordon et al., 2007; Palu et al., 2006; Hatha et al., 2005). Recent literature showed that 95.4% of the strains

associated with clinical cases correspond to four species, namely *A. caviae*, *A. dhakensis*, *A. veronii*, and *A. hydrophila* (Figueras & Beaz-Hidalgo, 2015; Fernandez-Bravo & Figueras, 2020). Despite the vast information being collated and presented on the AMR of motile aeromonads isolated from water, sediments, and seafood samples (Patil et al., 2016; Lijon et al., 2016; Gordon et al., 2007), there is a need for constant surveillance of the AMR patterns of MAS isolates from intensively farmed fish due to increased reports of infectious disease outbreaks despite antibiotic treatments.

Screening for the presence of virulence genes as a method to evaluate the potential virulence of aeromonads could be hypothetical, since virulence is a complex process, and empirical testing with an infection bioassay is often necessary for conclusive results. Nevertheless, screening for virulence genes has been used in many studies as a practical approach for evaluating the genetic potential of aeromonads to express virulence factors (Puthucheary et al. 2012). A number of putative virulence genes have been described in *Aeromonas* which are present in known human bacterial pathogens, are considered essential to establish infection in a susceptible host although the exact mechanism of how these virulence factors cause disease in susceptible host is still complex and not fully understood (Senderovich et al., 2021; Sen & Rodgers, 2004). The presence of virulence genes does not necessarily imply its expression in the host (Talagrand-Reboul, 2018). These virulence factors detected so far have been reported to contribute to biofilm formation, cell adherence, invasion, and cytotoxicity (Tomas, 2012), all important in the establishment of aquatic disease. Studies have also shown that only certain subsets of strains within a species, referred to as “pathotypes” produce disease

in particular hosts (Janda & Abbot, 2010). This could be due to the differences in the content of virulence-associated genes within the pathotypes compared with other strains. The detection of these virulence genes in a given strain of a particular *Aeromonas* species in a defined geographical area is necessary in determining potential pathogenicity and subsequent possible targets for vaccine (Khor et al., 2015).

Aeromonas virulence expression is complex, since several factors contribute significantly to the development of an infectious process (Pablos et al., 2009) as host becomes susceptible due to a compromised immune response caused by stresses (Dallaire-Dufresne et al., 2014). Colonisation and infection ability by the bacterium is due to the microorganism's structural components, toxins, and extracellular products (Hoel et al., 2017; Beaz-Hidalgo & Figueras 2013), acting jointly or individually (Igbinosa et al., 2017). Due to the diversity of the *Aeromonas* virulence complexes detected in aquatic strains, it has not yet been fully understood due to the unpredictability or differential expression of the virulence genes (Ottaviani et al., 2011). In the infectious process, the structural composition of a bacterial cell has a great influence on its ability to cause the disease. Several factors such as flagella, pili, proteins, and membrane antigens, amongst others, have been studied and identified roles described in bacterial pathogenicity, for example, locomotion, adhesion to host tissue, protection against bactericidal agents and immune system cells (Rasmussen-Ivey et al., 2016). Additionally, a great variety of extracellular products are generated among *Aeromonas* species (Praveen et al., 2016). These enzymatic proteins such as haemolysins, lipases and proteases as well as toxins have been studied over time due to their role in the infectious process

and host impairment (Beaz-Hidalgo & Figueras 2013). The transfer of the virulence factors produced by bacteria to the extracellular medium and/or to host cells is extremely important to the contamination and infection processes. Six different types of secretory systems have been detected in Gram-negative microorganisms (Liu, 2015); four of them were reported in the genus *Aeromonas*, described as types II, III, IV and VI (Beaz-Hidalgo & Figueras., 2013).

5.2.1 Study Aim

The objectives of the present study were to characterise and profile the antibiotic susceptibility and virulence genes of motile *Aeromonas* species recovered from clinically sick tilapia farmed in the Philippines, with a view to identifying targeted disease prevention and control measures against MAS in farmed tilapia species.

5.3 Materials and Methods

5.3.1 Identification of the isolates

The source and preliminary identification of the strains characterised in this chapter was reported in Chapter 3 (134-136). Preliminary identification of the isolates included conventional methods like Gram staining, oxidase, catalase, motility, and oxidation-fermentation while the molecular identification test was *16S rRNA* gene sequencing.

In total there were 14 A. veronii, 1 *A. jandaei*, 1 *A. caviae* and 1 *A. dhakensis*. The *Aeromonas* isolates were recovered both moribund and

apparently healthy tilapia from Taal Lake, Laguna Lake, Calauan and Nueva Ecija.

5.3.1.1 Biochemical profiles

The biochemical profiles of the different *Aeromonas* isolates were performed using API 20E identification kit (BioMérieux, France), following the manufacturer's instructions but modified for a temperature of incubation of 28° C and read after 48 hours (Crumlish *et al.,* 2002).

5.3.1.2 Molecular identification of the MAS strains

All motile *Aeromonas* isolates (n = 16) identified by *16S rRNA* gene sequencing were processed for *rpoD* sequence analysis. PCR amplification of the *rpoD* gene was performed as described by Yamamoto et al. (2000). PCR amplification was performed with a Biometra thermal cycler (Analytik Jena) by using 2X HS MyTaq mastermix (bioline UK) and the final concentrations of each component in a 25 ul reaction mixture were as follows: 12.5 µl of 2X HS MyTaq mastermix, 1.0 µl of 10 pmol of each oligonucleotide primer, 2.0 µl of 20 ng gDNA, and 8.5 µl of milliQ water. The primers for the PCR amplification were the 70F (5'-ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT-3') and 70R (5'-ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT-3') which amplifies approximately 820 bp gene segment. The annealing temperature ranged from 58 to 63 °C depending on the G+C content of the template DNA. Higher annealing temperatures were used for higher G-C contents. Amplified products were visualised on 1.5% (w/v) agarose gels with ethidium bromide (0.5 µg/ml), and PCR products purified using QIAquick PCR

Purification Kit (Qiagen). The total nucleotide sequences of the amplified fragments were sequenced commercially (Eurofins Genomics) by using sequencing primers 70Fs (5'-ACGACTGACCCGGTACGCATGTA-3') and 70Rs (5’-ATAGAAATAACCAGACGTAAGTT-3’) which correspond to the first 23 nucleotides of PCR primers 70F and 70R, respectively (Appendix 2.5). PCR primers and sequencing primers used in this study are summarized in **Error! Reference source not found.**. Sequences were aligned with ClustalW algorithm against phylogenetically related organisms available in GenBank in the National Centre for Biotechnology Information website (http: //www.ncbi. nlm.nih.gov). A phylogenetic tree based on the *16S rRNA* and *rpoD* genes for the different motile *Aeromonas* isolates was constructed using MEGA 10 software.

Table 5.1 PCR primers and sequencing primers for rpoD gene.

Primer	Target Gene	Sequence (5’-3’)	Length (mer)
70F	*rpoD*	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT	44
70Fs		ACGACTGACCCGGTACGCATGTA	23
70R	*rpoD*	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT	44
70Rs		ATAGAAATAACCAGACGTAAGTT	23

5.3.1.3 Protein spectral profiles of the MAS strains

Protein spectral profiles of the different *Aeromonas* (n = 16) isolates including the *A. hydrophila* NCIMB 9240 and *A. veronii* ATCC 35624 were determined using the ethanolic-formic acid extraction method as described in Chapter 4, with exception of a 24 h instead of 48 h incubation period. A

further modification was the use of 12 sequential dilutions for the *Aeromonas* species (1:1, 1:2.5. 1:5, 1:10, 1:20, 1:40…1:2560) all prepared in tetraplicates.

5.3.2 Viable growth in different incubation temperatures and salt concentrations

The method for the determination of temperature and salt tolerances of the *Aeromonas* isolates was the same as described in Chapter 4.

5.3.3 Histopathology

The details of the histopathology protocol is found in Chapter 3 (pages). H&E and Gram-stained tissue sections were examined using the digital slide scanner (ZEISS Axio Scan.Z1, ZEISS Germany).

5.3.4 Antibiotic susceptibility and antimicrobial resistance determinants

5.3.4.1 Antibiotic susceptibility assay

Susceptibility tests were performed with some modification by the disc diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI 2012) guidelines. All the isolates were tested for antibiotic sensitivity to antibiotics that are globally licensed for aquaculture which are amoxicillin (AML 10 µg), oxytetracycline (OT 30 µg), florfenicol (FFC 30 µg), sulfamethoxazole-trimethoprim (SXT 25 µg), oxolinic acid (OA 2 µg), enrofloxacin (ENR 5 µg), tetracycline (30 µg) and erythromycin (E 15). Additionally other antibiotics of human health significance were also tested which include ampicillin (25 µg), penicillin G (10 units), chloramphenicol (30 µg), and ampicillin (AMP; 25 µg)

(Oxoid UK). The antibiotics selected were those routinely screened in the diagnostic laboratory at the Institute of Aquaculture, University of Stirling and representative of antibiotics licensed for use in global aquaculture. The Kirby-Bauer method for antibiotic susceptibility testing was followed as described in Chapter 4 of this study.

5.2.5.2 Detection of antibiotic resistance genes

The presence of genes encoding resistance to tetracyclines (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tetM*), sulfonamides (*sul1*, *sul*2, *sul*3) and trimetophrim (*dfrA1*, *dfrA12*, *drfA17*) were detected by conventional one-step PCR using genomic DNA from Chapter 3 (pages). Extended Spectrum Beta-Lactamase (ESBL) resistance genes (*blaSHV*, *blaCTX-M*, *blaTEM*), plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*)), aminoglycoside resistance genes (*armA*, *aphaI-IAB*, *aac(3)-IIa*, *aac(6')-Ib*) and integrons (*IntII*, *IntI2,* Class 1 integron) were screened for all isolates (n = 16) regardless the results of antibiotic susceptibility testing. The list of PCR primers are shown in Table 5.2. The antimicrobial resistance genes amplified PCR products were sent for sequencing to confirm their identity as following methods described by Syrova et al. (2018).

Table 5.2 Primers used for PCR detection of different antimicrobial resistance genes in *Aeromonas* spp. isolates.

Targeted Gene	Primer Pair	Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
		Extended Spectrum B-Lactamase (ESBL) Resistance		
*blaTEM**	blaTEM-F	ATAAAATTCTTGAAGACGAAA	58	1080
	blaTEM-R	GACAGTTACCAATGCTTAATC		
*blaSHV**	blaSHV-F	TTATCTCCCTGTTAGCCACC	58	797
	blaSHV-R	GATTTGCTGATTTCGCTCGG		
*blaCTX-M**	blaCTX-M-F	CGCTTTGCGATGTGCAG	52	551
	blaCTX-M-R	ACCGCGATATCGTTGGT		
		Tetracycline Resistance		
*tetA**	tetA-F	GTAATTCTGAGCACTGTCGC	62	957
	tetA-R	CTGCCTGGACAACATTGCTT		
*tetB**	tetB-F	CTCAGTATTCCAAGCCTTTG	57	436
	tetB-R	CTAAGCACTTGTCTCCTGTT		
*tetC***	tetC-F	CTTGAGAGCCTTCAACCCAG	58	418
	tetC-R	ATGGTCGTCATCTACCTGCC		
*tetD***	tetD-F	AAACCATTACGGCATTCTGC	54	787
	tetD-R	GACCGGATACACCATCCATC		
*tetE**	tetE-F	GTGATGATGGCACTGGTCAT	62	1199
	tetA-R	CTGCCTGGACAACATTGCTT		
*tetG***	tetG-F	CAGCTTTCGGATTCTTACGG	56	844
	tetG-R	GATTGGTGAGGCTCGTTAGC		
*tetM****	tetM-F	ACACGCCAGGACATATGGAT	54	536
	tetM-R	ATTTCCGCAAAGTTCAGACG		
		Plasmid-Mediated Quinolone Resistance (PMQR)		
*qnrA**	qnrA-F	AGAAGGATTTCTCACGCCAGG	56	580
	qnrA-R	TGCCAGGCACAGATCTTGAC		
*qnrB**	qnrB-F	GATCGTGAAAGCCAGAAAGG	53	469
	qnrB-R	ACGATGCCTGGTAGTTGTCC		
*qnrS**	qnrS-F	GCAAGTTCATTGAACAGGGT	56	427
	qnS-R	TCTAAACCGTCGAGTTCGGCG		
		Aminoglycoside Resistance		
*armA**	armA-F	AGGTTGTTTCCATTTCTGAG	53	591
	armA-R	TCTCTTCCATTCCCTTCTCC		
*aphaI-IAB**	aphAI-IAB-F	AAACGTCTTGCTCGAGGC	53	461

	aphAI-IAB-R	CAAACCGTTATTCATTCGTGA		
*aac(3)-IIa**	aac(3)-IIa-F	ATGGGCATCATTCGCACA	55	484
	aac(3)-IIa-R	TCTCGGCTTGAACGAATTGT		
*aac(6')-Ib**	aac(6')-Ib-F	TTGCGATGCTCTATCAGTGGCTA	55	482
	aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT		
		Sulfonamide Resistance		
*sul1***	sul1-F	CTTCGATGAGAGCCGGCGGC	71	417
	sul1-R	GCAAGGCGGAAACCCGCGCC		
*sul2***	sul2-F	AGGGGGCAGATGTGATCGAC	54	249
	sul2-R	GCAGATGATTTCGCCAATTG		
*sul3***	sul3-F	GAGCAAGATTTTTGGAATCG	52	789
	sul3-R	CATCTGCAGCTAACCTAGGGCTTTGGA		
		Trimethoprim Resistance		
*dfra1***	dfra1-F	ACGGATCCTGGCTGTTGGTTGGACGC	65	254
	dfra1-R	CGGAATTCACCTTCCGGCTCGATGTC		
*dfra12***	dfra12-F	ACTCGGAATCAGTACGCA	50	462
	dfra12-R	GTGTACGGAATTACAGCT		
*dfra17***	dfra17-F	GATTTCTGCAGTGTCAGA	50	384
	dfra17-R	CTCAGGCATTATAGGGAA		
		Integrons		
*IntI1**	InII-F	CTACCTCTCACTAGTGAGGGGCGG	58	847
	InII-R	GGGCAGCAGCGAAGTCGAGGC		
*IntI2**	IntI2-F	GCAAATGAAGTGCAACGC	56	467
	IntI2-R	ACACGCTTGCTAACGATG		
Class 1 integron*	5'CS	GGCATCCAAGCAGCAAG	64	Variable
	3'-CS	AAGCAGACTTGACCTGA		

*Hossain et al. (2018), **Syrova et al. (2018), ***Skwor et al. (2020).

5.3.5 Detection of virulence determinants and virulence genes

Two methods were applied in this study: phenotypic expression of common virulence factors and detection of virulence genes.

5.3.5.1 Phenotypic virulence determinants

Phenotypic expression of putative virulence factors associated with pathogenic strains of *Aeromonas* were evaluated *in vitro* following the methods of (Sreedharan et al., 2011). In all assays *A. hydrophila* strain NCIMB 9240 was

applied as positive control and all inoculated agar plates were incubated at 28 °C.

Haemolysis. A pure colony of each isolate tested was streaked onto a 5 % (v/v) sheep blood agar plate incubated at 28 °C and observed daily for five days. Clearing around the bacterial colonies indicated positive haemolytic activity.

Extracellular Lipase. Lipase agar (Tween 80 agar) was prepared as per Plou et al. (1998). A positive result was indicated by a white halo of precipitation around colonies and a negative result by lack of this.

Extracellular Gelatinase. The agar was prepared as per Smith and Goodner (1958). A positive result was indicated by clearing around colonies and a negative result by lack of this.

Extracellular Protease. The agar was prepared as per Vermelho et al. (1996). A positive result was indicated by clearing around colonies after 24-48 h and a negative result by lack of this.

Extracellular DNAse. DNAse agar was prepared according to manufacturer's guide for DNAse Agar Base (CM0854) (Oxoid, UK) with 0.01 % toluidine blue O (w/v). A positive result was indicated by pink zones around colonies and a negative result by lack of this.

Biofilm Formation. The assay was performed using the Congo red agar method (Freeman et al., 1989). A positive result was indicated by black colonies with a dry crystalline consistency after 24-48 hours.

5.3.5.2 Detection Virulence Genes

All isolates 16 bacterial isolates were screened by conventional one step PCR for a total of 15 virulence genes (Table 5.3). Characteristics of primers and thermal cycling conditions used for the amplification of virulence genes are summarised in Table 5.3 with modifications in the annealing temperatures and time for PCR denaturation, annealing and extension which were performed at 15s, 15s and 30s, respectively and since optimisation of the PCR using these times and temperatures gave the best amplification bands.

Each PCR was performed in a 25 µl reaction mixture consisting of 12.5 µl of 2X HS MyTaq mastermix (bioline UK), 1.5 µl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 µl of DNA template, and Milli-Q water to volume. The PCR products were analysed by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE buffer with ethidium bromide (0.5 µg ml^{-1}), visualized on a UV transilluminator and documented. A GeneRuler™ 1kb Plus DNA Ladder (Thermo Scientific™, UK) was run together with the PCR products to enable estimation of the size of the amplified fragments.

Table 5.3 Primers used for PCR detection of different virulence genes in *Aeromonas* spp. isolates.

Virulence Factor	Gene	Primer	Nucleotide Sequence (5'- 3')	Target region (bp)	Annealing Temperature (°C)
Aerolysin*	*aer*	aerA_F	CCTATGGCCTGAGCGAGAAG	431	69
		aerA_R	CCAGTTCCAGTCCCACCACT		
Hemolysin	*hylA*	hylA-F	ATGAGTTTTGCCGATAGTTTATTTTTCCTGA	1320	67
A**		hylA-R	TTACGATTCCTGAGCGGGCTTGTCGGCCGGCGTG		
DNAse*	*exu*	exu_F	RGACATGCACAACCTCTTCC	323	61
		exu_R	GATTGGTATTGCCYTGCAAS		
Serine	*ser*	ser_F	CACCGAAGTATTGGGTCAGG	350	64
protease*		ser_R	GGCTCATGCGTAACTCTGGT		
Lateral	*lafA*	lafA_F	CCAACTTYGCYTCYMTGACC	738	62
flagella*		lafA_R	TCTTGGTCATRTTGGTGCTY		
Cytotoxic	*act*	act_F	AGAAGGTGACCACCACCAAGAACA	232	65
enterotoxin*		act_R	AACTGACATCGGCCTTGAACTC		
Elastase*	*ahyB*	ahyB_F	ACACGGTCAAGGAGATCAAC	513	62
		ahyB_R	CGCTGGTGTTGGCCAGCAGG		
ADP-ribosyl	*aexT/aexU*	aexT/aexU_F	TGGCVMTSAAAGAGTGGAT	225	60
transferase		aexT/aexU_R	GCARDGSRCCRTTGCCRGTC		
toxin***					
Lipase*	*lip*	lip_F	CAYCTGGTKCCGCTCAAG	247	63
		lip_R	GTRCCGAACCAGTCGGAGAA		
Heat-labile	*alt*	alt_F	TGACCCAGTCCTGGCACGGC	442	66
cyototonic		alt_R	GGTGATCGATCACCACCAGC		
enterotoxin*					
Heat-stable	*ast*	ast_R	TCTCCATGCTTCCCTTCCACT	331	65
cytotonic		ast_R	GTGTAGGGATTGAAGAAGCCG		
enterotoxin*					
DNA	*dam*	dam_F	ATGAAAAAAACACGCGCTTTTTTAAAATGG	873	65
adenine		dam_R	TCAGCCGAGTGGCGCCAGTTCGGCGTCG		
methyl-					
transferase**					
Enolase**	*enolase*	enolase_F	ATGTCCAAGATCGTTAAAGTGAT	1302	60
		enolase_R	TTAAGCCTGGTTCTTCACTTCTT		

T3SS membrane component**	*ascV*	ascV_F ascV_R	ATGAAGCCCGCTTCGCCTATCAA TCACAGGCAGACCCTTCCCAGC	2166	65
Cholesterol acyl transferase*	*gcaT*	gcaT_F gcaT_R	CTCCTGGAATCCCAAGTATCAG GGCAGGTTGAACAGCAGTATCT	237	65

* Nawaz et al (2010); **Khor et al (2015); ***Talagrand-Reboul et al. (2018).

5.4 Results

5.4.1 Identification of the isolates

All the bacterial isolates were identified as Gram-negative rods, catalase, and oxidase positive, motile and fermentative.

5.4.1.1 Biochemical profiles

As expected, the API 20E biochemical profiles gave a high level of variation observed between and within different *Aeromonas* species/strains (Table 5.4). Isolates 39 and 69 had their own unique profile were later identified as strains of *A. caviae* and *A. dhakensis*, respectively. For the rest of the isolates, which were later identified as *A. veronii*, there were five distinct biochemical profiles (Table 5.3). All the *A. veronii* isolates differed from the type strain *A. veronii* ATCC 35624 which was negative for ADH, but the Philippine strains were all ADH positive. Additionally, *A. veronii* 4 was negative for LDC, CIT and IND while the rest of Philippine *A. veronii* strains and the type strain were positive. The *A. veronii* 4 and 17 were negative for VP while the rest of *A. veronii* strains including the type strain were positive. For ODC, the *A. veronii* isolates 4, 17, 40 and 67 were negative while the type strain and the rest of *A. veronii* strains were positive. A positive result for AMY was shown by *A. veronii* isolates 23 and 51 while other *A. veronii* isolates and the type strain were

negative. The isolates *A. veronii* 17, 23 and 51 were positive for ARA which was negative in other *A. veronii* isolates and the type strain. The summary of the phenotypic and biochemical tests results on motile *Aeromonas* isolates are presented in Table 5.4.

Table 5.4 Phenotypic and biochemical characteristics of the different *Aeromonas* isolates.

Characteristics	***A. veronii*** **ATCC 35624**	***A. hydrophila*** **NCIMB 9240**	***A. caviae*** **39**	***A. dhakensis*** **69**	***AV*** **Profile 1 ***	***AV*** **Profile 2 ****	***AV*** **Profile 3 *****	***AV*** **Profile 4 ******	***AV*** **Profile 5 *******
Gram	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
O/F	+	+	+	+	+	+	+	+	+
MAC	+	+	+	+	+	+	+	+	+
Haemolysis	+	+	+	+	+	+	+	+	+
ONPG	+	+	+	+	+	+	+	+	+
ADH	**-**	+	+	+	**+**	**+**	**+**	**+**	**+**
LDC	**+**	+	-	+	**-**	**+**	**+**	**+**	**+**
ODC	**+**	+	-	+	**-**	**-**	**+**	**+**	**-**
CIT	**+**	+	-	+	**-**	**+**	**+**	**+**	**+**
H_2S	-	-	-	-	-	-	-	-	-
UREA	-	-	-	-	-	-	-	-	-
TDA	+	+	+	+	+	+	+	+	+
IND	**+**	+	+	+	**-**	**+**	**+**	**+**	**+**
VP	**+**	+	-	+	**-**	**-**	**+**	**+**	**+**
GEL	+	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	-	+	+	+	+
INO	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	-
RHA	-	-	-	-	-	-	-	-	-
SAC	+	+	+	+	+	+	+	+	+
MEL	-	-	-	-	-	-	-	-	-
AMY	**-**	-	+	+	**-**	**-**	**+**	**-**	**-**
ARA	**-**	+	+	-	**-**	**+**	**+**	**-**	**-**
NO_2	+	+	+	+	+	+	+	+	+

N_2	-	-	-	-	-	-	-	-	-

*AV Profile 1 = *A. veronii* isolate 4
**AV Profile 2 = A. veronii isolate 17
***AV Profile 3 = A. veronii isolates 23, 51
****AV Profile 4 = A. veronii isolates 26, 34, 41, 46, 47, 50, 64, 74
*****AV Profile 5 = A. veronii isolates 40, 67

An attempt was made to compare the biochemical profiles of the Philippine *A. veronii* strains with other published strains also using the same method (Table 5.5). In the reported literature, all *A. veronii* strains were negative for hydrogen sulphide production, urease, inositol, sorbitol, rhamnose, and melibiose. There is not one positive biochemical test that is common to all the *A. veronii* isolates in Table 5.5 although there is high rate of positivity (≥ 70%) noted for ONPG, ADH, LDC, CIT, IND, VP, GEL, GLU, MAN and SAC. From the API 20E results in this study and in other published literatures on aquatic *A. veronii*, this species can only be identified biochemically up to the genus level as *Aeromonas* sp. since the high positivity in the different biochemical tests were also similar to other *Aeromonas* species such as *A. hydrophila*, *A. caviae* and *A. dhakensis*.

Table 5.5 Summary of API 20E results for *Aeromonas veronii* isolates from farmed Nile tilapia in the Philippines and in other literature reports.

	Aeromonas veronii									
Reference*	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Number of Isolates	**14**	**22**	**1**	**35**	**5**	**9**	**5**	**3**	**1**	**5**
Country**	**PH**	**VT**	**TH**	**INT**	**SP**	**USA**	**GR**	**EG**	**SK**	**USA**
ONPG	**100**	**100**	**100**	**100**	**100**	**89**	**100**	**100**	**100**	**100**
ADH	**100**	**100**	**100**	**100**	**0**	**0**	**100**	**100**	**100**	**100**
LDC	**93**	**100**	**100**	**96**	**100**	**100**	**100**	**100**	**100**	**100**
ODC	71	0	0	0	100	100	0	100	0	0
CIT	**93**	**100**	**100**	**52**	**v**	**100**	**80**	**100**	**100**	**0**
H2S	0	0	0	n/a	n/a	0	0	0	0	0
URE	0	0	0	0	0	0	0	0	0	0
TDA	100	0	0	n/a	n/a	n/a	60	100	0	100
IND	**93**	**100**	**100**	**100**	**100**	**100**	**0**	**100**	**100**	**100**
VP	**86**	**0**	**0**	**88**	**100**	**89**	**70**	**v**	**100**	**100**
GEL	**100**	**100**	**100**	**100**	**100**	**89**	**50**	**100**	**100**	**100**
GLU	**100**	**100**	**100**	**92**	**100**	**100**	**100**	**100**	**100**	**100**
MAN	**93**	**100**	**100**	**100**	**100**	**100**	**80**	**100**	**100**	**100**
INO	0	0	0	0	0	0	0	0	0	0
SOR	0	0	0	0	0	0	0	0	0	0
RHA	0	0	0	0	0	0	0	0	0	0
SAC	**100**	**100**	**100**	**100**	**100**	**100**	**90**	**100**	**100**	**100**
MEL	0	0	0	0	0	0	0	0	0	0
AMY	14	0	0	0	100	n/a	0	v	0	0
ARA	21	0	0	12	0	0	0	0	0	0

(A) Current study (B) Hoai et al., 2019 (C) Dong et al., 2017 (D) Abbott et al., 2003 (E) Esteve et al., 2003 (F) Hickmann-Brenner et al., 1987 (G) Smyrli et al., 2017 (H) El Latif et al., 2019 (I) Yu et al., 2010 (J) Mohammed & Peatman, 2018.

(ONPG) β galactosidase; (ADH) Arginine dihydrolase; (LDC) Lysine decarboxylase; (ODC) Ornithine decarboxylase; (CIT) Citrate; (H2S) Hydrogen sulfide; (URE) Urease; (TDA) Tryptophan deaminase; (IND) Indole; (VP) Voges Proskauer; (GEL) Gelatinase; (GLU) Glucose; (MAN) Mannose; (INO) Inositol; (SOR) Sorbitol; (RHA) Rhamnose; (SAC) Sucrose; (MEL) Melibiose; (AMY) Amygdalin; (ARA) Arabinose.

(n/a) Data not available; (v) variable but no value provided. Numbers show percentage of positive strains.

**Country: PH = Philippines; VT = Vietnam; TH = Thailand; INT = International; SP = SPAIN; GR = Greece; EG = Egypt; SK = South Korea

5.4.1.2 Molecular identification

Identification of the MAS strains using the *16S rRNA* and *rpoD* gene was achieved and gave a 94% level of agreement using either of these methods (**Error! Reference source not found.**). An exception was observed with the isolate 4 in which *16S rRNA* identified it as *A. jandaei* while *rpoD* sequencing speciated it as *A. veronii*. The API 20E either shows an unacceptable profile or doubtful/wrong species identification. The API 20E identified eight *A. veronii* isolates as *Vibrio cholerae* and one isolate as *V. fluvialis*. Two of the *A. veronii* isolates (23 and 51) have an unacceptable profile while the rest of the *Aeromonas* isolates were identified *as A. hydrophila/caviae/sobria* complex. These results may be due to lack reactions of the strains on some biochemical substrates which aquatic strains are not able to metabolise. The summary of the identification test is shown in **Error! Reference source not found.**.

Table 5.6 Comparative identification results of the motile *Aeromonas* isolates from the three tests.

Isolate	Identification Test			
	API 20E	**ID Using Buller and Aeromonas profile in this study**	***16S rRNA* Gene Sequencing**	***rpoD* Gene sequencing**
Aeromonas veronii ATCC 35624	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
4	*Vibrio fluvialis*	*Aeromonas sp.*	*A. jandaei*	*A. veronii*
17	*Aeromonas hydrophila/caviae/sobria*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
23	*Indeterminate*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
26	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
34	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
39	*Aeromonas hydrophila/caviae/sobria*	*Aeromonas sp.*	*A. caviae*	*A. caviae*
40	*Aeromonas hydrophila/caviae/sobria*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
41	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
46	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
47	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
50	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
51	*Indeterminate*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
64	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
	Aeromonas			

67	*hydrophila/caviae/sobria*	*Aeromonas sp.*	*A. veronii*	*A. veronii*
69	*Aeromonas hydrophila/caviae/sobria*	*Aeromonas sp.*	*A. dhakensis*	*A. dhakensis*
74	*Vibrio cholerae*	*Aeromonas sp.*	*A. veronii*	*A. veronii*

The phylogenetic trees constructed, based on *16S rRNA* and *rpoD* gene sequences of the *Aeromonas* isolates and the reference strains of *Aeromonas* species, strongly supported the identification results from both molecular methods. In the *16S rRNA* tree, isolate 04 which is identified as *A. jandaei*, it can be seen it is grouped separately from two subgroups of *A. veronii* species (**Error! Reference source not found.**).

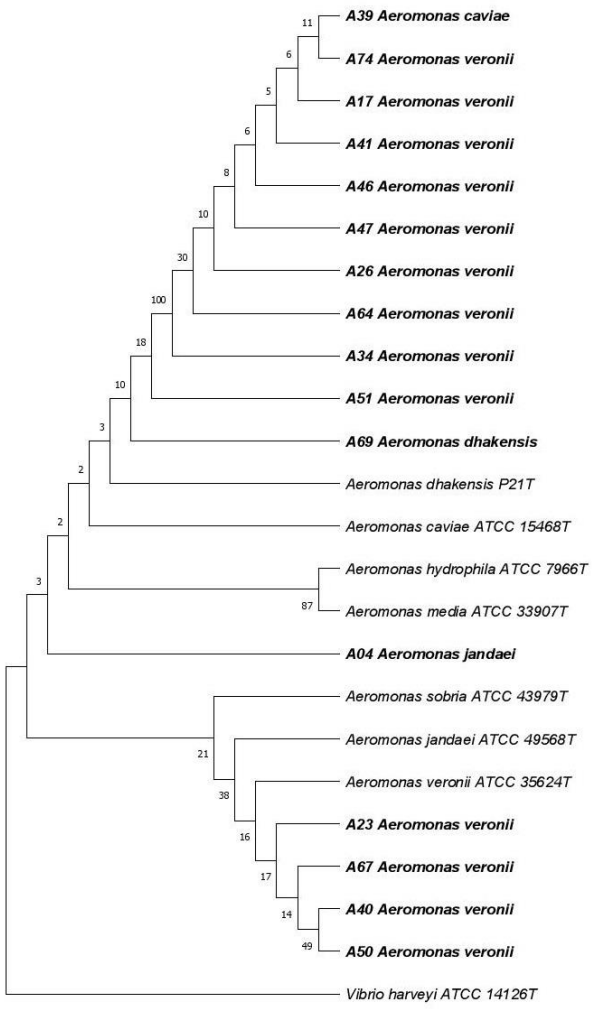

Figure 5.1 Phylogenetic tree was constructed based on the 16S rRNA of the motile *Aeromonas* isolates in this study and their closely related species. *Vibrio harveyi* ATCC 14126T was selected as an out-group. Percentage bootstrap values (1000 replicates) are shown at each branch point.

However, looking at the *rpoD* tree, isolate 4 which was identified as *A. veronii* is grouped with one of the two *A. veronii* subgroups (**Error! Reference source not found.**). Additionally, in the *rpoD* tree, isolate 41-*A. veronii* is positioned separately from both two *A. veronii* subgroups.

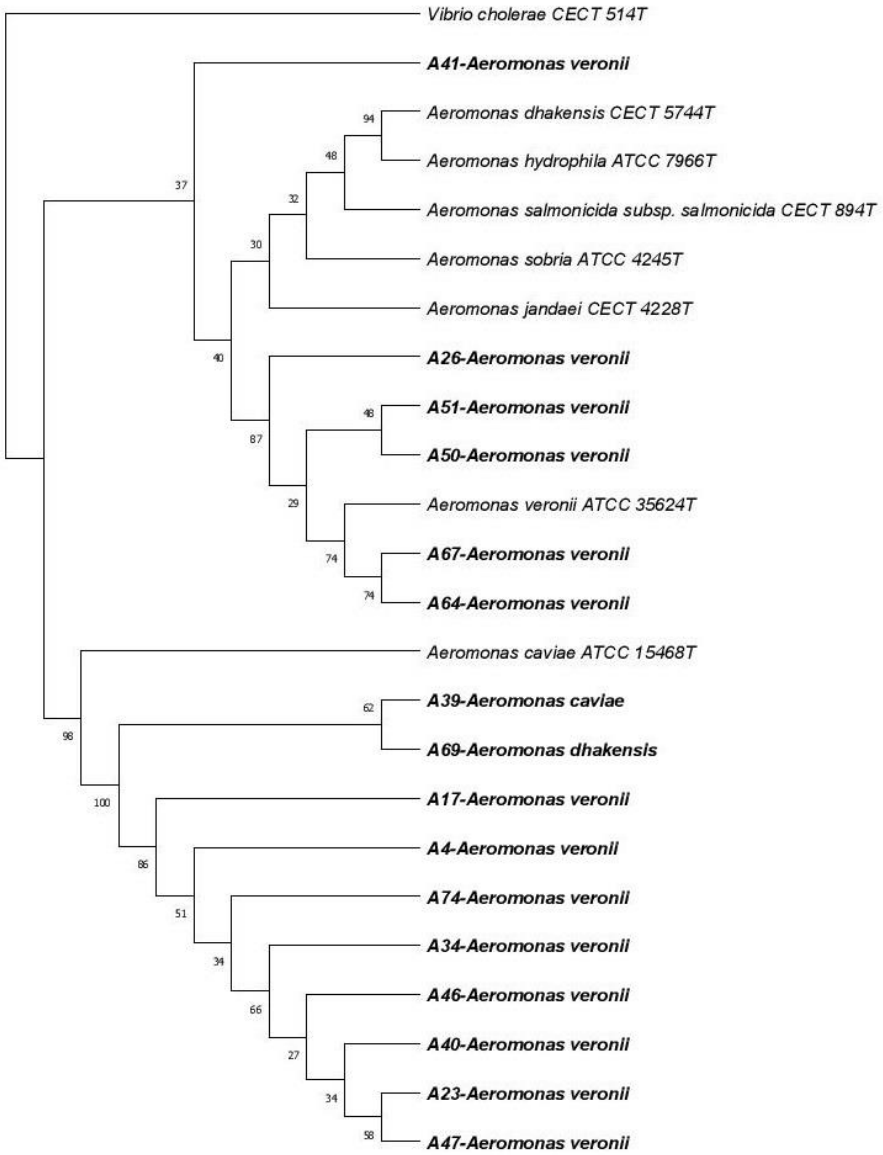

Figure 5.2 Phylogenetic tree was constructed based on the *rpoD* gene of the motile *Aeromonas* isolates in this study and their closely related species. *Vibrio cholerae* CECT 514T was selected as an out-group. Percentage bootstrap values (1000 replicates) are shown at each branch point.

5.4.1.3 Protein spectral profile the MAS strains

A single peak analysis was performed in the range of 4,000–8,000 Da, which included most of the potential biomarkers for bacterial identification, and the peak list of *A. veronii*, *A. caviae*, *A. dhakensis*, *A. veronii* ATCC 35624 and *A. hydrophila* NCIMB 9240 as compared with fish *Aeromonas* isolates in the study of Perez-Sancho et al. (2018). Figures taken directly from the MALDI-TOF MS instrument have been provided in greater scale in the Appendix 3 of this thesis.

From the MALDI TOF results, the protein spectral profiles obtained for motile *Aeromonas* isolates and the respective positive control samples were similar except for isolates *A. veronii* 34, 41 and 67. As seen from the spectral profiles in **Error! Reference source not found.**, there is no visual differences between different motile *Aeromonas* species tested in this study.

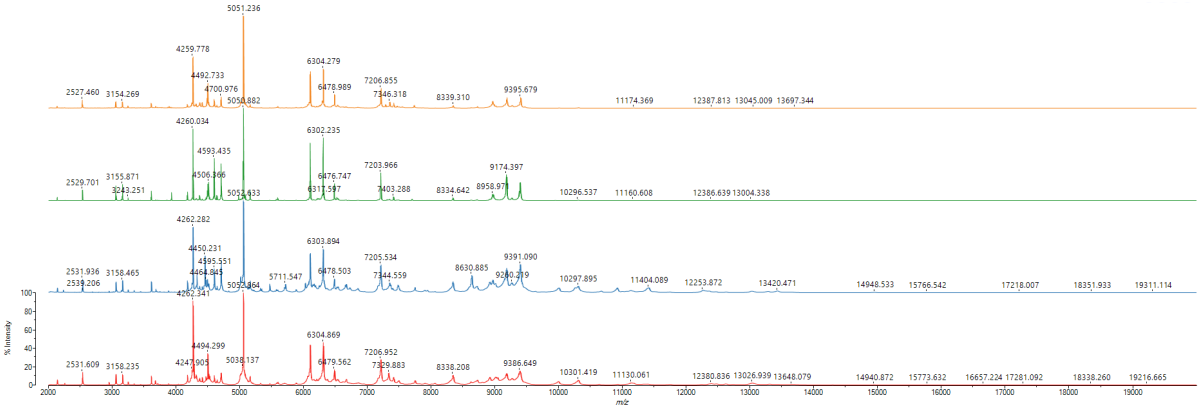

Figure 5.3 Depicts the optimal mass spectrum results achieved with isolate undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The four *Aeromonas* isolates were grown for 24 hours before subculture and were procured at subculture 2. The red spectrum displays isolate *A. veronii* ATCC 35624, the spectrum in blue shows isolate *A. hydrophila* NCIMB 9240, the green spectrum depicts isolate 39 and the orange spectrum exhibits isolate 69. (Appendix 3.3)

Moreover, in **Error! Reference source not found.** which shows spectral profiles from different *A. veronii* isolates, all have similar spectral profiles except for three *A. veronii* isolates which have additional unique peaks of their own.

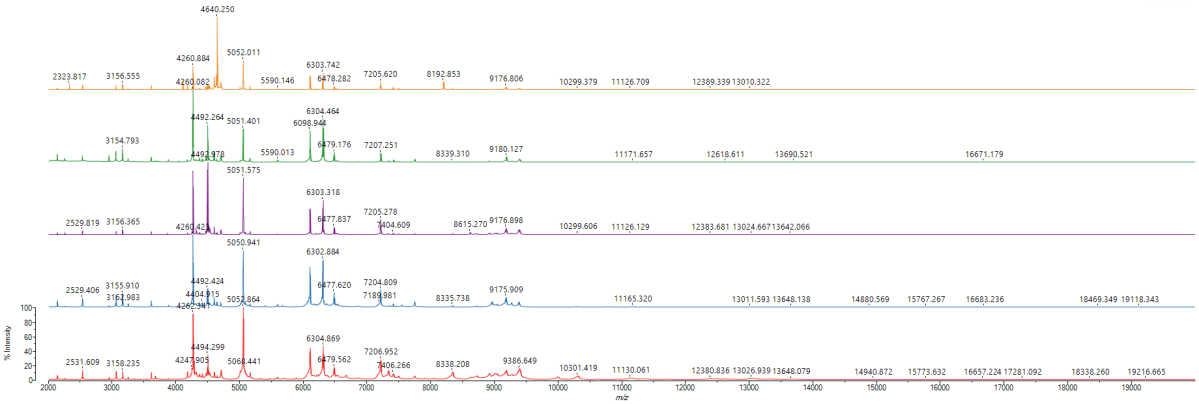

Figure 5.4 Depicts the optimal mass spectrum results achieved with isolate undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The five *Aeromonas* isolates were grown for 24 hours before subculture and were procured at subculture 2. The red spectrum displays isolate *A. veronii* ATCC 35624, the spectrum in blue shows isolate 4, the violet spectrum depicts isolate 23, the green spectrum depicts isolate 26 and the orange spectrum exhibits isolate 34. (Appendix 3.4)

Analysis of the peaks of the spectral profiles of the *Aeromonas* isolates and the controls all clearly showed identical peaks. In **Error! Reference source not found.**, all the *A. veronii* isolates with *A. caviae* (39), *A. dhakensis* (69) and *controls* (*A. hydrophila* NCIMB 9240 & *A. veronii* ATCC 35624) possessed identical peaks at 4260, 4492, 5050, 6097, 6302, 6480 and 7204.8 m/z.

Table 5.7 MALDI-TOF indicative peaks identified in motile *Aeromonas* isolates and type strains.

Isolate Number	*rpoD* ID	Peak analysis (m/z)										
		4260*	**4492**	**4640**	**4702***	**5050***	**5615**	**5771**	**6097**	**6302***	**6480***	**7204***
4	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
17	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
23	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
26	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
34	*A. veronii*	+	+	+	+	+	-	-	+	+	+	+
39	*A. caviae*	+	+	-	+	+	-	-	+	+	+	+
40	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
41	*A. veronii*	+	+	-	+	+	+	+	+	+	+	+
46	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
47	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
50	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
51	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
64	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
67	*A. veronii*	+	+	-	+	+	+	+	+	+	+	+
69	*A. dhakensis*	+	+	-	+	+	-	-	+	+	+	+
74	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+
NCIMB 9240	*A. hydrophila*	+	+	-	+	+	-	-	+	+	+	+
ATCC 35624	*A. veronii*	+	+	-	+	+	-	-	+	+	+	+

*Peak masses identified by Perez-Sancho et al. (2018).

Isolate 34 possessed a peak at approximately m/z 4640 which was absent in the rest while isolates 41 and 67 possessed peaks at m/z 5615 and 5770 which were not seen also with the other isolates (**Error! Reference source not found.**).

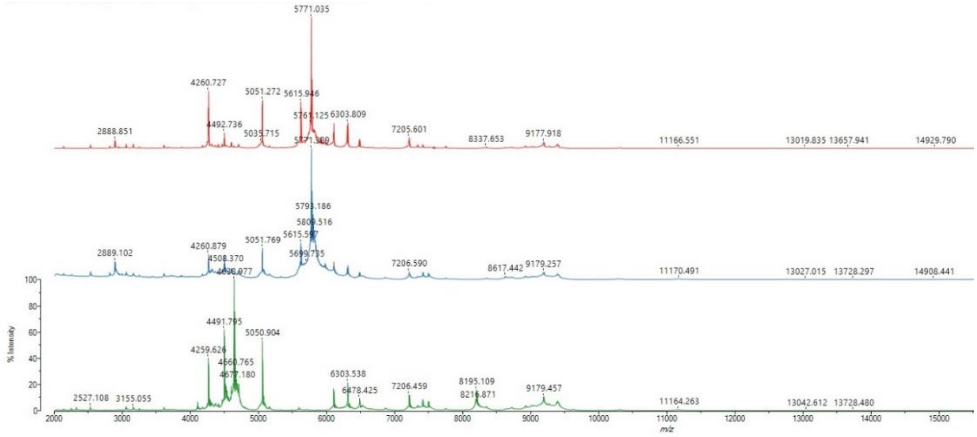

Figure 5.5 Depicts the optimal mass spectrum results achieved with isolate undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The five *Aeromonas* isolates were grown for 24 hours before subculture and were procured at subculture 2. The green spectrum displays isolate 34, the spectrum in blue shows isolate 41, and red green spectrum depicts isolate 67. (Appnedix 3.5)

Over-all the spectral profiles of *Aeromonas* spp. in this study together with the control reference strains were identical which made it difficult to speciate them by peak analysis alone. Three *A. veronii* isolates possessed unique peaks, however, these are not sufficiently strong enough to use for species identification, but this may give a unique pathogenic type within the species by possessing unique proteins.

5.4.2 Viable growth in different incubation temperatures and salt concentrations

All *Aeromonas* isolates (n = 16) were able to grow at 15 to 47° C except for isolates *A. caviae* 39, *A. veronii* 46, *A. veronii* 50 and *A. dhakensis* 69 which were only able to grow until 43° C (**Error! Reference source not found.**). For salinity tolerance, all *Aeromonas* isolates were able to grow at 0.5 to 4 % NaCl.

At 5 % NaCl isolates 39 and 69 showed viable growth while for 6 and 6.5 % NaCl only isolate 69 was able to grow (**Error! Reference source not found.**). These two isolates were identified as *A. caviae* and *A. dhakensis*, respectively.

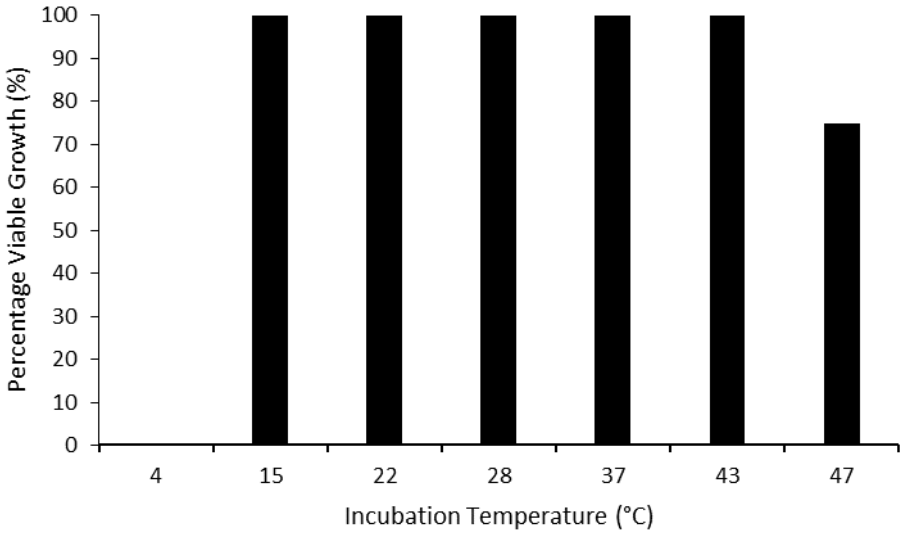

Figure 5.6 Percentage viable growth of the motile *Aeromonas* isolates at different incubation temperatures (°C).

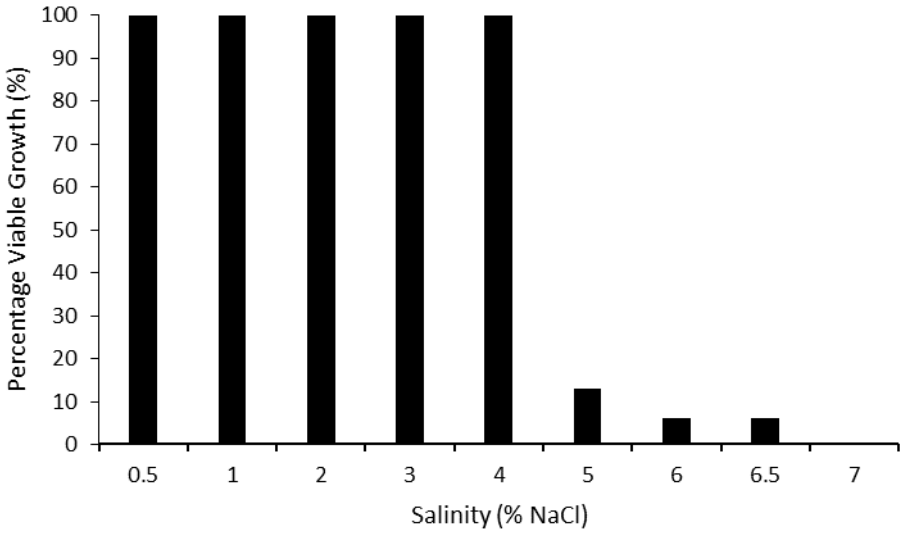

Figure 5.7 Percentage viable growth of the motile *Aeromonas* isolates at different salinity levels (% NaCl).

5.4.3 Histopathology

Microscopic examination of the tissues of the moribund and apparently healthy fish specimens where the motile *Aeromonas* isolates were recovered showed notable changes in the spleen, kidney, liver, and brain. Moderate splenitis was observed with numerous inflammatory cells aggregated around diffuse fibroid necrosis of ellipsoidal sheath in addition hemosiderosis. Addtionally massive congestion of the spleen was also observed (**Error! Reference source not found.**). Presence of Gram-negative rods was observed in the Gram-stained spleen (**Error! Reference source not found.**) of *A. veronii* isolate 17.

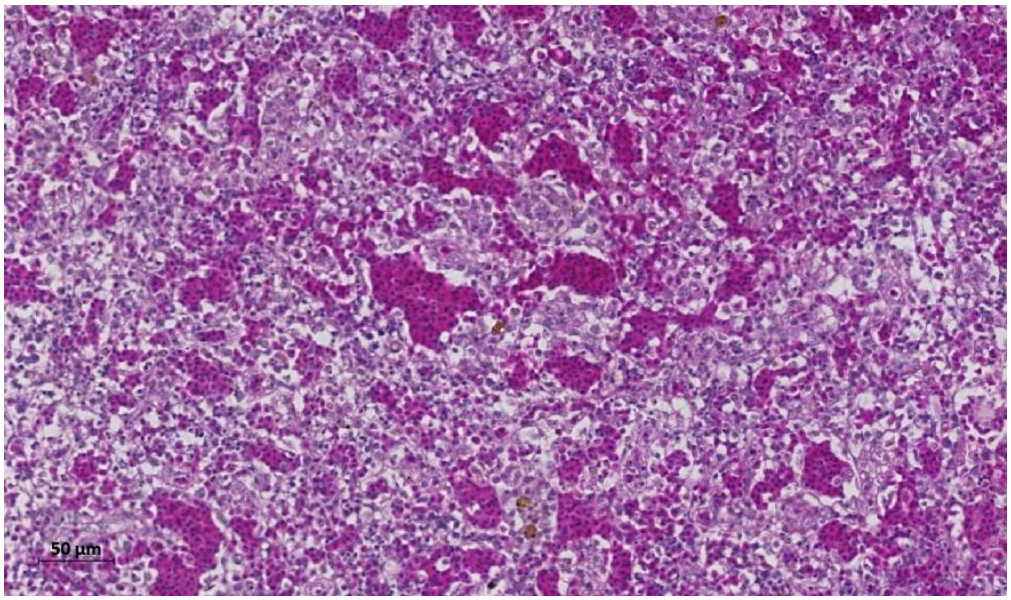

Figure 5.8 An H&E section of the spleen showing massive congestion.

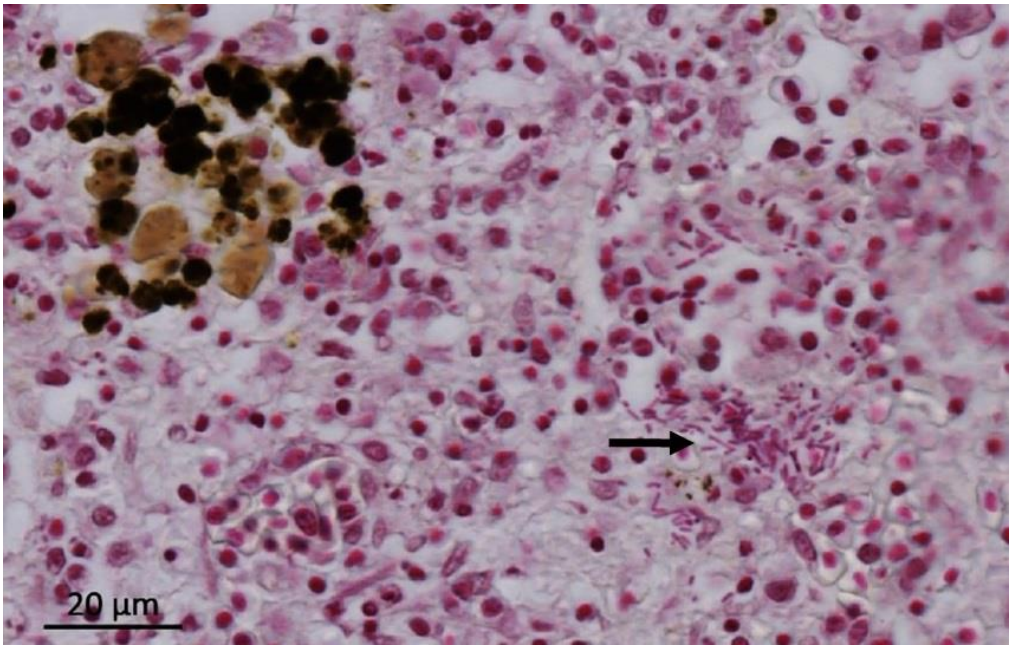

Figure 5.9 Gram-stained section of the spleen of *A. veronii* 17 showing numerous Gram-negative rods (arrow).

The liver showed multifocal hepatitis with necrosis, dissociation of the hepatocytes and congestion (**Error! Reference source not found.**). The kidney of most of the fish examined showed interstitial nephritis with diffuse coagulative necrosis of the tubular epithelium with most cells that are pyknotic and karyorrhectic (**Error! Reference source not found.**).

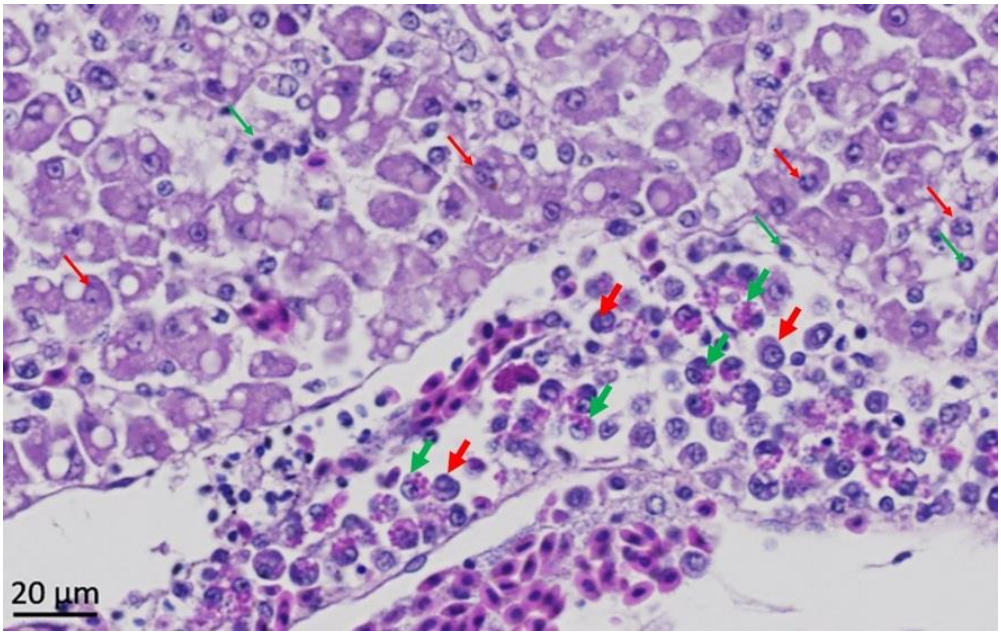

Figure 5.10 An H&E section of the liver showing necrosis characterised by margination of chromatin (thin red arrow), infiltration of lymphocytes (thin green arrow), and numerous macropahges (thick red arrow) and eosinophils (thick green arrow).

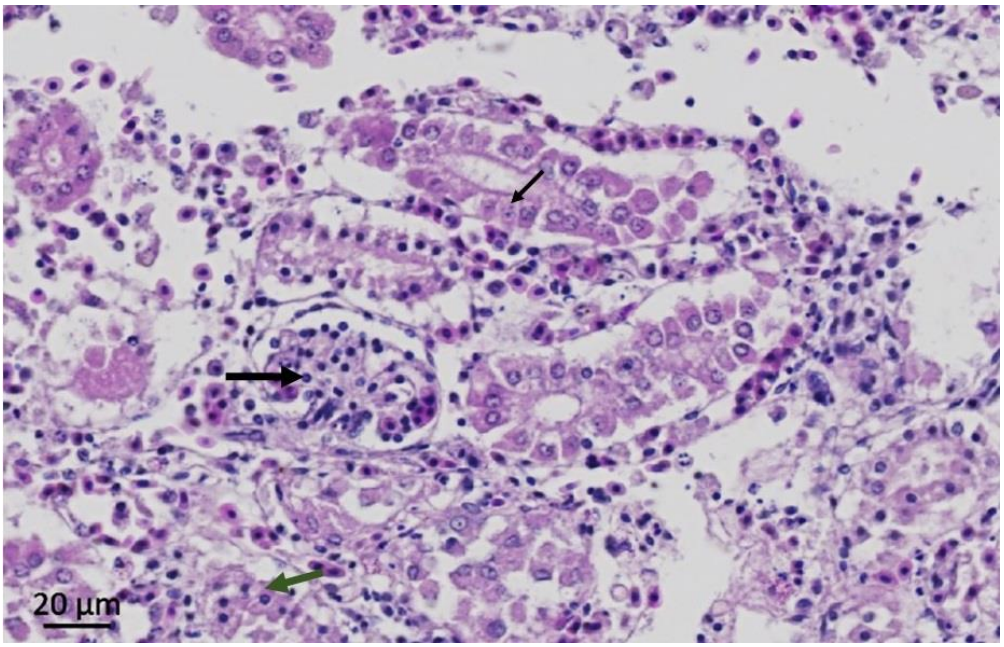

Figure 5.11 An H&E section of the kidney showing diffuse necrosis of the tubules with infiltration of inflammatory cells (thick arrow), and tubular cells that are pyknotic (green arrow) and karyorrhectic (thin arrow).

5.4.4 Antibiotic susceptibility and antimicrobial resistance determinants

5.4.4.1 Antibiotic susceptibility assay

The antibiotic susceptibility profiles for the 16 motile *Aeromonas* strains, representing 3 species of MAS (*i.e*., *A. veronii*, *A. caviae*, and *A. dhakensis*) is presented in **Error! Reference source not found.**. All the isolates were found to be susceptible to oxolinic acid, chloramphenicol, florfenicol and enrofloxacin. A 100% resistance was observed with amoxicillin and penicillin, 94% with ampicillin, 25% with both tetracycline and oxytetracycline, 38% with erythromycin and 63% with sulphamethoxazole-trimethoprim. Antibiotic resistance to three or more antibiotics was found in all isolates (100%, n=16) using the Kirby-Bauer disc diffusion method (

Table 5.8). Multiresistance defined as resistance to at least three antimicrobial classes (Schwarz et al., 2010) was found in 7 (44%) isolates.

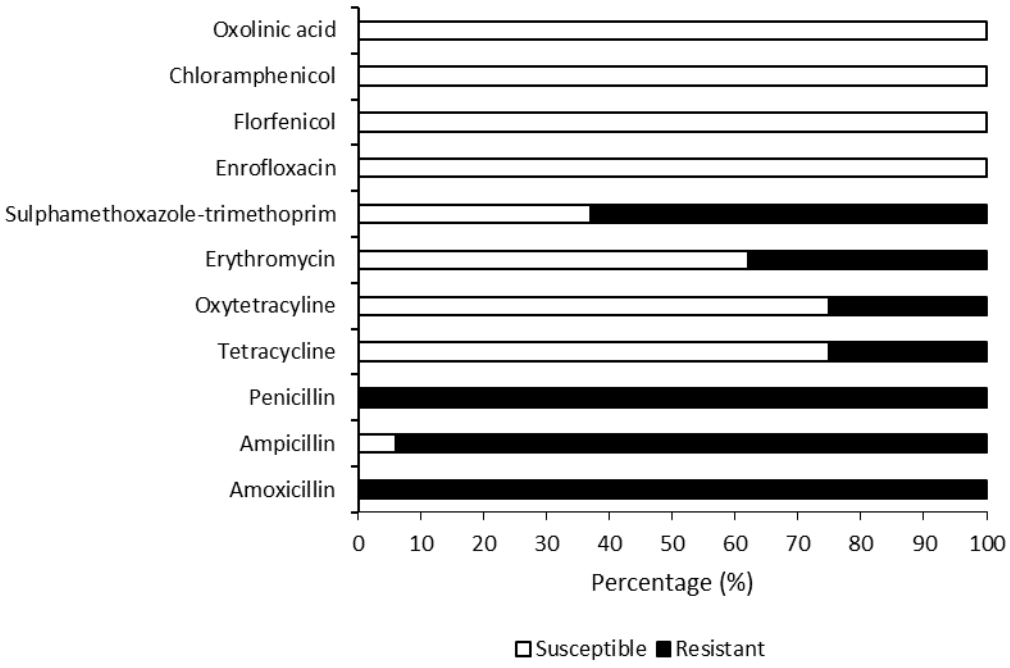

Figure 5.12 Antibiotic susceptibility patterns of motile *Aeromonas* isolated from farmed Nile tilapia in the Philippines.

5.4.4.2 Detection of antibiotic resistance genes

Resistance to oxytetracycline and tetracycline was identified in 4 (25%) isolates and was associated with the presence of *tetE* gene (Table 5.8). For sulfamethoxazole-trimethoprim, resistance was detected in 10 (63%) isolates and resistance genes *sul1* (Table 5.8) was only present in one isolate. All of the isolates were resistant to amoxicillin and penicillin. Resistance to other antimicrobials including ampicillin (94%) and erythromycin (38%) was found. No resistance was detected with enrofloxacin, oxolinic acid, florfenicol and chloramphenicol. Three *A. veronii* isolates carried class 1 integron-integrase

(*IntI1*) (Table 5.8) gene which is 100% identical to the integrase of *Escherichia coli* strain JJ1897 (Accession number CP013836.1). BLAST analysis of the amplified product for antimicrobial resistance genes confirmed their identity.

Table 5.8 Phenotypic vs. genotypic vs. multiresistance antibiotic profiles of the different motile *Aeromonas* spp. isolates.

Aeromonas Isolate	Multiresistance*	Resistant to**	Susceptible to**	AMR Gene/s Present
A. veronii 4	-	AML, AMP, P	TE, OT, E, SXT, ENR, FFC, C, OA	-
A. veronii 17	+	AML, AMP, P, E, SXT	TE, OT, ENR, FFC, C, OA	-
A. veronii 23	+	AML, AMP, P, TE, OT, E, SXT	ENR, FFC, C, OA	*tetE, sul1, IntI1*
A. veronii 26	-	AML, AMP, P	TE, OT, E, SXT, ENR, FFC, C, OA	-
A. veronii 34	-	AML, AMP, P, SXT	T, OT, E, ENR, FFC, C, OA	*Int1*
A. caviae 39	+	AML, AMP, P, E, SXT	TE, OT, ENR, FFC, C, OA	-
A. veronii 40	-	AML, P, TE, OT	AMP, E, SXT, ENR, FFC, C, OA	*tetE, IntI1*
A. veronii 41	+	AML, AMP, P, TE, OT	E, SXT, ENR, FFC, C, OA	*tetE*
A. veronii 46	-	AML, AMP, P, SXT	TE, OT, E, ENR, FFC, C, OA	-
A. veronii 47	+	AML, AMP, P, E, SXT	TE, OT, ENR, FFC, C, OA	-
A. veronii 50	-	AML, AMP, P, SXT	TE, OT, E, ENR, FFC, C, OA	-
A. veronii 51	-	AML, AMP, P, SXT	TE, OT, E, ENR, FFC, C, OA	-
A. veronii 64	+	AML, AMP, P, E, SXT	TE, OT, ENR, FFC, C, OA	-
A. veronii 67	-	AML, AMP, P, TE, OT	E, SXT, ENR, FFC, C, OA	*tetE*
A. dhakensis 69	+	AML, AMP, P, E, SXT	TE, OT, ENR, FFC, C, OA	-
A. veronii 74	-	AML, AMP, P	TE, OT, E, SXT, ENR, FFC, C, OA	-

* + = resistant to 3 or more antibiotic class; - = resistant to only 2 antibiotic class

**AML = amoxicillin; AMP = ampicillin; P = penicillin; TE = tetracycline; OT = oxytetracycline; E = erythromycin; SXT = sulfamethoxazole-trimethoprim; ENR = enrofloxacin; FFC = florfenicol; C = chloramphenicol; OA = oxolinic acid.

*** - = absent

5.4.5 Detection of virulence determinant and virulence genes

5.4.5.1 Phenotypic expression of virulence detected in vitro

Phenotypic virulence traits of Nile tilapia-borne aeromonads were observed by *in vitro* pathogenicity tests including *β*-hemolysis activity (**Error! Reference source not found.**A), biofilm formation (**Error! Reference source**

not found.B) and production of DNAse (**Error! Reference source not found.**C), gelatinase, lipase, and protease enzymes (**Error! Reference source not found.**D). In our study, 13 (81%) isolates were positive for lipase production and biofilm formation was found in 16 (100%) isolates. DNAse enzyme production and gelatinase activity were observed in 13 (81%) and 15 (94%) isolates, respectively. Also, 14 (88%) and 16 (100%) isolates were positive for protease and *β*-haemolysis, respectively. The result for the extracellular enzyme activities are summarised in **Error! Reference source not found.**.

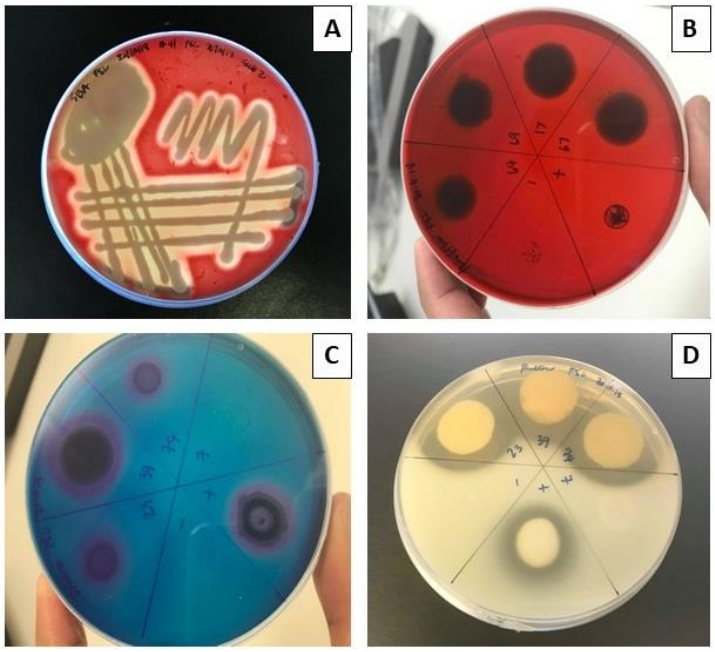

Figure 5.13 Some extracellular enzymes/virulence activity secreted by the *Aeromonas* isolates (A) biofilm formation; (B) extracellular protease; (C) extracellular DNAse; (D) haemolysis on 5% sheep blood agar.

5.4.5.2 Detection virulence genes

PCR amplification of the *act* gene which also detects the presence of *aer* and *hylA* showed 100% positivity. Separate detection of *aer* and *hylA* genes showed 12 isolates (75%) and only isolate 64 (*A. dhakensis*) were positive for the said virulence genes, respectively. Aside from *act* and *aer*, the most

frequent genes were *eno* (100%) encoding for enolase followed by *exu* (94%) for DNAse, and *ser* (75%) for serine protease. The prevalence of *aexT/aexU* coding for ADP-ribosyl transferase was 38 %, and for *lafA* lateral flagella and *gcaT* cholesterol acyl transferase was 31%. Only two isolates (39 and 69, 13%) were positive for the gene *ahyB* encoding elastase and. The genes *ascV* coding for T3SS membrane component, *lip* for lipase, *alt* for heat-labile cytotonic toxin, and *dam* for DNA adenine methyltransferase were detected at 6%. The gene *ast* for heat-stable cytotonic toxin was not detected in any isolate.

Table 5.9 Virulence Profile of motile *Aeromonas* isolates from farmed Nile tilapia in the Philippines.

Virulence Determinants	**Motile Aeromonas Isolates**																
	4	**17**	**23**	**26**	**34**	**39**	**40**	**41**	**46**	**47**	**50**	**51**	**64**	**67**	**69**	**74**	**Control**
In-Vitro Phenotypic Expression																	
Haemolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+ Ah
Protease	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+ Ah
Lipase	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+ Ah
DNAse	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+ Ah
Gelatinase	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+ Ah
Biofilm	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+ Ah
Virulence Genes																	
Aer	+	-	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+ Ah
hylA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+ Ah
Exu	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+ Ah
Ser	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+ As
lafA	-	-	-	-	+	-	-	+	-	+	-	-	-	+	+	-	+ Seq
Act	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+ Ah
ahyB	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+ Ah
aexT/aexU	-	-	-	+	-	-	-	-	-	+	+	-	+	-	+	+	+ As
Lip	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+ Ah
Alt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+ Ah
Ast	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ Ah
Dam	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+ Ah
Eno	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+ Ah
ascV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+ Seq
gcaT	-	-	-	-	-	+	-	+	-	-	-	+	+	-	+	-	+ Ah

* + = positive; - = negative, Ah = *Aeromonas hydrophila* NCIMB 9240; As = *Aeromonas salmonicida* NCIMB 1102; Seq = confirmed by gene sequencing

In an attempt to simplify the virulence profiles of these motile *Aeromonas* species the virulence profiles are shown in **Error! Reference source not found.**. The data show a combination of virulence factors detected in the *Aeromonas* isolates from farmed Nile tilapia (**Error! Reference source not found.**). It was clear that a range of virulence profiles were detected amongst MAS strains and species (**Error! Reference source not found.**), where the minimum number of virulence genes detected was 3 and the maximum was 12, which were found in isolate 4, identified as *A. veronii* and isolate 69 identified as *A. dhakensis* (**Error! Reference source not found.**). For Taal Lake isolates, there are six types of virulence genotypes, which include isolates 4, 17, 23, 26, 34, and 39, were all associated with diseased tilapia. Two of these genotypes *aer-exu-ser-act-eno* and *aer-exu-ser-act-aexT/aexU-lip-eno* were shared with *A. veronii* isolates from a different geographical site, Calauan Laguna. *Aeromonas* spp. with genotype containing an *aer-act-ser* (69%, n=16) subset of virulence genes is linked with diseased fish. However, an *aer-act* (isolate 4) or *act* only (isolate 17) containing genotype is also associated with disease. One isolate, the *A. dhakensis (*isolate 69) contains 12 virulence genes, which is the isolate highest number of virulence factors. *A. veronii* isolates from Laguna Lake and from Nueva Ecija have their own distinct virulence genotypes not shared with either Taal Lake or Calauan Laguna.

Table 5.10 Subsets of virulence genes in *Aeromonas* isolates from farmed Nile tilapia in the Philippines.

Subset of Virulence gene	Isolate/s	Species	Geographical Location
aer-act-eno	4	*A. jandaei*	Taal Lake
exu*-*act*-*eno	17	*A. veronii*	Taal Lake
aer*-*exu*-*ser*-*act*-*eno	23, 40	*A. veronii*	Taal Lake, Calauan Laguna
aer*-*exu*-*ser*-*act*-*aexT/aexU*-*lip*-*eno	26, 50, 74	*A. veronii,*	Taal Lake, Calauan Laguna
aer*-*exu*-*ser*-*lafA*-*act*-*eno	34	*A. veronii*	Taal Lake
exu*-*act*-*ahyB*-*lip*-*eno*-*gcaT	39	*A. caviae*	Taal Lake
exu*-*ser*-*lafA*-*act*-*lip*-*eno	67	*A. veronii*	Laguna Lake
exu*-*ser*-*lafA*-*act*-*eno*-*gcaT	41	*A. veronii*	Laguna Lake
aer*-*exu*-*ser*-*act*-*lip*-*dam*-*eno	46	*A. veronii*	Calauan, Laguna
aer*-*exu*-*ser*-*lafA*-*act*-*aexT/aexU*-*eno	47	*A. veronii*	Calauan, Laguna
aer*-*exu*-*ser*-*act*-*lip*-*eno*-*gcaT	51	*A. veronii*	Calauan, Laguna
aer*-*hylA*-*exu*-*lafA*-*act*-*ahyB*-*aexT/aexU*-*alt*-*dam*-*eno*-*ascV*-*gcaT	69	*A. dhakensis*	Calauan Laguna
aer*-*exu*-*ser*-*act*-*aexT/aexU*-*lip*-*eno*-*gcaT	64	*A. veronii*	Nueva Ecija

5.5 Discussion

Species identification in the MAS strains associated with infectious disease outbreaks in freshwater aquaculture systems is complex. This is well recognised within the sector, particularly when undertaking initial disease or diagnostic investigation where it is important to know the aetiological agent for subsequent treatment and or prevention. However, given the heterogeneity within the MAS combined with the ubiquitous nature of these bacterial species, understanding the pathogenesis has become rather challenging. In this present study, the most common bacterial species identified from the disease outbreaks from sampled tilapia farms was *A. veronii*. This was identified using a combination of methods where similar to previous reports (Dong et al., 2017; Amal et al., 2018; Mzula et al., 2020). In the study presented, a comparative

approach was adopted to identify the bacteria to species level. A greater level of agreement was found in speciation using the 2 difference molecular approaches compared with the biochemical assays alone. Recent work by Pakingking et al. (2020; 2015) found a similar range of bacterial species recovered from the pond water environment and the microbiota of farmed Nile tilapia in the Philippines, however, their identification methods relied on biochemical profiles. In this study alone we identified 5 variations in the biochemical patterns from 14 strains belonging to the same species. To provide confidence in the identification of pathogenic MAS species and strains a single approach is not recommended, instead a combination of approaches which would include traditional phenotypic test that are cheap and reliable but also give an immediate results with one of the 2 PCR methods would be recommended for diagnostic investigations.

Prior to API 20E identification, the isolates were already identified by *16S rRNA* gene sequencing which is found in Chapter 3. This test was performed to determine the biochemical profiles of the piscine associated motile *Aeromonas* isolates in order to develop a profile which can aid in their identification. In the result of this study, API 20E was not able to correctly identify the isolates at species level and in some instance at the genus level like where some *Aeromonas* were identified as *Vibrio*. Although misidentification was very high, comparing the results with previous studies on API 20E results of piscine motile aeromonads revealed high positivity in 10 of the tests and consistent negative results in 6 other tests. Given this data, biochemical profiles for motile aeromonads can be developed in order to aid their identification. The misidentification of motile *Aeromonas* by API 20E hinders the recognition of *Aeromonas* infections implicated in diseased fish and also in clinical cases in

immunodeficient hosts. Our results demonstrate that the use of API 20E system has some limits in the diagnostic of piscine motile *Aeromonas*, however, the use of this method in the current laboratory practice can still be employed as long as analysts take cognizance of the origin of bacterial isolates and use modified biochemical profiles that are best suited to identify piscine and environmental isolates using previous information and studies for the particular bacterial genera and species of interest.

The result of *16S rRNA* gene sequencing and the *rpoD* sequencing showed excellent agreement in the identification of the *Aeromonas* isolates. From the identification tests, it was revealed that most of the isolates are identified as *A. veronii*. Other species includes *A. caviae* and *A. dhakensis* which we report for the first time as associated with naturally diseased Nile tilapia in the Philippines. The correct identification rate with biochemical tests like API 20E has been shown to be very low as compared to molecular methods (Persson et al., 2015). Bacterial species identification has relied recently on the use of *16S rRNA* gene sequencing, however, for *Aeromonas* this gene is not the optimal target due to low discriminatory power and interspecies variation (Shin et al., 2015; Kupfer et al., 2006; Morandi et al., 2005; Martinez-Murcia et al., 1992). Several studies have described that some housekeeping genes in this tight taxonomic bacterial genus are able to differentiate them at the species level. These genes include RNA polymerase subunits B (*rpoB*) and D (*rpoD*), and the DNA gyrase subunit B (*gyrB*) (Persson et al., 2015; Martinez-Murcia et al., 2011; Lamy, Laurent & Kodjo, 2010). Infectious disease particularly MAS caused by motile aeromonads is associated primarily with *A. hydrophila* (Austin & Austin, 2016). However, this study showed that *A. veronii* dominated as the

recovered infectious etiologic agent as we are not able to isolate a single *A. hydrophila*. This observation is consistent with reports that natural infections caused by *A. hydrophila* with reliable taxonomic identification is relatively rare in tilapia (Dong et al., 2017). Moreover, the *Aeromonas* spp. were all recovered in freshwater tilapia farms in the island of Luzon particularly in the Taal Lake, Batangas and Laguna. This result on geographical occurrence is also similar to previous study of the authors for streptococcosis in farmed tilapia (Legario et al, 2020).

Bacterial identification using the *16S rRNA* gene is generally accepted as stable and specific biomarker due to its highly conserved nature with microheterogenous portions or polymorphisms (Alperi et al., 2008). These scattered nucleotide differences in the *16S rRNA* is the basis for its use in bacterial species delineation. However, this low level of microheterogeneities may lead to misidentification (Cilia et al., 1996; Marchandin et al., 2003; Morandi et al., 2005) particularly for species that are tightly defined such as those belonging to genus *Aeromonas* (Martinez-Murcia et al., 1992; Martinez-Murcia et al., 2007). The high interspecies similarity of the *16S rRNA* gene sequence among aeromonads, which ranges from 96.7 to 100% (Martínez-Murcia et al. 2007), in addition to overlapping biochemical profiles and minimal information about the correlation between phenotypic and genotypic identification makes its taxonomy complex (Ormen et al. 2005).

The taxonomy of the genus *Aeromonas* has undergone continuous changes due to addition of new species and new diagnostic criteria such as the use of housekeeping genes (Martinez-Murcia et al., 2007; Saavedra et al., 2006). The *rpoD* gene (Martinez-Murcia et al., 2007; Soler et al., 2004), among

other housekeeping genes (Kupfer et al., 2006; Saavedra et al., 2006; Yanez et al., 2003), has proven to be an excellent molecular tool for inferring the taxonomy of *Aeromonas* (Alperi et al., 2008). In this study, results from 16S *rRNA* and *rpoD* showed the same identification except for one isolate. Isolate 04 was identified in 16S rRNA as *A. jandaei* while in *rpoD* it was *A. veronii*. Looking at the phylogenetic relationship of the isolate in both phylogenetic trees, it can be assumed that identification based on *rpoD* is more accurate since it is grouped within the subgroup of the *A. veronii* in the *rpoD* tree while in *16S rRNA* tree it was distantly positioned from *A. jandaei* ATCC 49658T.

The use of MALDI-TOF MS has been successful in the identification of bacterial genera of relevance to aquaculture (Regecova et al., 2014) and could presumably efficiently identify *Aeromonas* species (Benagli et al., 2012; Chen et al., 2014; Deng et al., 2014; Donohue et al., 2006; Donohue et al., 2007; Lamy et al., 2011; Rychert et al., 2015). However, there are no specific studies that include a large number of *Aeromonas* isolates from diseased fish. The detection and identification of genus- and species-specific mass peaks can be useful for bacterial identification (Holland et al., 1999, 2000). The high consistency of the mass peaks in the spectral profiles among *A. veronii*, *A. hydrophila*, *A. caviae* and *A. dhakensis* in this study suggests a low variability in their MALDI-TOF proteome which will make speciation of piscine motile *Aeromonas* difficult using this method alone. This close-similarity among MALDI-TOF spectral profiles from the motile *Aeromonas* isolates in this study, is likely related with the strong phylogenetic relatedness within the genus *Aeromonas* (Martin-Carnahan & Joseph, 2005). Therefore, these peaks could be considered potential genus-characteristic ions but not species-characteristic mass peaks. However, some

specific mass ions were observed only in the spectra of three *A. veronii* isolates which may indicate variability in the proteome within the species in line with their high intra-species variability in the genus *Aeromonas* (Mandrell et al., 2005) that would limit their use as species-characteristic mass peaks. Comparison of the mass spectra and mass list obtained by MALDI-TOF MS for very closely related microorganism like *Aeromonas* is hindered by experimental parameters such as bacterial culture conditions, protein extraction protocol or sample preparation protocol and the equipment as it affects the reproducibility obtained (Jackson et al., 2005; Williams et al., 2003). The culture medium used in this study was TSA while in the study of Perez-Sancho et al. (2018) it was blood agar. Additionally, dilution of the extracted protein was performed in this study which was not done by the work of Perez-Sancho et al. (2018). In this study, dilution of the protein extracts allowed more identification of mass peaks since some of the mass peaks were not identifiable at higher protein concentrations. These two differences could explain why some the mass peaks identified in this study were absent or not consistently present in the work of Perez-Sancho et al. (2018). Results in the present study highlight the significant and unpredictable effect of sample dilution when using MALDI-TOF MS. The protein spectral profile of a sample shows some peaks which are only seen at lower dilutions. This hinders the identification/assessment of biomarkers using this platform. Single sample dilutions when analysed resulted in substantial discrepancies and that potential biomarkers might be detectable at certain concentration ranges only and that valuable information is lost when too restricted dilution ranges are analysed (Albalat et al., 2013). The data in the present study presented a problem of using a single sample dilution in the

identification of motile *Aeromonas* by protein spectral profile biomarkers using MALDITOF. The sample composition and dilution severely affect peptide quantification, therefore analysing samples by serial dilutions is recommended.

The observations of using MALDI-TOF to speciate *Aeromonas* isolates in this study is similar to the work of Perez-Sancho et al. (2018) where they found the utility of MALDI-TOF MS for the identification of *Aeromonas* at the genus level but it has a poor performance in accurate identification at species level of *Aeromonas* species implicated in fish disease. Some of the peak masses (4256, 4700, 5049, 6304 and 6480 m/z) of the motile *Aeromonas* in this study were also identified in the fish *Aeromonas* isolates in the study of Perez-Sancho et al., (2018).This is in contrast to the study of Shin et al., (2017) on clinical isolates of *Aeromonas* where they found good correlation in species identification with MALDI-TOF and housekeeping gene sequencing although gene sequencing with phylogenetic analysis is still the most accurate. Therefore, further studies with standardized experimental protocols will be necessary before well-defined potential species-specific peaks for piscine motile *Aeromonas* can be assigned. Moreover, it will also be valuable to determine the molecular weight of the specific peak masses and their peptide sequence or identity in order to know whether these expressed proteins play crucial roles in the pathogenesis of motile aeromonads in fish.

The identification of motile *Aeromonas* associated with fish epizootics can be performed using the conventional biochemical methods or API 20E test kit as long as the fish health laboratory is aware of its limitation and instead use species profiles from published studies of *Aeromonas* recovered from fish. This is especially relevant to poor countries that lack the capacity to perform

advance identification methods like PCR and gene sequencing. However, for accurate identification and epidemiological surveillance the use of housekeeping gene sequencing like *rpoD* is recommended. Whilst MALDI-TOF is becoming popular for bacterial identification due to its ease and low cost of consumables (Rosa et al., 2019), at this stage it is not yet recommended for use in the species identification of piscine motile *Aeromonas* due to lack of species-characteristic mass peaks.

The growth of the *Aeromonas* isolates at different temperature and salinity levels were consistent with the previous study of Knochel (1990). Motile aeromonas isolates from environments with temperatures above 25° C have optimal growth at temperature range of 10-37° C and salinity of 0-4% NaCl which is very similar with isolates from this study where they grew optimally at 15-37°C and 0-4%. The ability of the isolates to tolerate wide range of temperature and salinity supports their growth making these bacteria formidable pathogens with persistent viability and presence in diverse aquatic ecosystems. The ecological background of the isolates which is from tropical waters influenced their thermal and salinity growth range which is indicative of their potential to cause infection to their susceptible host (Knochel, 1990).

Our results revealed that the motile aeromonads isolated from farmed Nile tilapia displayed a significant proportion of phenotypic virulence traits. These phenotypic traits are related to the detected virulence genes that could lead to the pathogenicity of the *Aeromonas* strains. Biofilm formation acts against chemotaxis and the action of antibiotics while extracellular lipase enzyme interrupts the normal immune functions (Tomás 2012). The β-haemolysis activity is related to the enterotoxin production (Arslan & Kucuksari

2015). DNase, protease and gelatinase enzymes play important roles in the degradation of nucleic acids, protein and gelatine, respectively, during infection and invasion processes (Pemberton *et al*. 1997).

Aeromonas isolates in the present study are potential pathogens due to the high number of virulence genes present although there is not a definitive link between the presence of specific toxin genes and clinical presentation (Roges et al., 2020; Hoel et al., 2017). The mechanism of pathogenesis in *Aeromonas* is complex and still not well understood as its virulence is multifactorial and partially elucidated (Talagrand-Rebould et al., 2018; Khor et al., 2015; Sen & Rodgers, 2004) although numerous virulence factors have been described (Tomas, 2012). The expression of the putative virulence-associated factors in *Aeromonas* appears to be affected by environmental conditions (Tso & Dooley 1995, Merino et al. 1998), making the detection of true virulent strains difficult. These putative virulence factors that have been detected and studied include haemolysins, cytotoxins, enterotoxins proteases, elastase, lipases, DNAses and adhesins (lateral flagella) (Ran et al., 2018; Nawaz et al., 2010; Kirov et al., 2002). The identification of these virulence factors in a pathogen is necessary to determine the potential pathogenicity and can be used as target for vaccines (Khor et al., 2015). In our study, all *Aeromonas* isolates possessed the cytotoxic enterotoxin *act* gene while 75% of them were positive for the aerolysin *aer* gene. These two genes play an important role in the establishment of infections (Chopra et al., 1996; Ran et al., 2018). Aerolysin is a pore-forming toxin that is secreted via the Type 2 secretion system (T2SS) of *Aeromonas* which causes the destruction of the eukaryotic cell membrane and triggers osmotic lysis (Abrami et al., 2000; Rossjohn et al., 1988). Moreover, *Aeromonas*

aerolysin induces lesions in the intestine enabling it to cross epithelial barrier and causes systemic invasion (Ran et al., 2018). The *act* gene is the most cytotoxic enterotoxin, in addition to its enterotoxic activity, has haemolytic and cytotoxic activities and unlike other enterotoxins it is exported via T2SS (Rasmussen-Ivey et al., 2016; Chopra & Houston, 1999; Xu et al., 1998). These toxins trigger fluid accumulation in the intestinal loop (Nawaz, et al., 2010) which will eventually cause ascites which is one of the most prevalent clinical signs of the tilapia samples in this study. Moreover, due to its lytic activity on erythrocytes the fish becomes anaemic which is evidenced by extreme gill pallor of all moribund samples. The observed expression of β-haemolytic activity of all the motile Aeromonas isolates in this study can be correlated positively either to the presence of the *act* or *aer* genes or both since both genes have haemolytic function. All the isolates in this study contained enolase *eno* gene which aside from its glycolytic activity has been linked in the pathogenesis of some bacteria. Surface-expressed enolase binds to plasminogen which subsequently activates the fibrinolytic system and promotes bacterial penetration of the barriers of infected host (Sha et al., 2009). This activity of enolase may aid in the dissemination of *Aeromonas* bacteria in the tissues and organs thus contributing to septicaemia. The presence of T3SS ADP-ribosylating toxins *aexT/aexU* was detected in five *A. veronii* and one *A. dhakensis* isolates. These toxins are involved in the delivery of toxins directly into the host cells and induce apoptosis (Senderovich et al., 2012). Some studies have shown that presence of virulence genes are species-dependent (Nawaz et al., 2010; Sun et al., 2016) as in the case of *aexT* which was found almost exclusively in *A. veronii* (Martino et al., 2011) as also observed in our

study. The primers designed for ADP-ribosylating toxins detection matches the homologous parts shared by *aexT* and *aexU* genes (Talagrand-Rebould et al., 2018), thus positivity for *A. dhakensis* may be due to presence of *aexU* but not *aexT*. The presence of either of these toxins does not necessarily confers the isolate pathogenic since presence of Type 3 secretion system (T3SS) components like *ascV* is required for the delivery of *aexT/aexU*. The presence of a virulence gene does not imply its expression because it may be involved in a non-pathogenic mutualistic interaction (Silver et al., 2007) albeit the *A. veronii* isolates positive for *aexT/aexU* were all recovered from diseased fish. In this view, screening of *aexT/aexU* by PCR resulting to positive result should be followed by PCR *aexT* gene alone.

The polar monotrichous flagellum of motile *Aeromonas* which is responsible for its swimming motility is important not only in the initial approach of bacterial cells but also to the attachment to surfaces (Kirov et al., 2002). Additionally, some strains produce inducible lateral flagella which are involved in swarming motility, a phenomenon associated with the expansion of colonisation surfaces, biomass production and biofilm formation (Santos et al., 2010). Bacteria in biofilms are more resistant to host defences and antimicrobial agents than they are as free-floating planktonic cells and express more virulent phenotypes as a result of gene activation through bacterial communication ("quorum sensing") or gene transfer (Costerton et al., 1999). Both flagellar systems are associated with the colonisation process and biofilm formation, however lateral flagella are more involved in swarming which makes colonisation of the gastrointestinal tract efficient thus is critical in virulence (Kirov et al., 2002). In this study, all the *Aeromonas* isolates exhibited biofilm

formation however only five isolates composed of 4 *A. veronii* and 1 *A. dhakensis* were positive for the lateral flagella *lafA* gene indicating that polar flagellum contributes to biofilm formation and that the lateral flagella confer increased virulence to the strain. In the recent study of Yang et al. (2021), virulent, attenuated and avirulent *A. veronii* strains all contained polar and lateral flagella genes which suggest that some unknown mechanisms may be involved for their expression to contribute to virulence. Although the genomes of some *A. veronii* strains are publicly accessible, there is a need to perform genetic manipulations to evaluate the contribution of polar and lateral flagella to virulence (Rasmussen-Ivey et al., 2016).

Genotyping the *Aeromonas* isolates based on their subset of virulence genes revealed some leading observations. All *A. veronii* genotypes containing *aer-act-ser* genes were recovered from diseased fish. The cytotoxic enterotoxin and aerolysin are important factors for the establishment of infection in *Aeromonas* pathogenesis. Moreover, serine protease is vital for the expression of aerolysin gene. However, some genotypes of *A. veronii* like the *exu-act-eno* was associated with a diseased fish while those with *exu-ser-lafA-act-lip-eno* and *exu-ser-lafA-act-eno-gcaT* are from apparently healthy samples but upon histopathological examination the fish showed signs of cellular and tissue damage. The two *A. veronii* genotypes, *aer-exu-ser-act-eno* and *aer-exu-ser-act-aexT/aexU-lip-eno,* in Taal Lake shared with Calauan, Laguna isolates is notable since the Calauan hatchery supplies the tilapia fry in grow-out cages in Taal Lake. This indicates that there is movement of bacterial pathogens between the two sites. Significantly, Laguna Lake and Nueva Ecija *A. veronii* isolates have their own distinct virulence genotypes since these areas do not

source out their fry from Calauan hatcheries. The two other *Aeromonas* spp. isolates namely *A. caviae* and *A. dhakensis* have also their own distinct virulence profiles. The *A. dhakensis* isolate showed the most numerous virulence genes, however, it was recovered from tilapia fry which we cannot determine to be diseased although it came from a hatchery where the broodstocks were having disease outbreak. Looking at the genospecies of the *Aeromonas* isolates, the *aer* and *act* genes were consistently present in isolates recovered from diseased tilapia presenting numerous gross and internal clinical signs while those that have only the *act* gene were from apparently healthy tilapia although histopathologically they also exhibited signs of tissue/cellular damage. The high proportion of these two genes *aer* (75%) and *act* (100%) among *Aeromonas* strain in this study is similar to the previous studies of Nawaz et al. (2010) and Khor et al. (2015) where they also were present in high percentage in *A. veronii* isolates from diseased catfish and in aquatic *Aeromonas* spp., respectively. Based on this result, *act* gene may be integral in the pathogenicity of aquatic *Aeromonas* especially of the *A. veronii* and that the presence of *aer* enhances the severity of the infection as displayed by the gross clinical signs. As these two genes exhibits haemolytic, cytotoxic and enterotoxic activities which are important in establishment of infection (Nawaz et al., 2010), their presence in a particular *Aeromonas* strain can be an indication of the virulence of the strain. The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads (Santos et al., 1999). Over-all this shows a significant variability in terms of the pathogenic potential of the isolates especially on the *A. veronii* strains. The different combinations of virulence genes in different geographical areas suggests that different

mechanisms may be used by these *Aeromonas* spp. to colonise and cause infection or to mutualistically co-exist with the fish host. The expression of virulence factors is linked to gene regulation cascades associated with interactions of microorganisms with the environment in which they are found (Rasmussen-Ivey, 2016). These findings need to be evaluated carefully in terms of the pathogenesis of *Aeromonas* spp. in fish since the exact relationship between presence of virulence genes or subset of virulence genes and the ability of a particular genospecies to cause disease has not been fully established.

Increasing reports of *A. veronii* and other motile aeromonads cases have indicated that their virulence and antibiotic resistance are both gradually increasing (Shameena et al., 2020; Zhang et al., 2019). In this study, the phenotypic and genotypic characteristics of the *Aeromonas* isolates correlated well with the observed clinical signs of the moribund tilapia such as dropsy, ascites, lethargy, inflammation of the internal organs particularly the spleen, kidney and liver, and body redness. Moreover, histopathology revealed cellular changes such as necrosis, inflammation, haemorrhages, infiltration of immune response cells among others. More and more countries have recognised the threat of *A. veronii* and have classified the bacteria as a highly virulent *Aeromonas* capable of infecting both humans and aquatic species (Borchardt et al., 2003; World Health Organization, 2011). Hence, more research into motile aeromonads particularly *A. veronii* is of importance considering both its economic value in aquaculture and public health

There is a widespread belief that the prophylactic use of antibiotics in aquaculture, aside from its systemic application in treatment of fish diseases,

enhances growth and reduces mortality thereby increasing production and revenues (Pridgeon & Klesius, 2013). These compounds are commonly administered through fish feed for the prevention and/or treatment of *Aeromonas*-related diseases such as motile *Aeromonas* septicaemia (MAS), haemorrhagic septicaemia, ulcer disease, and red-sore disease (Swann & White, 1991). The prevention and treatment of fish diseases through extensive application of antimicrobial agents contributes to the development of antibiotic-resistant bacterial strains (Rhodes et al., 2000). Most commonly used antibiotics in aquaculture around the world include sulphonamides potentiated with trimethoprim, ormethoprim, oxytetracycline, florfenicol, and erythromycin (Serrano, 2005). Consequently, resistance to antibiotics has been widely spread in aquaculture environments, which has been reported in many studies (Petersen et al. 2002; Colquhoun et al. 2007; Newaj-Fyzul et al. 2008; Sørum 2008; Shah et al. 2012). As a result of these concerns, the usage of antibiotics as growth promoters has been strictly regulated or even banned in many countries (Piotrowska & Popowska, 2014). In this study, the *Aeromonas* isolates were resistant to β-lactam antibiotics since the genus *Aeromonas* has an intrinsic resistance to this compound (Senderovich et al., 2012). One particular isolate of concern was the *A. veronii* 23 because it has resistance to four antibiotic classes. Moreover, this isolate harbours two plasmid-mediated resistance genes and one class 1 integron. Having these mobile elements that can carry antibiotic resistance may become problem in aquaculture and also in humans in zoonotic infections. Other three *A. veronii* isolates also harbours class 1 integron which may also allow them to obtain or transfer antibiotic resistance not only among *Aeromonas* species but other bacterial species that

are of concern not only in aquaculture but in human health. Whilst there is an observed increase antibiotic resistance among motile *Aeromonas*, the Philippine strains are currently still susceptible to oxolinic acid, enrofloxacin, florfenicol and chloramphenicol. However, this should not make the aquaculture industry complacent but instead be vigilant and adhere to good antibiotic practice since these antibiotics may become ineffective if the bacterial pathogens acquire resistance.

Although antibiotics may ultimately contribute to aquaculture productivity, there is a rising concern that the extensive application of antibiotics in aquaculture selects for antibiotic resistant bacteria as well as antibiotic resistance genes (ARGs), especially when inadequate- or over-doses of antibiotics are administered (Kolar et al., 2001). These can ultimately contribute to antibiotic resistance on a global level through water discharge and fish consumption. Nonetheless, data on bacterial community composition and antibiotic resistance dynamics in aquaculture environments subjected to antibiotic use are still limited. Antimicrobial resistance in *Aeromonas* can either be chromosomally mediated or by mobile elements like plasmids, transposons and integrons (Stratev & Odeyemi, 2016). Whilst chromosomally mediated resistance is intrinsic in a particular bacterial genus, acquired resistance is via mobile elements that facilitates its rapid spread of resistance genes across bacterial genera and species (Stratev & Odeyemi, 2016). These mobile elements could be transmitted via three pathways namely transformation, transduction and conjugation (Romero et al., 2012). Therefore, it is very important to differentiate intrinsic from acquired resistance in studying antibiotic response profile of pathogens since it is the acquired resistance that is of

concern in therapeutic management of infections. In this study, most of the isolates were resistant to β-lactams antibiotics as *Aeromonas* species are known to be intrinsically resistant to this antibiotic class due to their production of multiple inducible, chromosomally encoded β-lactamases (Senderovich et al., 2012; Ndi & Barton, 2011; Fosse, Giraud-Morin & Madinier, 2003). We were not able to detect by PCR the plasmid-mediated β-lactam resistance genes namely *blaTEM*, *blaSHV* and *blaCTX-M* which are implicated in extended spectrum β-lactamase (ESBL) resistance. Therefore the observed phenotypic resistance to the β-lactam antibiotics in our isolates is due to their intrinsic resistance explaining the high levels of amoxicillin, ampicillin and penicillin resistance in the Nile tilapia associated strains (Bakken et al., 1988). Resistance to tetracycline and oxytetracycline was detected in four *A. veronii* isolates which was encoded by the plasmid-mediated *tetE* gene which studies have shown to be a dominant tetracycline resistance gene in aeromonads from freshwater fish aquaculture (Piotrowska & Popowska, 2014; Han et al., 2012; Cizek et al., 2010; Nawaz et al., 2006). Majority of the isolates showing phenotypic resistance to SXT did not have the sulfonamide and trimethoprim resistance genes except for *A. veronii* A23. This suggests the presence of other resistance mechanisms such as other described resistance genes or mutation in chromosomal genes encoding dihydropteroate synthetase (Giordanetto et al., 2005). Erythromycin resistance was detected in 38% of the isolates, however, we did not assay to detect resistance genes for macrolides since it is only primarily used against Gram-positive bacteria in shrimp farms in the Philippines. All isolates were susceptible to chloramphenicol, florfenicol, enrofloxacin and oxolinic acid. These antibiotics, except for chloramphenicol, which is banned in

the Philippine aquaculture, can be future alternatives to β-lactams antibiotics and tetracyclines for treatment of MAS in tilapia fisheries.

In this study, we detected the presence of class 1 integron *IntI1* coding for integrase in three *A. veronii* isolates. Integrases are responsible for the mobility of antibiotic resistance cassettes found in integrons. Integrons are a class of site-specific recombination elements which contain a variable region where antibiotic resistance genes are found organised as resistance cassettes (Francia et al, 1999). These integrons are important contributors of antibiotic resistance in members of the *Aeromonadaceae* (Jacobs & Chenia, 2007). One of the isolates with *IntI1* was also found with *sul1* gene which is involved in sulfonamide resistance while the other two isolates have none. Other antibiotic resistance genes may be present in the two other *IntI1* genes but were not included in the AMR genes assayed in this study. The presence of a single gene cassette *sul1* correlates with studies that integrons from fish pathogenic bacteria primarily contain a single gene cassette (L'abee-Lund & Sorum, 2001). Class 1 integrons are known to harbor *sul1*, and the close association between *sul1* and class 1 integrons has been reported earlier (Antunes et al., 2005). Linkage between antibiotic use and resistance has been demonstrated for aquaculture-associated bacteria (Verner-Jeffreys et al., 2009; Tamminen et al., 2010; Laganà et al., 2011; Scarano et al., 2014), and similar data has been reported in animal husbandry facilities. These findings have resulted in calls to ban non-medical use of antibiotics (Meek et al., 2015). However, with intensification of aquaculture production to meet food security, and the emergence of infectious bacterial diseases, the ban on the use of antibiotics will be vehemently contested. Instead, an updated data on antibiotic efficacy and

vigilant monitoring of antibiotic resistance in bacterial pathogens affecting aquaculture sector can be done to guide fish farmers in a regional scope.

In accordance with histopathological changes, as observed in the current study, our findings were consistent with previous studies that showed similar histopathological lesions due to MAS (Hassan et al., 2017). These observations were supportive to the motile aeromonads' virulence toxins particularly the aerolysin and cytotoxic enterotoxin which were present in the *Aeromonas* strains in this study. These two toxins induce apoptosis of epithelial cells and macrophages (Krzyminska et al. 2012) and pore formation in the membranes of affected cells (Heuzenroeder et al. 1999; Wong et al. 1998) causing cellular degeneration and necrosis. In the study of Sreedharan et al. (2013), the *A. veronii* displayed cytotoxicity towards the Hep-2 cell line. Krzyminska et al. (2012) showed that the interaction of the *act* from *A. veronii* with epithelial cells resulted in extensive generation of reactive oxygen species (ROS) and nitric oxide radicals (NO-) and caused cytotoxicity which further supports the ability of *act*-positive *Aeromonas* strains to cause cell damage and death. All of the *Aeromonas* strains in the current study were beta-haemolytic and it was shown in a previous study (Singh & Snayal, 1992) that this activity increases fluid accumulation in the organs of experimentally infected animals explaining the inflammatory changes observed in the tissues of the affected fish and the high incidence of dropsy in moribund tilapia. Additionally, all of the *Aeromonas* strains in this study also possessed the *exu* gene which codes for the DNAse which causes cells to become pyknotic and karyorrhectic leading to cell death and necrosis. The extracellular products, enzymes, and enterotoxins produced by motile aeromonads that together led to a systemic damage to the internal

organs, mainly, liver, kidney and spleen with eventual death (Beaz-Hidalgo & Figueras, 2013; Citarasu et al., 2011; Hassan et al., 2017). To confirm the virulence of the *Aeromonas* strains recovered from tilapia, an *in-vivo* infection study is necessary. Moreover, both an in-vitro and in-vivo virulence gene expression of the different virulence factors of fish motile *Aeromonas* spp. can help understand the establishment of infection and progression of MAS in fish.

5.6 Conclusion

In summary the comparison of the different identification methods showed the superiority of molecular sequencing specifically *16S rRNA* and *rpoD* genes over the API 20E and MALDI-TOF in species identification of the piscine motile *Aeromonas*. However, the API 20E and conventional biochemical tests can still be utilised in identification by fish health laboratories especially in developing countries if the profiles are compared with those of piscine motile Aeromonas. The use of MALDI-TOF identification for piscine *Aeromonas* in this study is limited only to the genus level, however refinement of the technique may be able to discriminate species in phylogenetically tight bacterial groups.

The antibiotic response profile and ARG results showed resistance profiles in motile *Aeromonas* spp. recovered from farmed Nile tilapia in the Philippines. Although it may not be possible to ban the use of antibiotics in tilapia farming, an accurate dosing guideline can be developed for antibiotic treatment of fish if clear understanding of the antibiotic response profile (susceptibility, resistance, and presence of ARGs) of the pathogen is established within the framework of complex aquaculture ecosystems in the

country. The varied virulence profiles of the *Aeromonas* isolates show the complex pathogenic mechanisms they employ to cause disease in fish, and their ability to adapt to the conditions of the environment. Detection and typing based on virulence profile will be an important data for development of diagnostic methods for rapid strain identification and for the development of therapeutic strategies like vaccines.

CHAPTER 6.

General Discussion

6.1 Context of this study

The stagnation and myriad of problems the Philippine tilapia sector is currently experiencing is a warning for an eventual collapse of the industry if not addressed properly and immediately (Guerrero, 2019). The effect of this scenario for the Philippines will be devastating socially and economically since tilapia aquaculture maintains a key role in rural communities, where it directly and indirectly contributes towards improving the welfare of the poorest farmers and communities. At the same time, it provides a source of essential micro and macronutrients to households thus directly supporting food security in the Philippines, which had higher prevalence of undernourishment and higher prevalence of severe food insecurity compared with the world, Asia and Southeast Asia (FAO, 2020).

The aim of the present study was to provide an in depth understanding of the health management practices and approaches adopted within the existing range of tilapia farming systems practised in the Philippines. This was achieved by using a range of methods, including a field-based survey to gather farm-based information and biological samples for diagnostic investigation (Chapter 2 and 3, respectively). This provided high quality diagnostic data from which in

depth analysis was performed to determine the disease status of the animals farmed (Chapter 3) and characterisation of two aquatic bacterial pathogens known to cause mass mortalities and morbidities in global aquaculture (Chapter 4 and 5). By using an integrated diagnostic approach, we can correlate what the farmers see and experience, with the actual cause of the fish loss at the time. The laboratory-based studies provided essential information on the pathogenesis which can support the development of efficacious health management strategies to reduce the fish losses in a more cost-effective way that supports the further development of the sector.

6.2. Summary and conclusions

At the time of the survey, only anecdotal or very limited data was available on the health management practises but perhaps more importantly, on the cause of fish losses experienced and reported by the farmers. Ideally a longitudinal survey, perhaps as part of an active surveillance programme would provide robust data on health management practises. This was out with the scope of the current study and instead a cross sectional survey was performed. Previous studies in aquaculture have used both cross sectional (del Pozo et al., 2009; Huang et al., 2020) and longitudinal approaches (del Pozo et al., 2010; Walker et al., 2011; Mitchell et al., 2012) and there are strengths and weaknesses in both applications. Cross-sectional studies are observational studies that make few assumptions about the disease status of the population of interest, allowing large numbers of potential risk factors to be screened (del Pozo, 2009). A cross-sectional design was used in this study, since limited

information was available on infectious bacterial diseases in tilapia within the Philippines, allowing the prevalence to be established (Dohoo et al., 2003, Thrusfield, 2006).

The key findings from the cross-sectional survey applied in this study identified the lack of a systematic and comparable biosecurity approach within the farming systems. Rarely is there a “one-size-fits-all” biosecurity plan that can be applied to all farms in a sector, but there are similar risks and threats, which once identified then the health management approaches can be modified depending on the farming practises (Young et al., 2015). A good example from the current study was this inability of the tilapia farmers practising submerged cages or those with water depth 2 m or more in this study to determine disease or mortality in the stocks. Here the farmers correctly stated that they would not be able to reliably account for the fish mortalities or even recognised morbidity until it was too late due to water depth and the fact that dead/sick fish would sink to the bottom without being observed. However, this would not be unique to tilapia cage farming and in fact it would be similar to any cage farming system practised in deep water.

One approach adopted by the salmonid sector to help monitor their fish stocks in the cages is the inclusion of cameras – these are in the cages and the fish can be monitored 24 h with video recordings (Petrell et al., 1997). Tilapia being an inexpensive fish, especially compared with farmed salmonids, the use of camera in cages will be impractical and uneconomical generally, however, a monthly sampling of fish stocks to determine growth and health conditions using behaviour and clinical signs which in this study showed the farmers were capable of could be applied. From this survey, it was mentioned that some farm

workers were selling the moribund fish for human consumption in the local market as an additional source of income for their salary. Although in the Philippines raw tilapia is never consumed, it still poses zoonotic hazard from handling of the raw product as some fish bacterial pathogens such as *S. iniae* and *Aeromonas* spp. are known to infect humans (WHO, 1999). A particular case of epidemic occurred in Singapore in 2015 where *S. agalactiae* sequence type 283 caused infections in humans by consumption of raw fish (Kalimuddin et al., 2017). This *S. agalactiae* ST 283 were recovered from both freshwater and saltwater fishes one of which was tilapia (Chau et al., 2017). Though there are no reports in the country of fish bacteria infecting humans, this does not mean that it has not occurred or may not occur thus a proactive approach is necessary to prevent any disease outbreaks. The selling of moribund fish by the farm workers to the market must be prohibited such that pertinent laws or regulations should be passed. Moreover, this practice can be avoided if tilapia farm owners increase the salary, or the local government extend financial aid or incentive to these farm workers.

Nearly all the farmers included in the study did use commercial feeds for their fish stocks. It is fair to say that as the global aquaculture industry has intensified and become a more commercially viable product for the international market, so too has the aquafeed sector grown. Most intensively farmed fish are now fed with commercial fish pelleted diets where the micro and macro nutrients are balanced. The average cost of feed during the production cycle can be as much as 60% of the total production costs, and so it would be sensible to use well balanced diets with a well-managed farming system and yet the data from the farm level survey in chapter 2 did not support that these were

always well-managed systems. In fact, the general lack of any biosecurity practises would suggest that improvement could be made, which would be relatively cost-effective and have immediate benefit to the individual farms as well as strengthening the over-all sector over time. This may include the recommended stocking density, procurement of disease-free fry, monthly health inspection of stocks, and consistent water/nutrient quality monitoring of the culture environment.

Whilst the water quality data provided from the field-base survey in Chapter 2 agreed with other tilapia farming systems, the persistent use of sinking type of feed and excessive feeding rations observed from the data in the farms based in Taal Lake may contribute towards poor environmental health in the farms. This in turn could promote poor fish health and leave the animals immunocompromised and vulnerable to opportunistic diseases. Survey data for water quality monitoring showed that hatcheries do not perform any water quality monitoring while all of the grow-out farms measured aspects of quality primarily salinity, temperature, DO and pH. The farmers were most concerned when the DO level of the water dropped in critical value as this will cause massive fish kills. Data from the field survey showed that farmers in general tended to overstock their systems and in fact some were willing to tolerate 70% losses in stock. It may be that the importance of overstocking, poor water quality parameters in general, except for DO, is not considered as important by the farmers given the perception that tilapia are a robust fish species able to tolerate wide range of environmental conditions. Although, they consider DO as important the farmers were helpless to maintain optimum level as the lake is a common resource and regulation of feeding and illegal cages were difficult to

monitor and control. However, being able to tolerate is not the same as optimal. A follow-on study would support the need for more refined or regular measurements of water quality in freshwater lake cages and brackishwater ponds related to disease outbreaks.

One of the more positive findings from the survey data in chapter 2 was the attendance of farmers at trainings, seminars, and their ability to recognise disease in their stocks. However, analysis of the questionnaire-based data showed awareness of the abnormalities in their fish stocks, but unfortunately this did not translate to preventive action. Perhaps, as has been seen in other intensive freshwater farming systems, one of the issues to solving these problems is the lack of efficacious solutions available to farmers, irrespective of where they get their information from. However, although these farmers were adept in recognising disease in their stocks, some other aspects of fish husbandry and health management were lacking. This can be seen in the survey where farmers were developing their own strain of tilapia which they believed were more robust and resistant to diseases. However, these unguided breeding can result to strains having lower genetic diversity resulting to undesirable growth characteristics and depressed fitness which can instead lead to decline in productivity (Nguyen, 2016; Fessehaye et al., 2007). Additionally, in response to the diseases they observed, some were preparing farm-made medicated feeds using antibiotics not licensed for use in aquaculture.

For any management or intervention strategy to be effective, whether this is measured by increased fish production output or prevention/control of disease and mortality outbreaks, depend upon sound information. Aquaculture

farm records can be a reliable form of information where analyses can be made to address problems encountered in the culture. In this study, most of the tilapia farms did not keep mortality records which was understandable since these farmers do not understand the value of such records in fish health management. Moreover, this supports the observation that the Philippine tilapia farming sector lacks application of effective biosecurity measures. Keeping mortality records is important since it can alert farmers to a problem and they can develop remedies by operational intervention (Ellis et al., 2012) in their husbandry such as reduction of feeding, change of nets or application of antibiotics through medicated feeds. Unlike production and stocking density records, the lack of mortality records in the farms in this study is due to the farmers' belief that mortalities do not hugely affect their production, even when the data contradicted this perception.

Although no records of mortality were kept in the tilapia farms in this study, farmers were able to take note of unusual high number of diseased or dead fish during the warmer months of the year which was during March to June. During this time, higher water temperatures were recorded, and this may contribute indirectly to the observed disease and mortality outbreaks. This observation is similar to those in other tilapia producing countries such as Brazil, Egypt and Vietnam (Roriz et al., 2017; Elsheshtawy et al., 2019; Liao et al., 2020). As the water temperature in the surveyed farms did not exceed beyond the tolerable limit for tilapia, the increase as stated previously may indirectly contribute to the problem by increasing the respiratory rate of planktonic organisms and other animals in the eutrophic lake causing a further drop in the DO causing additional stress to fish stocks. Additionally, higher

temperature increases the expression of virulence factors of aquatic bacterial pathogens such as *S. agalactiae* (Kayansamruaj et al., 2014) thus causing higher infection rate in severely stressed fish. Whilst disease and mortality outbreaks peaked during these warm months, farmers observed that infectious diseases persisted throughout the production cycle albeit in lower numbers which is like the perennial disease problems reported in South China tilapia farms (Liu et al., 2013). This low level of diseases in fish stocks during other months were considered normal by the Philippine farmers, although, from a viewpoint of food security and safety this is indicative of lack of appropriate fish health management (Segner et al., 2012). The increase in water temperature together with low water quality and highly stocked farming system may be the factors contributing to the disease outbreaks during warm months of the year (Zamri-Saad et al., 2014; Chitmanat et al., 2016).

Of course, the recognised limitation of this study was the small sample size of the number of fish farms. The farms included in the study, were chosen as they represent the varied production units in the Philippine sector. However, given the geographical distribution of the farms and the Philippines itself, a large survey was not possible in this study. However, by choosing representative farming and production systems, the data generated could be applied to the wider sector. However, further works such as pathogenicity and molecular epidemiology of the pathogens are required prior to exploration of any health management strategies being implemented from this study.

Understanding the drivers for disease outbreaks in aquaculture is complex and multifactorial. In chapter 3, samples were taken at each farm and a description from the farmers of the clinical abnormalities observed grossly,

matched well with the clinical presentation of the moribund fish and the disease diagnosis. It is critical that samples are routinely taken to confirm the health status or during a morbidity event the cause of the disease outbreak. In farmed tilapia, there are reports of emerging (*A. veronii*, Dong et al., 2017), (*S. iniae* and *S. agalactiae*, Alsaid et al., 2013) and existing disease conditions. All of these will require a different health management approach, which need not to be costly and can be simple as verification of health status of the fry pre-stocking. Often farmers will perform some health evaluation in the stocks purchased prior to stocking (Phan et al., 2009). These rarely require laboratory-based methods and are often developed by farmers as a practical way for them to check the health of the fry or juveniles. These types of practises vary between different farming sectors and species. Correlation with laboratory-based methods is rare although more recently there has been an increase in uptake of laboratory-based pathogen detection methods in aquatic stocks prior to stocking in the farm. A good example of this is the use of an accelerated colorimetric loop-mediated isothermal amplification (LAMP) assay for the detection of *S. iniae* and *S. agalactiae* for screening broodstock and fry before stocking and for monitoring fish health in grow-out ponds (Suebsing et al., 2013). It was clear from the work performed in this study that such practises are not performed and yet could be encouraged via training programmes which farmers already attended.

The diagnostic approach used in chapter 3 confirmed the disease status of the fish sampled and confirmed a bacterial aetiology. Farmed tilapia suffered high levels of losses due to bacterial infections compared with other pathogens (Kayansamruaj et al., 2020) and so the bacterial aetiology and the

species identified in this study was not unexpected, but until this work has been completed the disease status was unknown in these farming systems. As stated previously, without these diagnostic data the farmers run risk of high production costs as they may be inadvertently treating when the actual cause of the disease is nutritional or water quality and not pathogen driven.

When disease outbreaks occur however, it is critical to confirm the cause and the data from the work presented in this chapter, showed primarily Gram-negative and Gram-positive bacterial diseases. It was interesting from a disease perspective that both *S. iniae* and *S. agalactiae* were detected in the systems but by far a higher percentage of tilapia suffering from streptococcosis were infected with *S. agalactiae*. Additionally, motile aeromonads were also recovered from diseased tilapia and the species primarily involved was *A. veronii* contrary to the previous published studies in the Philippines on motile aeromonad septicaemia in freshwater fishes where the primary aetiologic agent was *A. hydrophila* (Lio-Po et al., 1992; 1998; Yambot, 1998). Bacterial isolates were identified using both conventional and molecular methods. The conventional morphological and biochemical methods were able to discriminate bacterial isolates at the genus level only, albeit putatively. The *16S rRNA* gene sequencing, considered as the gold standard in bacterial identification, was able to confirm the putative identification from the traditional identification results and was able to identify the different bacterial isolates up to the species level.

In some samples from diseased fish, either an *S. agalactiae*/*A. veronii* or *S. iniae*/*A. veronii* complex were recovered. It may well be that these were, co-infections or where fish became infected at the same time to the 2 bacterial species, or it may be results of subsequent infections as most samples showed

marked clinical signs of infection which ranged from acute to chronic. Further work was not performed to explore this phenomenon, but future studies could incorporate experimental challenges with both pathogens performed under a sequential time. There are more reported cases of co-infections emerging in aquaculture such as that of *Edwardsiella ictaluri* and *A. hydrophila* (Crumlish et al., 2017), *E. ictaluri* and *Flavobacterium columnare* (Dong et al., 2017), *S. agalactiae*, *S. iniae*, *Francisella noatunenis* and *A. hydrophila* (Delphino et al., 2019) to name a few. Co-infections have a fundamental effect and can alter the course and the severity of different fish diseases (Kotob et al., 2017). Confirmation of a coinfection would be critical to the development of any future health management strategies developed.

A co-infection of *S. agalactiae* and *S. iniae* was not observed in this study but has been reported (Featherstone, 2014). Recovery of both *S. iniae* and *S. agalactiae* from fish sampled during this study could have been performed by using chromogenic agar that differentially show *S. iniae* and *S. agalactiae* in the single agar plate, however, the work performed in chapter 3 was not to confirm co-infection but instead to recover and identify the bacterial species associated with the disease at the time of sampling. Nevertheless, now that the presence of both pathogens has been identified from the worked performed in this study, it would be sensible for any screening programmes in the future to incorporate a chromogenic agar for viable bacterial recovery and confirm the presence of the 2 bacterial species. The importance of this work would be to support the future development of the health management strategies as current vaccination programmes are not in place in the Philippine tilapia sector but would be a sensible approach going forward. It is recognised

that the 2 vaccines available to protect against streptococcal infections do not cross-protect (Wang et al., 2014).

An alternative method for rapid detection of the streptococci or aeromonas pathogens can be done by species-specific PCR using extracted genomic DNA from fish tissue (Rodkum et al., 2012; Cui et al., 2019; Persson et al., 2015). However, the value of this would be more limited compared with the gene sequencing methods like *16S rRNA*. Species-specific PCR is only for detection but not for phylogenetic analysis which is important for epidemiological study as sequencing identification methods do not only identify species but also can give show evolutionary or genetic variation within a species.

The recovery of *L. garvieae* from diseased tilapia is the first record in the Philippines. This bacterium has been reported to infect and cause mass mortality in several fish species including tilapia (Evans et al., 2009; Anshary et al., 2014). *L. garvieae*-infected fish exhibited similar clinical signs typical of streptococcosis. This is very important since lactococcosis and streptococcosis clinical signs are identical (Vendrell et al., 2006), misidentification of the aetiologic agent can lead to treatment failure and production losses in the farms.

Results of histopathology from different organs of the moribund fish showed abnormalities and tissue damage typical of bacterial septicaemia. Histologically, the moribund tilapia tissue samples showed congestion, haemorrhages, inflammation, degeneration, and necrosis in several internal organs, particularly the liver, heart, spleen, kidney, and brain as systemic phase of the septicaemic disease. These histological lesions that were indicative of

bacterial infection are consistent with those described by Jantrakajorn (2014) in Nile tilapia infected naturally with *S. agalactiae* and *S. iniae*, and also of motile aeromonas by El-Gohary et al. (2020). Parasitic infection typical of the myxosporean parasite *Myxobolus* sp. was detected in the gill muscles of some moribund fish samples. This is also the first report of the myxosporean parasite affecting farmed Nile tilapia in the Philippines, although its impact on the mortality outbreaks was not determined in the current study.

To understand the pathogenesis of streptococcal and motile aeromonas infections of diseased farmed tilapia in chapter 3, further identification and characterisation were performed to the bacterial isolates in chapters 4 and 5. Currently, the immediate and inexpensive diagnosis of bacterial infections in infected fish is difficult as fish exhibit similar clinical signs regardless of the etiological agent. Reliance on a single laboratory-based method to identify the bacterial species is not possible and a combination of tests are always performed. In this study, conventional morphological, biochemical and molecular methods were used for species level identification of the isolates but also to determine unique characteristics of the bacterial pathogens associated with disease outbreaks in these farming systems.

In chapter 4, identification of *S. agalactiae* using the API20 STREP, species-specific duplex PCR and *16S rRNA* gene sequencing gave excellent agreement except for *S. agalactiae* serotype Ib strains which were metabolically impaired as they had very slow growth. Additionally, molecular serotyping of *S. agalactiae* was performed which determined that there were two serotypes of *S. agalactiae* namely serotype Ia and serotype Ib circulating within the tilapia farming systems in the Philippines. The predominance of *S. agalactiae* serotype

Ia over serotype Ib is consistent with reports in SEA (Anshary, et al., 2014; Barkham et al., 2019; Jantrakajorn et al., 2014; Kayansamruaj et al., 2019; Syuhada et al., 2020) but contrary to previous reports that *S. agalactiae* serotype Ib is the main serotype causing streptococcosis in Philippine tilapia farms. For *S. iniae*, there was also excellent agreement with species-specific duplex PCR and *16S rRNA*, however, the API20 STREP database cannot identify it. This limitation in *S. iniae* identification using the API can be sorted out using biochemical profiles of piscine *S. iniae* from published studies or literatures (El Aamri et al., 2010). An additional test which was useful to differentiate *S. agalactiae* from *S. iniae* is the starch hydrolysis test. An attempt was also used to speciate and subtype streptococci isolates using protein spectral profiles from MALDI-TOF MS which resulted in the visual differentiation of *S. agalactiae* and *S. iniae* similar to the observations of Piamsomboon et al. (2020). Although an indication that MALDI-TOF MS can differentiate *S. agalactiae* serotype Ia from serotype Ib, and that it allows speciation of *S. iniae* and *S. agalactiae*, it is premature to conclude because of the limited sample size and few number of *S. agalactiae* serotypes.

From the results of the different identification tests for streptococci, it is safe to recommend the use of Gram staining, oxidase, catalase, API 20STREP or tube biochemical tests, species-specific duplex PCR and starch hydrolysis as rapid and inexpensive tests for the identification of *S. agalactiae* and *S. iniae* in cases of streptococcosis outbreaks in finfish aquaculture. This could be taken up by fish diagnostic laboratories especially in the developing countries. However, in the case of *S. agalactiae* the determination of serotype is important in lieu of control and treatment since vaccine from different serotype does not

offer cross-protection to the fish (Evans et al., 2004). Serotype determination is not expensive as it is done only by conventional multiplex PCR method.

The histopathology of the tilapia samples where *S. agalactiae* and *S. iniae* were recovered presented lesions typical of bacterial septicaemia. Meningitis and encephalitis were observed in 80% of the moribund samples where both streptococci bacteria were recovered. From the study of Chen et al. (2007), they have noted that *S. agalactiae* infected tilapia fish contained large numbers of the bacteria in tissues and in circulation as compared with *S. iniae*. This finding of the streptococci tropism for brain tissue is typically decribed for streptococcosis (Eldar et al., 1995; Abuseliana et al., 2010; Baums et al., 2013). Histopathological abnormalities observed in the brain, kidney, spleen, heart and liver tissues were suggestive of septicaemia as observed in natural and experimental streptococcal infection in accordance with those described in fish (Austin & Austin, 2007; Musa et al., 2009; Evans et al., 2006; Abdullah et al., 2013; Suanyuk et al., 2005; Shoemaker et al., 2001; Rahmatullah et al., 2017). These lesions may be due to the high number of bacteria producing toxins and enzymes that also stimulates the immune response of infected fish. Over-all, in this study the histopathology confirmed the virulence of both Philippine isolates of *S. agalactiae* and *S. iniae* to induce systemic infection in tilapia.

As infection bioassay was not possible during the study, the antibiotic response profile, antibiotic resistance genes and virulence factors were determined in order establish the pathogenic nature of the streptococci isolates. Both *S. iniae* and *S. agalactiae* serotype Ia were susceptible to assayed antibiotics which are licensed for use in global aquaculture except for oxolinic acid for which they are inherently resistant. The *S. agalactiae* serotype

Ib antibiotic profile was similar to the two strains except for its resistance to sulphamethoxazole-trimethoprim. These results are similar to previous reports in Thailand (Jantrakajorn et al., 2014; Dangwetngam et al., 2016; Kannika et al., 2017; Klingklib & Suanyuk, 2017) and may indicate a low reliance to antibiotics in tilapia farms evidenced by a low level of antibiotic resistance. No antibiotic resistance genes were detected in the isolates in this study; however, this does not mean that they will be not able to develop or acquire one so prudent and judicious use of antimicrobials is still recommended.

All the *S. iniae* isolates had identical virulence profiles in containing the necessary genes to establish infection in fish (Baums et al., 2013). These are the adhesins, invasins and immune evasins virulence genes. Similar types of virulence genes were also detected in *S. agalactiae* isolates except that the serotype Ib lacked the *cylE* gene involved in haemolysis. The loss of this particular gene, coupled with reduced metabolic activity, can be an evolutionary strategy in order to establish persistent infection in the host by becoming less virulent (Bull, 1994; Frank, 1996; Ebert & Bull, 2003; Rosinski-Chupin et al., 2013). Studies have shown that highly haemolytic GBS were easily cleared by the host immune response unlike the non-haemolytic strain which has higher survival and can persist causing subclinical and chronic infection (Li et al., 2014).

During bacterial culture, some of the *S. agalactiae* exhibited pin-pricked-sized colonies which were described as non-haemolytic and had reduced metabolic activity as compared to other isolates. These same *S. agalactiae* serotype Ib isolates were the strains that the API20 STREP was not able to identify. This morphological variant was also observed in clinical human GBS in

the study of Banno et al. (2014). These are described as small colony variants (SCV) which are a slow-growing subpopulation of bacteria with distinctive phenotypic and pathogenic traits that are involved in chronic and recurrent infections (Proctor et al., 2006; Johns et al., 2015; Tikhomirova et al., 2018). This morphological switch may be a strategy employed by bacteria to increase intracellularity in host macrophage thus evading clearance from the host's immune system producing persistent infections. These SCV are noteworthy because this is a form of the pathogenic bacteria that produces subacute infections leading to recurrent and chronic infections, reduced antibiotic susceptibility and resistance to oxidative burst (Proctor et al., 2006; Banno et al., 2014; Painter et al., 2017). So far this is the first report of SA-SCV in piscine *S. agalactiae*. Currently, there is no study regarding the SCV form of piscine *S. agalactiae* and their implications in pathogenesis in fish.

Identification of motile *Aeromonas* species using conventional and biochemical methods did not show excellent agreement with the *16S rRNA* and *rpoD* gene sequencing. A greater agreement was found between *16S rRNA* and *rpoD* gene sequencing in the identification compared with the biochemical assay. Some studies recommend the use of the housekeeping genes like *rpoD* over *16S rRNA* because it has higher discriminatory power and interspecies variation (Shin et al., 2015), however, the amount of *rpoD* sequences in the gene bank database is much smaller the reason why most diagnostic laboratories still use *16S rRNA* for pathogen identification purposes. The difficulty lies within the genus *Aeromonas* itself as it is composed of phylogenetically tight members but showing highly heterogeneous biochemical and pathogenic characteristics even within species level. A good solution to the

identification of motile aeromonads is the development and use of multiplex PCR which can discriminate different species. The motile aeromonas recovered were *A. veronii*, *A. caviae* and *A. dhakensis*, all of which were first report in the Philippines infecting farmed Nile tilapia. An attempt was made to use MALDI-TOF MS to identify piscine motile *Aeromonas* as previous studies reported that this method can discriminate them to the species level (Donohue et al., 2006; Benagli et al., 2012; Piamsomboon et al., 2020). However, results of this study demonstrated the utility of MALDI-TOF MS in identifying piscine *Aeromonas* to the genus level only since the protein spectral profiles of the samples were identical across the genus making species identification impossible. An important observation in this study is that species specific protein biomarkers detected by previous studies (Donohue et al., 2006; Benagli et al., 2012; Perez-Sancho et al., 2018) were present in the lower dilutions of the sample from the different *Aeromonas* species. Therefore, it is recommended that further investigation should be done on the use of different dilutions in sample analysis for bacterial identification using the MALDI-TOF MS.

The dominance of *A. veronii* in aquatic disease outbreaks, which was also reported from this study, was not surprising as numerous reports have identified this bacterium as the dominant aetiologic agent responsible for haemorrhagic septicaemia resulting to mortality outbreaks in many fish species (Hoai et al., 2019; Dong et al., 2017; Smyrli et al., 2017; Zhu et al., 2016; Sreedharan et al., 2011). The current reports of the dominance of *A. veronii* in diseased fish could be to previous misidentification of the bacteria as *A. hydrophila* since old studies were still reliant on conventional methods of identification which was proven in this study as unreliable. Moreover, the high

prevalence of aerolysin virulence gene and its expression in *A. veronii* might explain its high presence in natural infections in contrast to *A. hydrophila*. The aerolysin of *A. veronii* was shown to induce intestinal lesions enabling it to cross intestinal barrier and invade the host systemically which is in contrast to the low activity and expression of aerolysin from *A. hydrophila* contributing to its low virulence via natural route of infections (Ran et al., 2018). However, this needs further investigation as the pathogenesis of motile aeromonads are highly diverse and complex. This indicates that *A. veronii* is an important motile aeromonas species that contributes to diseases in farmed Nile tilapia in the Philippines aside from the well described *A. hydrophila*.

The motile *Aeromonas* isolates in this study are potential virulent strains due to their production of extracellular enzymes and the presence of various virulence factors. Two virulence genes namely *aer* which codes for aerolysin and *act* for cytotoxic enterotoxin were detected in 75% and 100% the isolates, respectively. The high proportion of these two genes among *Aeromonas* strain in this study is like the previous studies of Nawaz et al. (2010) and Khor et al. (2015) where they also were present in high percentage in *A. veronii* isolates from diseased catfish and in aquatic *Aeromonas* spp., respectively. Based on this result, *act* gene may be integral in the pathogenicity of aquatic *Aeromonas* especially of the *A. veronii* and that the presence of *aer* enhances the severity of the infection as displayed by the gross clinical signs. As these two genes exhibits haemolytic, cytotoxic and enterotoxic activities which are important in establishment of infection (Nawaz et al., 2010), their presence in a particular *Aeromonas* strain can be an indication of the virulence of the strain. The

production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads (Santos et al., 1999).

The motile *Aeromonas* isolates were genotyped based on the virulence genes they possessed. Results showed remarkable variability in the virulence gene content within the *A. veronii* group which indicates significant variability in terms of their pathogenic strategy, however, analysis showed that the presence of *act* and *aer* genes resulted in higher proportion of moribund fish showing numerous gross clinical signs. A careful evaluation should be made in terms of pathogenesis of *Aeromonas* spp. in fish since the exact relationship of virulence genes or subset of virulence genes and the ability of a particular genospecies to cause disease has not been fully established. The different combinations of virulence genes in different geographical areas may also suggests that different mechanisms may be used by these *Aeromonas* spp. to colonise and cause infection or to mutualistically co-exist with the fish host (Silver et al., 2007; Rasmussen-Ivey et al., 2016).

The Philippine motile *Aeromonas* strains in this current study showed susceptibility to oxolinic acid, enrofloxacin, florfenicol and chloramphenicol while some showed resistance to oxytetracycline, tetracycline and sulphamethoxazole-trimethoprim. Almost all were resistant to β-lactam antibiotics since the genus *Aeromonas* has an intrinsic resistance to this compound (Senderovich et al., 2012*).* Plasmid-mediated β-lactam resistance genes responsible for extended spectrum β-lactamase (ESBL) resistance were not detected confirming that the observed phenotypic resistance to β-lactam antibiotics were of intrinsic in origin. The *tetE* gene was detected in *A. veronii* strains which is responsible to the observed resistance to oxytetracycline and

tetracycline. This conforms to previous studies showing that the *tetE* gene is the dominant tetracycline resistance gene in aeromonads from freshwater fish aquaculture (Piotrowska & Popowska, 2014; Han et al., 2012; Cizek et al., 2010; Nawaz et al., 2006). Majority of the isolates showing phenotypic resistance to sulphamethoxazole-trimethoprim did not have the specific resistance gene suggesting that there might be other genes involved or that there is mutation in chromosomal genes encoding dihydropteroate synthetase (Giordanetto et al., 2005). Moreover, class 1 integron *IntI1* coding for integrase was detected in some *A. veronii* isolates which is concerning since this enzyme allows for the transfer of antibiotic resistance cassettes within *A. veronii* and other motile *Aeromonas* species. Over-all the results showed piscine *Aeromonas* species in this current study exhibited emerging resistance to antibiotics especially the *A. veronii* strains. As there is no vaccine available to treat motile aeromonas septicaemia because of the heterogeneity of the aetiologic agents involved, the control of this bacterial infection is still reliant on the use of antibiotics and good husbandry practices. The aquaculture industry should be vigilant and adhere to good antibiotic practice as antibiotic usage may become ineffective as the bacterial pathogens acquire resistance.

In accordance with histopathological changes, as observed in the current study, our findings were consistent with previous studies that showed similar histopathological lesions due to MAS (Hassan et al., 2017). These observations were supportive to the motile aeromonas virulence toxins particularly the aerolysin and cytotoxic enterotoxin which were present in the *Aeromonas* strains in this study. The extracellular products, enzymes, enterotoxins, and antibiotic resistance produced by motile aeromonads that together contributed

to their virulence leading to a systemic damage to the internal organs, mainly, liver, kidney, and spleen with eventual death (Beaz-Hidalgo & Figueras, 2013; Citarasu et al., 2011; Hassan et al., 2017).

6.3 Future work

The work presented in this thesis contributes to a growing body of knowledge in tilapia infectious bacterial diseases applicable to the Philippines. Moreover, it established that at the time of sampling, the disease and mortality outbreaks observed by tilapia farmers is primarily due to streptococcosis and motile aeromonas septicaemia. Field survey in the different tilapia farms revealed a lack of robust biosecurity measure. The continued growth of the Philippine population will put more pressure on tilapia production such that the degree of intensification will increase with the eventual collapse of the sector if not addressed correctly and immediately due to interrelated problems of environmental pollution, recurrent fish kills and disease/mortality outbreaks. It is therefore imperative that tilapia production should expand to brackishwater and seawater to relieve the freshwater lakes and other freshwater areas from high number of cages and pens, and ecological deterioration. As the pathogens are already established in the sector, research work on the development of autogenous vaccines for streptococcosis using the isolates in this study be done to help lessen its impact on the already stagnating tilapia production in the country. Such research will continue to enhance our understanding on the pathogenesis of the piscine streptococcal species so that effective and appropriate control measures and strategies can be established. Additional

investigation on the diversity and virulence of motile aeromonas species involved in motile aeromonas septicaemia in tilapia will add existing data on the control of this highly challenging bacterial genus. All of these will help improve the tilapia farming sector of the Philippines to become sustainable and profitable at the same time supporting food security and safety of the Filipino consumers.

Suggestions for future work include:

- ***Implement long term, routine field sampling to determine the presence and dynamics of infectious bacterial diseases in Philippine tilapia aquaculture sector*.** This can be done by establishing aquatic diagnostic laboratories in different regions that is capable of doing surveillance and disease diagnostics.
- ***Development of a national biosecurity measure in the tilapia sector***. This can be achieved by assisting hatcheries and grow-out farms of developing specific pathogen-free broodstocks and fry. Additionally, to initiate annual training programs for farmers in health management and record keeping related to husbandry and health. Output from this thesis would contribute towards development of a tilapia health management manual and biosecurity, written in language that can be understood by tilapia farmers.
- ***Investigations of the nature of infectious co-infections as it contributes to severity of infectious diseases affecting farmed tilapia***. This can be done by doing infection bioassays using the different bacterial pathogens recovered in this study. A sequential experimental infection approach can further elucidate

the pathogenesis of streptococcal and aeromonas diseases starting from establishment of bacteria in tissues to progression to acute stage.

- ***Development of autogenous vaccine against streptococcosis.*** A polyvalent vaccine with *S. agalactiae* serotypes Ia and Ib, and *S. iniae* will be ideal since there is no cross-protection from these pathogens. The vaccine should be a type that can be administered by bath or feed or both as intra-peritoneal administered vaccine is uneconomical for tilapia species.
- ***Investigate the pathogenicity of the different bacterial isolates in different tilapia breeds or strains.*** As expansion of tilapia farming to brackishwater and seawater will be inevitable in the near future, it will be a good proactive work to determine and understand the pathogenicity of these bacteria especially the streptococci as they are also known to infect tilapia reared in seawater and other marine fishes.
- ***Further work on the nature and role of the small colony variants of S. agalactiae***. Experimental studies on the metabolic and genotypic characteristics of small colony variant *S. agalactiae* will contribute to the understanding on its role in subclinical or chronic infections in aquatic animals especially finfishes.
- ***Further investigations on the motile aeromonas septicaemia (MAS) affecting Philippine freshwater fishes***. As motile aeromonads are genetically and pathogenetically diverse, there is a need to further investigate of the main species involved in the

current disease outbreaks associated with MAS and whether the species involved are host specific.

REFERENCES

Abbott, S. L., Cheung, W. K., Kroske-Bystrom, S. Malekzadeh, T., & Janda, J. M. (1992). Identification of *Aeromonas* strains to the genospecies level in the clinical laboratory. *Journal of Clinical Microbiology*, 30, 1262–1266.

Abbott, S. L., Cheung, W. K. W., & Janda, J. M. (2003). The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. *Journal of Clinical Microbiology*, 41(6), 2348–2357. Borrell, *et al*., 1998.

Abdelhamed, H., Ibrahim, I., Baumgartner, W., Lawrence, M.L. and Karsi, A. (2017). Characterization of Histopathological and Ultrastructural Changes in Channel Catfish Experimentally Infected with Virulent *Aeromonas hydrophila*. Front. Microbiol. 8:1519. doi: 10.3389/fmicb.2017.01519.

Abdelsalam, M., Asheg, A. & Eissa, A.E. (2013). *Streptococcus dysgalactiae*: An emerging pathogen of fishes and mammals. Int J Vet Sci Med. 1:1–6.

Abdullah, S., Omar, N., Yusoff, S.M., Obukwho, E.B., Nwunuji, T.P., Hanan, L. & Samad, J. (2013). Clinicopathological features and immunohistochemical detection of antigens in acute experimental *Streptococcus agalactiae* infection in red tilapia (*Oreochromis* spp.). *SpringerPlus* 2, 1– 7.

Abu-elala, N.M., Abd-Elsalam, R.M. & Younis, N.A. (2020). Streptococcosis, Lactococcosis and Enterococcosis are potential threats facing cultured Nile tilapia (*Oreochomis niloticus*) production. Aquaculture Research. 51(10): 4183-4195.

Abu Nor, N., Zamri-Saad, M., Md Yasin, I.-S., Salleh, A., Mustaffa-Kamal, F., Matori, M.F. & Azmai, M.N.A. (2020). Efficacy of Whole Cell Inactivated *Vibrio harveyi* Vaccine against Vibriosis in a Marine Red Hybrid Tilapia (*Oreochromis niloticus* × *O. mossambicus*) Model. *Vaccines*. 8(4):734.

Abuseliana, A., Daud, H., Aziz, S.A., Bejo, S.K., Alsaid, M. (2010). *Streptococcus agalactiae* the etiological agent of mass mortality in farmed red tilapia (*Oreochromis* sp.). Journal of Animal and Veterinary Advances, 9:2640-2646.

Achtman, M. (2008). Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu Rev Microbiol. 262:53–70.

Adams, A. & Thompson, K.D. (2011). Development of diagnostics for aquaculture: challenges and opportunities. *Aquaculture Research* 42, 93– 102.

Adanir, D. & Turutoglu, H. (2007). Isolation and antibiotic susceptibility of *Aeromonas hydrophila* in a carp (*Cyprunus carpio*) hatchery farm. Bull Vet Inst Pulawy 51:361–364.

ADB. (2005). An impact evaluation study on the development of genetically improved farmed tilapia and their dissemination in selected countries, 77. Manila: Asian Development Bank.

ADB. (2005b). An evaluation of small-scale freshwater rural aquaculture development for poverty reduction. Manila, Asian Development Bank. 161 pp.

Adiba, S., Nizak, C., van Baalen, M., Denamur, E., & Depaulis, F. (2010). From grazing resistance to pathogenesis: The coincidental evolution of virulence factors. *PLoS ONE* 5: e11882.

Agnew, W., & Barnes, A. C. (2007). *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Vet. Microbiol.* 122, 1– 15.

Ahmed, M. & Lorica, M. H. (2002). 'Improving Developing Country Food Security through Aquaculture Development: Lessons from Asia', Food Policy 27: 125-41.

Aizawa, N., Masuda, M. & Ito, L.S. (2014). Current situation of freshwater aquaculture in the lower Amazon River and the potentiality of development. Tropics, 23: pp. 127-134.

Akter, T., Foysal, M.J., Alam, M., Ehsan, R., Paul, S.I., Momtaz, F., Siddik, M.A.B., Tay, A.C.Y., Fotedar, R., Gupta, S.K., Islam, T. & Rahman, M.M. (2020). Involvement of Enterococcus species in streptococcosis of Nile tilapia in Bangladesh Aquaculture, 531, pp. 1-8

Albert, M., Ansaruzzaman, M., Talukder, K., Chopra, A., Kühn, I., Rahman, M., & Möllby, R. (2000). Prevalence of enterotoxin genes in *Aeromonas* spp.

isolated from children with diarrhea, healthy controls, and the environment. *Journal of Clinical Microbiology*, 38, 3785–7790.

Ali, A., Hassan, D., Saleha, A.A., Siti-Khairani, B. & Milud, A. (2010). *Streptococcus agalactiae* the etiological agent of mass mortality in farmed red tilapia (*Oreochromis* sp.). *Journal of Animal and Veterinary Advances* 9, 2640– 2646.

Allan, B. J., & Stevenson, R. M. (1981). Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. *Canadian Journal of Microbiology*. 27, 1114–1122.

Alperi, A., Figueras, M. J., Inza, I., & Martínez-Murcia, A. J. (2008). Analysis of *16S rRNA* gene mutations in a subset of *Aeromonas* strains and their impact in species delineation. *International Microbiology*, 11, 185–194.

Alsaid, M., Mohd, H., Mohamed, N., Khairani, S., Mohamed, Y., Farag, A., Hayati, R. (2013). Pathological findings of experimental *Streptococcus agalactia*e infection in red hybrid tilapia (*Oreochromis* sp.). International Conference on Chemical, Agricultural and Medical Sciences (CAMS), Dec. 29–30. Kuala Lumpur (Malasia), pp. 70-73, 10.15242/IICBE.C1213075.

Alsaid, M., Daud, H., Mohamed, N., Bejo, S. K., Mohamed, Y., & Abuseliana, A. (2013). Environmental factors influencing the susceptibility of red hybrid tilapia (*Orechomis* sp.) to *Streptococcus agalactiae* infection. Advanced Science Letters, 19(12), 3600– 3604.

Aly, S. M. & Albutti, A. (2014). Antimicrobials Use in Aquaculture and their Public Health Impact. Journal of Aquaculture Research & Development, vol. 5, no. 4.

Amal, M.N.A. & Zamri-Saad, M. (2011). Streptococcosis in tilapia (*Oreochromis niloticus*): a review. Pertanika J. Trop. Agric. Sci. 34(2): 195-206.

Amal, M.N.A., Koh, C.B., Nurliyana, M., Suhaiba, M., Nor-Amalina, Z., Santha, S. *et al*. (2018). A case of natural co-infection of tilapia lake virus and *Aeromonas veronii* in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) farm experiencing high mortality. *Aquaculture* 485: 12–16.

Amphan, S., Unajak, S., Printrakoon, C., & Areechon, N. (2019). Feeding-regimen of β-glucan to enhance innate immunity and disease resistance of Nile

tilapia, *Oreochromis niloticus* Linn. against *Aeromonas hydrophila* and *Flavobacterium columnare*. *Fish & Shellfish Immunology*, 87, 120– 128.

Angka, S. L. (1990). The pathology of the walking catfish, Clarias batrachus (L.), infected intraperitoneally with *Aeromonas hydrophila*. Asian Fish. J. 3, 343-351.

Angienda, P.O., Aketch, B.O. & Waindi, E.N. (2010). Development of all-male fingerlings by heat treatment and the genetic mechanism of heat induced sex determination in Nile tilapia (*Oreochromis niloticus* L.). International Journal of Biological and Life Sciences 6 (1), 38–43.

Anguita, J., Aparicio, L. B. R., & Naharro, G. (1993). Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. *Applied and Environmental Microbiology*, 59(8), 2411–2417.

Anshary, H., Kurniawan, R.A., Sriwulan, S., Ramli, R. & Baxa, D.V. (2014) Isolation and molecular identification of the etiological agents of streptococcosis in Nile tilapia (*Oreochromis niloticus*) cultured in net cages in Lake Sentani, Papua, Indonesia. *Springerplus* 3, 627.

Aoki, T. (1999). Motile aeromonads (*Aeromonas hydrophila*). In: Woo PTK, Bruno DW (eds) Fish diseases and disorders, Viral, bacterial and fungal infections, vol 3. CABI Publishing, London, pp. 427–453.

Aravena-Roman, M., Inglis, T.J.J., Henderson, B., Riley, T.V. & Changa, B.J. (2012). Antimicrobial susceptibilities of *Aeromonas* strains isolated from clinical and environmental sources to 26 antimicrobial agents. Antimicrob Agents Ch 56:1110–1112.

Areechon, N., Kannika, K., Hirono, I., Kondo, H. & Unajak, S. (2016). Draft genome sequences of *Streptococcus agalactiae* serotype Ia and III isolates from tilapia farms in Thailand. Genome Announc. 4, 5132–5133.

Armistead, B., Oler, E., Waldorf, K.A. & Rajagopal, L. (2019). The Double Life of Group B Streptococcus: Asymptomatic Colonizer and Potent Pathogen. Journal of Molecular Biology.

Armwood, A.R., Camus, A.C., López-Porras, A., Ware, C., Griffin, M.J. & Soto, E. (2019). Pathologic changes in cultured Nile tilapia (*Oreochromis niloticus*)

associated with an outbreak of *Edwardsiella anguillarum*. *J. Fish Dis.* 42:1463–9.

Arslan, S. and Kucuksari, R. (2015). Phenotypic and genotypic virulence factors and antimicrobial resistance of motile *Aeromonas* spp. from fish and ground beef. *J Food Saf* 35, 551– 559.

Arthur, J. R.; Lumanlan-Mayo, S. (1997). Checklist of the parasites of fishes of the Philippines. *FAO Fish. Tech. Pap.* 369, 102.

Asao, T., Kinoshita, Y., Kozaki, S., Uemura, T., & Sakaguchi, G. (1984). Purification and some properties of *Aeromonas hydrophila* hemolysin. *Infection and Immunity*, 46, 122–127.

Asencios, Y. O., Sánchez, F. B., Mendizábal, H. B., Pusari, K. H., Alfonso, H. O., Sayán, A. M., … Chaupe, N. S. (2016). First report of *Streptococcus agalactiae* isolated from *Oreochromis niloticus* in Piura, Peru: Molecular identification and histopathological lesions. *Aquaculture Reports*, 4, 74– 79.

Ash, C., Martínez-Murcia, A. J., & Collins, M. D. (1993). Identification of *Aeromonas schubertii* and *Aeromonas jandaei* by using a polymerase chain reaction-probe test. *FEMS Microbiology Letters*, 108, 151–156.

Assis, G.B.N., Tavares, G.C., Pereira, F.L., Figueiredo, H.C.P. & Leal, C.G. (2017). Natural coinfection by *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus* L.). Journal of Fish Diseases 40: 51–63.

Atyah, M. A. S., M. Zamri-Saad, and A. Siti-Zahrah. (2010). First report of methicillin-resistant *Staphylococcus aureus* from cage-cultured Tilapia (*Oreochromis niloticus*). Veterinary Microbiology 144: 502– 504.

Austin, B., & Adams, C. (1996). Fish pathogens, In B. Austin, M. Altwegg, P. J. Gosling, and S. W. Joseph (ed.). The genus *Aeromonas*. John Wiley and Sons, New York, N.Y. (pp. 197–243).

Austin B. & Austin D.A. (2007). Bacterial fish pathogens: disease of farmed and wild fish. Springer Science & Business Media.

Austin, B., & Austin, D. (2012). Bacterial Fish Pathogens; Diseases of Farmed and Wild Fish. 5th ed. Springer-Verlag, Dordrecht, The Netherlands.

Austin, B., & Austin, D. A. (2016). Bacterial fish pathogens: Disease of farmed and wild fish (6th ed.). Retrieved from https://doi.org/10.1007/978-3-319-32674-0

Austin, B. (2017). Antibiotics and Disinfectants in in Diagnosis and Control of Diseases of Fish and Shellfsh. John Wiley & Sons, Ltd. pp. 263–278.

Austin, B. (2019). Methods for the diagnosis of bacterial fish diseases. Mar Life Sci Technol 1:41–49.

Axelsson, M., Altimiras, J. & Claireaux, G. (2002). Post-prandial blood flow to the gastrointestinal tract is not compromised during hypoxia in the sea bass *Dicentrarchus labrax*. Journal of Experimental Biology 205(18): 2891–2896.

Azad, I.S., Al-Marzouk, A., James, C.M., Almatar, S., Ai-Gharabally, H. & Qasem J.A. (2012). Outbreak of natural Streptococcosis in hatchery produced silver pomfret (*Pampus argenteus* Euphrasen) larvae in Kuwait. *Aquaculture* 330, 15– 20.

Bach, R. P., Chen, P. K. & Chapman, C. B. (1978). Changes in the spleen of the channel catfish Ictalurus punctatus Rafinesque induced by infection with Aeromonas hydrophila. J. Fish Dis. 1, 205–217. doi: 10.1111/j.1365-2761.1978. tb00023.

Bacharach, E., Mishra, N., Briese, T., Zody, M.C., KembouTsofack, J.E., Zamostiano, R. *et al*. (2016). Characterization of a novel orthomyxo-like virus causing mass die-offs of tilapia. *MBio* 7: e00431–16.

Baiano, J.C.F. & Barnes, A.C. (2009). Towards control of *Streptococcus iniae*. *Emerging Infectious Diseases* 15: 1891– 1896.

Bachrach, G., Zlotkin, A., Hurvitz, A., Evans, D.L. & Eldar, A. (2001). Recovery of *Streptococcus iniae* from diseased fish previously vaccinated with a streptococcus vaccine. *Applied and Environmental Microbiology* 67, 3756 – 3758.

Baeck, G.W., Kim, J.H., Gomez, D.K. & Park, S.C. (2006). Isolation and characterization of *Streptococcus* sp. from diseased flounder (*Paralichthys olivaceus*) in Jeju Island. J Vet Sci. 7:53–58.

Bagarinao, T. & Lantin-Olagauer, I. (1999). The sulphide tolerance of milkfish and tilapia in relation to fish kills in farms and natural waters in the Philippines. Hydrobiologia 382, 137– 150.

Baliao, D. D., de los Santos, M. A., Franco, N. M., & Jamon, N. R. S. (2000). Net cage culture of tilapia in dams and small farm reservoirs. Tigbauan, Iloilo, Philippines: SEAFDEC Aquaculture Department.

Baliao, D.D. and Dosado, N.S. (2011). Tilapia cage farming in freshwater reservoir using artificial diets during dry and wet seasons. Philippine Agric Scientist, 94 (1): 38-45.

Banno, H., Kimura, K., Tanaka, Y., Kitanaka, H., Jin, W., Wachino, J., Yamada, K., Shibayama, K. & Arakawa, Y. (2014). Characterization of Multidrug-Resistant Group B Streptococci with Reduced Penicillin Susceptibility Forming Small N0n-Beta-Hemolytic Colonies on Sheep Blood agar Plates. Journal of Clinical Microbiology 52(6): 2169-2171.

Banno, H., Kimura, K., Tanaka, Y., Sekizuka, T., Kuroda, M., Jin, W., … Arakawa, Y. (2017). Analysis of multidrug resistant group B streptococci with reduced penicillin susceptibility forming small, less hemolyitc colonies. *PLoS One*, 12(8), e0183453.

Barkham, T., Zadoks, R.N., Azmai, M.N.A., Baker, S., Bich, V.T.N., Chalker, V., et al. (2019). One hypervirulent clone, sequence type 283, accounts for a large proportion of invasive *Streptococcus agalactiae* isolated from humans and diseased tilapia in Southeast Asia. PLoS Negl Trop Dis 13(6): e0007421.

Barnes, A.C. & Ellis, A.E. (2003). Variation in arginine dihydrolase activity in *Streptococcus iniae* may be an artefact of the assay. *Bulletin of the European Association of Fish Pathologists* 23, 163– 166.

Barony G.M., Tavares G.C., Assis G.B.N., Luz R.K., Figueiredo H.C.P. & Leal C.A.G. (2015). New hosts and genetic diversity of *Flavobacterium columnare* isolated from Brazilian native species and Nile tilapia. *Diseases of Aquatic Organisms* 117, 1– 11.

Barrow, G.I. & Feltham, R.K.A. (2003). Cowan and Steel's Manual for the identification of the Medical Bacteria, 3rd edition. Cambridge University Press, Cambridge, U.K.

Basiao, Z.U., Arago, A.L. & Doyle, R.W. (2005). A farmer-oriented Nile tilapia, *Oreochromis niloticus* L., breed improvement in the Philippines. Aquac. Rep., 36: 113-119.

Baums, C., Hermeyer, K., Leimbach, S., Adamek, M., Czerny, C., Hörstgen-Schwark, G., … Steinhagen, D. (2013). Establishment of a model of *Streptococcus iniae* meningoencephalitis in Nile Tilapia (*Oreochromis niloticus*). *Journal of Comparative Pathology*, 149, 94– 102.

Bayliss, R., Clarke, C., Oakley, C.M., Whitfield, A.G. & Young, S.E. (1984). The bowel, the genitourinary tract, and infective endocarditis. *Br Heart J* 51, 339– 345.

Beaz-Hidalgo, R., Magi, G. E., Balboa, S., Barja, J. L., & Romalde, J. L. (2008). Development of a PCR protocol for the detection of *Aeromonas salmonicida* in fish by amplification of the fstA (ferric siderophore receptor) gene. *Veterinary Microbiology*, 128, 386– 394.

Beaz-Hidalgo R. & Figueras M.J. (2012). Molecular detection and characterization of furunculosis and other *Aeromonas* fish infections. In: *Health and Environment in Aquaculture* (ed. by E. Carvalho), pp. 97– 132.

Beckmann, C., Waggoner, J.D., Harris, T.O., Tamura, G.S., & Rubens, C.E. (2002). Identification of novel adhesins from Group B streptococci by use of phage display reveals that C5a peptidase mediates fibronectin binding. *Infect Immun* 70: 2869– 2876.

Behera, B. K., Pradhan, P. K., Swaminathan, T. R., Sood, N., Paria, P., Das, A., … Jena, J. K. (2018). Emergence of tilapia lake virus associated with mortalities of farmed Nile tilapia *Oreochromis niloticus* (Linnaeus 1758) in India. *Aquaculture*, *484*, 168– 174.

Belem-Costa, A. & Cyrino, J. P. (2006). Antibiotic resistence of *Aeromonas hydrophila* isolated from *Piaractus mesopotamicus* (Holmberg, 1887) and *Oreochromis niloticus* (Linnaeus, 1758). *Sci Agric, 63*, 281–284.

Bernoth, E. (1997). Furunculosis: the history of the disease and of disease research. In: Bernoth EM, Ellis AE, Midtlyng PJ, Olivier G, Smith P (eds.) Furunculosis: Multidisciplinary fish disease research Academic press London. (pp. 1–20).

Berridge, B.R., Fuller, J.D., de Azavedo, J., Low, D.E., Bercovier, H., & Frelier, F. (2001). Development of a specific nested oligonucleotide PCR primer for S. iniae 16s-23s ribosomal DNA intergenic spacer. *Journal of Clinical Microbiology*, *36*, 2778-2781.

Beveridge, M.C.M. & Phillips, M.J. (1993). Environmental impact of tropical inland aquaculture. In: Pullin RSV, Rosenthal H, Maclean JL, editors. ICLARM Conference Proceedings 31. Proceedings of the Environment and Aquaculture in Developing Countries; 1990 September 17-22; Bellagio, Italy: International Center for Living Aquatic Resources Management. p. 213-236.

Bevis, R., 1994. The effect of artificial nests on reproductive performance in the Nile tilapia (*Oreochromis niloticus*, L.) spawned in net hapas. M. Sc. Thesis. Asian Institute of Technology, 111 pp.

BFAR-PHILMINAQ. (2007). *Managing aquaculture and its impacts: a guidebook for local governments.* Diliman Quezon City, Bureau of Fisheries and Aquatic Resources (BFAR)-PHILMINAQ Project. 80 pp.

BFAR (Bureau of Fisheries and Aquatic Resources). (2016). Philippine fisheries, profile for 2015. Quezon City, Philippines: Department of Agriculture.

Bhujel, R.C. (2013). On-farm feed management practices for Nile tilapia (*Oreochromis niloticus*) in Thailand. On-farm feed and feed management in aquaculture. FAO Fisheries and Aquaculture Technical Paper No. 583. pp. 159-189. FAO, Rome.

Bigalbal, N.R.B., Lopez, G.D.V., Torres, F.S.B. & Santos, M.D. (2018). Stock assessment of commercially important fish fauna in Manila Bay, Philippines. Transactions of the National Academy of Science and Technology Philippines 4(1): 167.

Bilen, A.M. & Bilen, E. (2013). Effects of diet on the fatty acids composition of cultured sea bass (*Dicentrarchus labrax*) liver tissues and histology compared with wild sea bass caught in Aegean Sea. Mar. Sci. Technol. Bull. 2, 13–19.

Bjornsdottir, B., Fridjonsson, O. H. Magnusdottir, S., Andresdottir, V. Hreggvidsson, G. O. & Gudmundsdottir, B. K. (2009). Characterisation of an extracellular vibriolysin of the fish pathogen *Moritella viscosa*. *Veterinary Microbiology,* 136, 326–334.

Boehm, A.B. & Sassoubre, L.M. (2014). *Enterococci* as indicators of environmental fecal contamination. Enterococci From Commensals to Lead. Causes Drug Resistant Infection (2014), pp. 1-21

Boerlage, A.S., Dung, T. T., Hoa, T. T., Davidson, J., Stryhn, H. & Hammell, K. L. (2017). Production of red tilapia (*Oreochromis* spp.) in floating cages in the Mekong Delta, Vietnam: mortality and health management. *Diseases of Aquatic Organisms* 124: 131– 144.

Boomker, J., Imes, G.D., Cameron, C.M., Naude, T.W., & Schoonbee, H.J. (1979). Trout mortalities as a result of *Streptococcus* infection. *Onderstepoort*, *Journal of Veterinary Research*, *46*, 71-77.

Bondad-Reantaso, M.G. & Arthur, J.R. (1989). Trichodinids (Protozoa: Ciliophora: Peritrichida) of Nile Tilapia (*Oreochromis niloticus*) in the Philippines. *Asian Fisheries Sciences*, vol. 3, no. 1, p. 27-44.

Bondad-Reantaso, M.G., Subasinghe, R.P., Arthur, J.R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z. & M. Shariff. (2005). Disease and health management in Asian aquaculture. Vet. Parasitol., 132 (3), 249-272.

Borrell, N., Figueras, M. J., & Guarro, J. (1998). Phenotypic identification of *Aeromonas* genomospecies from clinical and environmental sources. *Canadian Journal of Microbiology*, 44, 7–12.

Bosshard, P.P., Zbinden, R., Abels, S., Boddinghaus, B., Altwegg, M. & Bottger, E.C. (2006). 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory. J Clin Microbiol. 44:1359–1366.

Bowater, R.O., Forbes-Faulkner, J., Anderson, I.G., Condon, K., Robinson, B., et al. (2012). Natural outbreak of *Streptococcus agalactiae* (GBS) infection in wild giant Queensland grouper, *Epinephelus lanceolatus* (Bloch), and other wild fish in northern Queensland, Australia. J Fish Dis 35: 173–186.

Bromage, E.S., Thomas, A. & Owens, L. (1999). *Streptococcus iniae*, a bacterial infection in barramundi *Lates calcarifer*. Dis Aquat Organ. 36:177–181.

Bromage, E.S., Owen, L. (2002). Infection of barramundi (*Lates calcarifer*) with *Streptococcus iniae*: effects of different routes of exposure. *Dis Aquat Org*. 52: 199– 205.

Brown, S.P., Cornforth, D.M., & Mideo, N. (2012). Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends Microbiol* 20: 336– 342.

Bryan, J.D., & Shelver, D.W. (2009) *Streptococcus agalactiae* CspA is a serine protease that inactivates chemokines. *J Bacteriol* 191: 1847– 1854.

Buchanan, J.T., Stannard, J.A., Lauth, X., Ostland, V.E., Powell, H.C., Westerman, M.E. & Nizet, V. (2005). *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. *Infect Immun* 73, 6935– 6944.

Bull, J.J. . (1994). Virulence. Evolution, 5, pp. 1423-1437.

Buller, N. B. (2004). *Bacteria from fish and other aquatic animals: A practical identification manual*. Oxfordshire, UK: CABI Publishing.

Bullock, G.L. (1981). Streptococcal infections of fishes. *Fish Disease Leaflet* (pp. 1-7). United States Department of the Interior, Fish and Wildwife Service.

Burr, S. E., Stuber, K., Wahli, T., & Frey., J. (2002). Evidence for a type III secretion system in *Aeromonas salmonicida* subsp. *salmonicida*. *Journal of Bacteriology*, 184, 5966–5970.

Buscetta, M., Papasergi, S., Firon, A., Pietrocola, G., Biondo, C., Mancuso, G., Midiri, A., Romeo, L. *et al*. (2014). FbsC, a novel fibrinogen-binding protein, promotes *Streptococcus agalactiae*-host cell interactions. *J Biol Chem* 289, 21003– 21015. doi:10.1074/jbc.M114.553073.

Byers, H. K., Cipriano, R. C., Gudkovs, N., & Crane, M. S. (2002). PCR-based assays for the fish pathogen *Aeromonas salmonicida* II. Further evaluation and validation of three PCR primer sets with infected fish. *Diseases of Aquatic Organisms*, 49, 139–144.

Camus, A.C., Durborow, R.M., Hemstreet, W.G., Thune, R.L. & Hawke JP (1998). Aeromonas bacterial infections: motile aeromonad septicaemia (No. 478). Southern Regional Aquaculture Center.

Canals, R., Ramirez, S., Vilches, S., Horsburgh, G., Shaw, J. G., Tomás, J. M., & Merino, S. (2006). Polar flagellum biogenesis in *Aeromonas hydrophila*. *Journal of Bacteriology*, 188, 542–555.

Carrasco, S.E., Chomel, B.B., Gill, V.A., Kasten, R.W., Maggi, R.G., Breitschwerdt, E.B., Byrne, B.A., Burek-Huntington, K.A. *et al.* (2014). Novel *Bartonella* infection in northern and southern sea otters (*Enhydra lutris kenyoni* and *Enhydra lutris nereis*). *Vet Microbiol* 170, 325– 334.

Cascón, A., Anguita, J., Hernanz, C., Sánchez, M., Fernández, M., & Naharro, G. (1996). Identification of *Aeromonas hydrophila* hybridization group 1 by PCR assays. *Applied and Environmental Microbiology*, 62, 1167–1170.

Castilho, M., Castro, T., Araújo, V., Trajano, R., Santos, P., Pimenta, P. & Freitas-Almeida, A. (2009). High frequency of hemolytic and cytotoxic activity in *Aeromonas* spp. isolated from clinical, food and environmental in Rio de Janeiro, Brazil. *Antonie Van Leeuwenhoek*, 96(1), 53–61.

Carriero, M.M., Mendes-Maia, A.A., Moro-Sousa, R.L. & Henrique-Silva, F. (2016). Characterization of a new strain of *Aeromonas dhakensis* isolated from diseased pacu fish (*Piaractus mesopotamicus*) in Brazil. J Fish Dis. doi:10.1111/jfd.12457

Caulton, M. S. (1982). Feeding, metabolism and growth of tilapias - some quantitative considerations. pp. 157–184. *In*: Pullin R.S.V. and Lowe-McConnell R.H. (eds), The Biology and Culture of Tilapia. ICLARM, Manila, The Philippines.

Chang, P. & Plumb, J. (1996). Effects of salinity on Streptococcus infection of Nile tilapia, *Oreochromis niloticus*. J Appl Aquaculture. 6:39–45.

Chattopadhyay, D., Carey, A.J., Caliot, E., Webb, R.I., Layton, J.R., Wang, Y., Bohnsack, J.Adderson, E.E. & Ulett, G.C. (2011). Phylogenetic lineage and pilus protein Spb/SAN1518 affect opsonin-independent phagocytosis and intracellular survival of Group B Streptococcus. Microbes and Infection 13, 369-382.

Chau, M. L., Chen, S. L., Yap, M., Hartantyo, S. H. P., Chiew, P. K. T., Fernandez, C. J., Wong, W. K., Fong, R. K., Tan, W. L., & Tan, B. Z. Y. (2017). Group B Streptococcus infections caused by improper sourcing and handling of fish for raw consumption, Singapore, 2015–2016. *Emerging Infectious Diseases*, 23(12), 2002– 2010.

Chellaram, C. & John A. (2015). Insecticidal Activity of an Epibiotic *Bacillus kochii* from Gorgonian Coral, *Junceella juncea* (Pallas, 1776). Journal of Pure and Appplied Microbiology. Vol. 9 (3). p. 2459-2464.

Chen, C.Y., Chao, C.B. & Bowser, P.R. (2007). Comparative histopathology of *Streptococcus iniae* and *Streptococcus agalactiae*-infected tilapia. *Bulletin of the European Association of Fish Pathologists* 27, 2– 9.

Chen, P.L., Lamy, B. & Ko, W.C. (2016). *Aeromonas dhakensis*, an increasingly recognized human pathogen. Front Microbiol 7:793.

Chen, Y.S., Chang, C.H., Pan, S.F., Wang, L.T., Chang, Y.C., Wu, H.C. & Yanagida, F. (2013). *Lactococcus taiwanensis* sp. nov., a lactic acid bacterium isolated from fresh cummingcordia. *Int J Syst Evol Microbiol* 63, 2405– 2409.

Chen, S.L. (2019). Genomic Insights Into the Distribution and Evolution of Group B Streptococcus. Front. Microbiol. 10:1447

Chervinski, J. (1982). Environmental physiology of tilapias. In: Pullin, R.V.S. and Lowe-McConnell, R.H. (eds). The biology and Culture of Tilapias. ICLARM Conference Proceedings No. 7, ICLARM, Manila, Philippines, pp. 119-128.

Chopra, A. K., Vo, T. N., & Houston, C. W. (1992). Mechanism of action of a cytotonic enterotoxin produced by *Aeromonas hydrophila*. *FEMS Microbiology Letters*, 70, 15–19.

Chopra, A. K., Houston, C. W., Peterson, J. W., & Jin, G. F. (1993). Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. *Canadian Journal of Microbiology*, 39, 513–523.

Chopra, A., Pham, R., & Houston, C. (1994). Cloning and expression of putative cytotonic enterotoxin-encoding genes from *Aeromonas hydrophila*. *Gene*, 139, 87–91.

Chopra, A. K., Peterson, J. W., Xu, X. J., Coppenhaver, D. H., & Houston, C. W. (1996). Molecular and biochemical characterization of a heat-labile cytotonic enterotoxin from *Aeromonas hydrophila. Microbial Pathogenesis*, 21, 357–377.

Chopra, A. K., & Houston, C. W. (1999). Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microbes and Infection*, 1, 1129–1137.

Chirila, F., Fit, N., Nadas, G., Negrea, O. & Ranga, R. (2008). Isolation and characterization of an *Aeromonas hydrophila* strain in a crap (*Cyprinus carpio*) toxemia focus. Bulletin UASVM, Vet Med 65:244–247.

Chitmanat, C., Lebel, P., Whangchai, N., Promya, J. & Lebel, L. (2016). Tilapia diseases and management in river-based cage aquaculture in northern Thailand. *Journal of Applied Aquaculture*, 28 (1), 9– 16.

Chu, C., Huang, P., Chen, H., Wang, Y., Tsai, I., Lu, C. & Chen, C. (2016). Genetic and pathogenic difference between *Streptococcus agalactiae* serotype Ia fish and human isolates. BMC Microbiology. 16:175. doi 10.1186/s 12866-016-0794-4.

Cipriano, R.C., Bullock, G.L. & Pyle, S.W. (1984). *Aeromonas hydrophila* and motile aeromonad septicaemias of fish.

Clarridge, J.E., Attorri, S.M., Zhang, Q. & Bartell, J. (2001). 16S ribosomal DNA sequence analysis distinguishes biotypes of *Streptococcus bovis*: *Streptococcus bovis* Biotype II/2 is a separate genospecies and the predominant clinical isolate in adult males. J Clin Microbiol. 39:1549–1552.

Coche, A.G. (1982). Cage culture of tilapias. In Biology and culture of the tilapias, edited by R.S.W. Pullin and R.H. Lowe-McConnell. ICLARM Conf.Proc. (7):205–46.

Colorni, A., Diamant, A., Eldar, A., Kvitt, H., & Zlotkin, A. (2002). *S. iniae* infections in Red Sea cage cultured and wild fish. *Diseases of Aquatic Organisms*, *49*, 165-170.

Conroy, G. (2009). Estreptocococis en tilapia: prevalencia de las especies de *Streptococcus* en América Latina y sus manifestaciones patológicas. En: Memorias de *Streptococcus* en peces de aguas cálidas. 3, 15–20.

Coull, J. R. (1993). 'Will the Blue Revolution Follow the Green Revolution? The Modern Upsurge of Aquaculture', Area 25 (4): 350-7.

Counihan-Edgar, K.L., Gill, V.A., Doroff, A.M., Burek, K.A., Miller, W.A., Shewmaker, P.L., Jang, S., Goertz, C.E. *et al.* (2012). Genotypic characterization of *Streptococcus infantarius* subsp. *coli* isolates from sea otters with infective endocarditis and/or septicemia and from environmental mussel samples. *J Clin Microbiol* 50, 4131– 4133.

Coz-Rakovac, R., Strunjak-Perovic, I., Hacmanjek, M., Popovic, N.T., Lijep, Z., Sostaric, B. (2005). Blood chemistry and histological properties of wild and cultured sea bass (*Dicentrarchus labrax*) in the North Adriatic Sea. Vet. Res. Commun. 29, 677–687.

Crane M & Hyatt, A. (2011). Viruses of fish: an overview of significant pathogens. Viruses 3: 2025–2046.

Croce, B. & Stagg, R.M. (1997) Exposure of Atlantic salmon parr (*Salmon salar)* to a combination of resin acids and a water-soluble fraction of diesel fuel oil: A model to investigate the chemical causes of pigmented salmon syndrome. Environ Toxicol Chem 16(9): 1921-1929.

Crumlish, M., Dung, T.T., Turnbull, J.F., Ngoc, N.T.N. & Ferguson, H.W. (2002). Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. Journal of Fish Diseases. 25, 733-736.

Crumlish, M., Thanh, P.C., Koesling, J., Tung, V.T. & Gravningen, K. (2010). Experimental challenge studies in Vietnamese catfish, *Pangasianodon hypophthalmus* (Sauvage), exposed to *Edwardsiella ictaluri* and *Aeromonas hydrophila*. J Fish Dis 33, 717– 722.

Cruz, P.S., Andalencio, M.N., Bolivar, R.B. & Fitzsimmons, K. (2008). Tilapia-shrimp polyculture in Negros Island, Philippines: a review. J World Aquacult Soc 39: 713-725.

Cruz-Papa, D.M.A.D., Candare, C.M.G., Cometa, G.L.S., Gudez, D.E.G., Guevara, A.M.I.T., Relova, M.B.T.G. & Papa, R.D.S. (2014). *Aeromonas hydrophila* Bacteriophage UP87: an alternative to antibiotic treatment for motile *Aeromonas* septicemia in Nile tilapia (*Oreochromis niloticus*). Philipp Agric Sci 97:96–101.

Cui, M., Zhang, H., Li, J., Liu, R., Wu, M., Xu, D. & Zhang, Q. (2019). Differential PCR detection of *Streptococcus agalactiae* and *Streptococcus iniae* in tilapia (*Oreochromis niloticus*) by a single primer pair. J. Fish. Dis. 42, pp. 1211-1216.

Dallaire-Dufresne, S., Tanaka, K.H., Trudel, M.V., Lafaille, A., Charette, S.J. (2014). Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis, Vet. Microbiol. 169 (1–2) 1–7, https://doi.org/10.1016/j.vetmic.2013.06.025.

Dangwetnam, M., Suanyuk, N., Kong, F. & Phromkunthong, W. (2016). Serotype distribution and antimicrobial susceptibilities of *Streptococcus agalactiae* from infected cultured tilapia (*Oreochromis niloticus*) in Thailand: Nine-year Perspective. Journal of Medical Microbiology. 65, 247-254.

Daood, N. (2012). Isolation and antibiotic susceptibility of *Aeromonas* spp. from freshwater fish farm and farmed carp (Dam of 16 Tishreen, Lattakia). J Basic Sci 28:27–39

Darwish, A.M., & Griffin, B.R. (2002). Study shows oxyetracycline controls *Streptococcus* in tilapia. *Global Aquaculture Advocate*, *5*, 34-35.

Darwish, A.M., & Hobbs, M.S. (2005). Laboratory efficacy of amoxicillin for the control of *S. iniae infection* in blue tilapia. *Journal of Aquatic Animal Health*, *17*, 197-202.

Das, A., Rathore, A., Jananil, C., Hemanth, C. & Balakrishnan, R.A. (2013). Diagnosis of motile *Aeromonas sobria* from cat fishes with septicemia by PCR. J Agri Vet Sci 2:87–91.

Daudpota, A.M., Abbas, G., Kalhoro, I.B., Shah, S.S.A., Kalhoro, H., Rehman, M.H. & Ghaffar, A. (2016). Effect of feeding frequency on growth performance, feed utilization and body composition of Nile tilapia, *Oreochromis niloticus* cultured in low salinity water. Pakistan J. Zool., 48: 171-177.

Deasy, B. M., M. C. Rea, G. F. Fitzgerald, T. M. Cogan &T. P. Beresford. (2000). A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus. Syst. Appl. Microbiol.*23:510-522.

De Figueiredo, J., & Plumb, J. A. (1977). Virulence of different isolates of *Aeromonas hydrophila* in channel catfish. *Aquaculture*, 11, 349–354.

Delannoy, C.M.J., Crumlish, M., Fontaine, M.C., Pollock, J., Foster, G., Dagleish, M.P., Turnbull, J.F. & Zadoks, R.N. (2013). Human *Streptococcus agalactiae* strains in aquatic mammals and fish. BMC Microbiol. 13, 41.

Delannoy, C. M., Crumlish, M., Fontaine, M. C., Pollock, J., Foster, G., Dagleish, M. P., … Zadoks, R. N. (2013). Human *Streptococcus agalactiae* strains in aquatic mammals and fish. BMC Microbiology, 13, 41.

Delannoy, C.M., Zadoks, R.N., Crumlish, M., Rodgers, D., Lainson, F.A., Ferguson, H.W., Turnbull, J. & Fontaine, M.C. (2016). Genomic comparison of virulent and non-virulent *Streptococcus agalactiae* in fish. Journal of Fish Diseases 39, 13-29.

Del-Pozo J., Crumlish M., Ferguson H.W. & Turnbull J.F. (2009). A retrospective cross-sectional study on "*Candidatus arthromitus*" associated rainbow trout gastroenteritis (RTGE) in the UK. Aquaculture 290, 22– 27.

Del-Pozo J., Crumlish M., Ferguson H.W., Green D.M. & Turnbull J.F. (2010). A prospective longitudinal study of "*Candidatus arthromitus*"-associated rainbow trout gastroenteritis in the UK. Preventive Veterinary Medicine 94, 289– 300.

Del-Pozo, J., Crumlish, M., Turnbull, J. F., & Ferguson, H. W. (2010). Histopathology and ultrastructure of segmented filamentous bacteria-associated rainbow trout gastroenteritis. *Veterinary Pathology*, 47(2), 220– 230.

Del Rio-Zaragoza, O.B., Fajer-Avila, E.J. & Almazán-Rueda, P. (2010). Haematological and gill responses to an experimental infection of dactylogyrid monogeneans on the spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869). *Aquac Res*. 41: 1592– 1601.

Delphino, M., Barone, R. S. C., Leal, C. A. G., Figueiredo, H. C. P., Gardner, I. A., & Gonçalves, V. S. P. (2019). Economic appraisal of vaccination against *Streptoccocus agalactiae* in Nile tilapia farms in Brazil. Preventive Veterinary Medicine, 162, 131– 135.

Delphino, M.K.V.C., Leal C.A.G., Gardner I.A., Assis G.B.N., Roriz G.D., Ferreira F., Figueiredo H.C.P., & Gonçalves V.S.P. (2019). Seasonal dynamics of bacterial pathogens of Nile tilapia farmed in a Brazilian reservoir. Aquaculture., 498, pp. 100-108.

Deng, J., Fu, L., Wang, R., Yu, N., Ding, X., Jiang, L., . . . Che, X. (2014). Comparison of MALDI-TOF MS, gene sequencing and the Vitek 2 for identification of seventy-three clinical isolates of enteropathogens. *Journal of Thoracic Disease*, *6*, 539–544.

Dias, M.K.R. et. al. (2016). Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon. Vet Microbiol 188:12–15.

Didinen, B. I., Yardimci, B., Onuk, E., Metin, S., & Yildirim, P. (2014). Naturally *Lactococcus garvieae* infection in rainbow trout (*Oncorhyncus mykiss*

Walbaum, 1792): New histopathological observations, phenotypic and molecular identification. *Revue Medicine Veterinaire*, 165(1–2), 12– 19.

Dodson, S., Maurer, J. & Shotts, E. (1999). Biochemical and molecular typing of *Streptococcus iniae* isolated from fish and human cases. J Fish Dis. 22:331–336.

Dohoo, I., Martin, W., & Stryhn, H. (2003). Veterinary Epidemiological Research AVC Publishing, Charlottetown, Prince Edward Island, Canada.

Dong, H.T., Nguyen, V.V., Phiwsaiya, K., Gangnonngiw, W., Withyachumnarnkul, B., Rodkhum, C. & Senapin, S. (2015). Concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand. Aquaculture 448, 142– 150.

Dong, H.T., Nguyen, V.V., Le, H.D., Sangsuriya, P., Jitrakorn, S., Saksmerprome, V. et al. (2015). Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. Aquaculture 448: 427–435.

Dong, H.T., Techatanakitarnan, C., Jindakittikul, P., Thaiprayoon, A., Taengphu, S., Charoensapsri, W. et al. (2017). *Aeromonas jandaei* and *Aeromonas veronii* caused disease and mortality in Nile tilapia (*Oreochromis niloticus*). Journal of Fish Diseases 40: 1395–1403.

Dong, H. T., Senapin, S., Jeamkunakorn, C., Nguyen, V. V., Nguyen, N. T., Rodkhum, C., … Rattanarojpong, T. (2019). Natural occurrence of edwardsiellosis caused by *Edwardsiella ictaluri* in farmed hybrid red tilapia (*Oreochromis* sp.) in Southeast Asia. *Aquaculture*, 499, 17– 23.

Donohue, M. J., Smallwood, A. W., Pfaller, S., Rodgers, M., & Shoemaker, J. A. (2006). The development of a matrix-assisted laser desorption/ionization mass spectrometry-based method for the protein fingerprinting and identification of *Aeromonas* species using whole cells. *Journal of Microbiol Methods*, *65*, 380–389.

Donohue, M. J., Best, J. M., Smallwood, A. W., Kostich, M., Rodgers, M., & Shoemaker, J. A. (2007). Differentiation of *Aeromonas* isolated from drinking water distribution systems using matrix-assisted laser desorption/ionization-mass spectrometry. *Analytical Chemistry*, *79*, 1939–1946. https://doi.org/10.1021/ac0611420

Doran, K.S., Liu, G.Y. & Nizet, V. (2003). Group B streptococcal beta-hemolysin-cytolysin activates neutrophil signalling pathways in brain endothelium and contributes to development of meningitis. J. Clin Invest 112: 736-744.

Dorsch, M., Ashbolt, N. J., Cox, P. T., & Goodman, A. E. (1994). Rapid identification of *Aeromonas* species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental isolates. *Journal of Applied Bacteriology*, 77, 722–726.

Duan, Y., Dong, X., Zhang, X. & Miao, Z. (2011). Effects of dissolved oxygen concentration and stocking density on the growth, energy budget and body composition of juvenile Japanese flounder, *Paralichthys olivaceus* (Temminck et Schlegel). Aquaculture Research 42(3): 407–416.

Ebert, D. & Bull, J.J. 2003. Challenging the trade-off model for the evolution of virulence: is virulence management feasible? Trends Microbiol., 11, pp. 15-20

Eggset, G., Bjornsdottir, R., McQueen Leifson, R., Arnesen, J.A., Coucheron, D.H. & Jorgensen, T.O. (1994). Extracellular glycerophospholipid: cholesterol acyltransferase from *Aeromonas salmonicida*: activation by serine protease. *Journal of Fish Diseases*, 17, 17 29.

Eguia, M. R. R., & Taniguchi, N. (2006). DNA markers help manage Nile tilapia stocks. *Global Aquaculture Advocate*, *9*(4), 48-50.

Eguia, R.V. & Romana-Eguia, M.R.R. (2007). Tilapia Farming in Ponds and Cages. *SEAFDEC/AQD Extension Manual No. 36*. Iloilo, SEAFDEC Aquaculture Department. 40 pp.

Eknath, A.E. & Acosta, B.O. (1998). Genetic improvement of farmed tilapias (GIFT) project. Final report. March 1988 to December 1997. International Center for Living Aquatic Resources Management, Makati City, the Philippines.

Eldar. A., Bejerano, Y., & Bercovier, H. (1994). *Streptococcus shiloi* and *Streptococcus difficile*, two new streptococcal species causing a meningoecephalitis in fish. *Currrent Microbiology*, *28*, 139-143.

Eldar A., Bejerano Y., Livoff A., Hurvitz A. & Bercovier H. (1995). Experimental streptococcal meningo-encephalitis in cultured fish. *Veterinary Microbiology* 43, 33– 40.

Eldar, A.C., Frelier, P.F., Asanta, L., Varner, P.W., Lawhon, S. & Bercovier, H. (1995). *Streptococcus shiloi*, the name for an agent causing septicemic infection in fish, is a junior synonym of *Streptococcus iniae*. *International Journal of Systemic Bacteriology* 45, 840– 842.

Eldar, A. & Ghittino, C. (1999). *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases. Diseases of Aquatic Organisms. 36, pp. 227-231.

Eley, R.L., Carroll, J.H. & De Woody, D. (1972). Effects of caged catfish culture on water quality and community metabolism of a lake. Proc.Okla.Acad.Sci., 52:10–5

Ellis, A. E., Vale, A. do, Bowden, T. J., Thompson, K., & Hastings, T. (1997). *In vivo* production of A-protein, lipopolysaccharide, iron-regulated outer membrane proteins and 70-kDa serine protease by *Aeromonas salmonicida* subsp. *salmonicida*. *FEMS Microbiology Letters*, 149, 157–163.

Ellis, T., Berrill, I., Lines, J., Turnbull, J. & Knowles, T. (2012). Mortality and fish welfare. Fish Physiology and Biochemistry 38: 189– 199.

Elsheshtawy, A., Yehia, N., Elkemary, M., & Soliman, H. (2019). Investigation of Nile tilapia Summer Mortality in Kafr El-Sheikh Governorate, Egypt. *Genetics of Aquatic Organisms*, 3(1), 17– 25. https://doi.org/10.4194/2459-1831-v3_1_03.

Eltholth, M., Fornace, K., Grace, D., Rushton, J. & Häsler, B. (2015). Characterisation of production, marketing and consumption patterns of farmed tilapia in the Nile Delta of Egypt. Food Policy 51, 131–143,

El Aamri, F., D. Padilla, F. Acosta, M. J. Caballero, J. Roo, J. Bravo, J. Vivas, & F. Real. (2010). First report of *Streptococcus iniae* in Red Porgy (*Pagrus pagrus*, L.). Journal of Fisheries Diseases 33:901–905.

El Latif, A.M.A., Elabd, H., Amin, A. *et al.* (2019). High mortalities caused by *Aeromonas veronii*: identification, pathogenicity, and histopathological studies in *Oreochromis niloticus*. *Aquacult Int* 27, 1725–1737.

El-Ghany, N.A.A., El-khatib, N.R. & Salama, S.S. (2014). Causes of mortality in discus fish (*Symphysodon)* and trials for treatment. Egypt J Aquac 4:1–12.

El-Gohary, F. A., Zahran, E., El-Gawad, E. A., El-Gohary, A. H., Abdelhamid, F. M., El-Mleeh, A., Elmahallawy, E. K., & Elsayed, M. M. (2020). Investigation of the prevalence, virulence genes, and antibiogram of motile aeromonads Isolated from Nile tilapia fish farms in Egypt and assessment of their water quality. *Animals*, 10, 1432.

El-Sayed, N. & El-Gheit, A. (2005). Streptococcus infections among farmed and wild tilapia fish in Egypt. Egypt. J. Exp. Biol., 1, pp. 1-7.

El-Sayed, A. F. M. (2006). Tilapia Culture in Salt Water: Environmental Requirement, Nutritional Implications and Economic Potentials. En. Editores: L. Elizabeth Cruz Suarez, Denis Ricque Marie, Mireya Tapia Salazar, Martha G. Nieto Lopez, David A. Villareal Cavazos, Ana C. Puello Cruz y Armando Garcia Ortega. Avances en Nutricion Acuicola VIII. VIII Simposium Internacional de Nutricion Acuicola. 15-17 Noviembre. Universidad Autonoma de Nuevo Leon, Mexico. ISBN 970-694-333-5.

El-Sayed, A.F.M. (2019). Tilapia culture, 2nd ed. Cambridge, MA: Academic Press.

El-Shafey, A.A.M. (1998). Effect of ammonia on respiratory functions of blood of *Tilapia zilli*. *Comparative Biochemistry and Physiology*, 121A, 305– 313.

El-Sherif, M. S., & A. M. EL-Feky. (2008). Effect of ammonia on Nile Tilapia (*O. niloticus*) performance and some haematological and histological measures. Eighth International Symposium on Tilapia in Aquaculture. Cairo, Egypt.

Eldar, A.C. & Ghittino C. (1999). *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases. *Diseases of Aquatic Organisms* 36, 227– 231.

Engle, C.R. (2006). Marketing and Economics. pp. 619-644. In C.E. Lim, C.D. Webster (Editors). Tilapia Biology, Culture and Nutrition. Haworth Press Inc., New York, USA. 645 pp.

Epidemiological Surveillance System. (2007). Epidemiological comment on reported diseases and Microbiological Information System, Spain. Year 2006 Boletín Epidemiológico Semanal, 15. pp. 109-114.

Escandor, J. (2015). Lake Buhi ravaged by ₱14.5M fish kill. Retrieved from buhiforum.wordpress.com

Esteve, C., Valera, L., Gutierrez, C., Ventosa, A. (2003). Taxonomic study of sucrose-positive *Aeromonas jandaei*-like isolates from faeces, water and eels: emendation of *A. jandaei* Carnahan et al. 1992 Int. J. Syst. Evol. Microbiol. 53: 1411-1419.

Euzéby, J.P. (1998). Taxonomic note: necessary correction of specific and subspecific epithets according to Rules 12c and 13b of the International Code of Nomenclature of Bacteria (1990 revision). Int. J. Syst. Bacteriol. 48: 1073–1075.

Evans, J.J., Shoemaker, C.A. & Kelsius, P.H. (2000). Experimental *Streptococcus iniae* infection of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) and tilapia (*Oreochromis niloticus*) by nares inoculation Aquaculture, 198, pp. 197-210.

Evans, J.J., Klesius, P.H., Gilbert, P.M., Shoemaker, C.A., Al-Sarawi, M.A., Landsberg J., Durendez, R., Al-Marzouk, A., & Al-Zenki, S. (2002). Characterization of β-hemolytic group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* and and mullet, *Liza klunzingeri*, in Kuwait. *Journal of Fish Diseases*, *25*, 505-513.

Evans, J.J., Klesius, P.H., & Shoemaker, C.A. (2004a). Starch hydrolysis testing of multiples isolates for rapid differentiation of *S. iniae*. *Bulletin of European Association of Fish Pathology*, *24*, 231-239.

Evans, J.J., Klesius, P.H. & Shoemaker, C.A. (2004b). Efficacy of *Streptococcus agalactiae* (Group B) vaccine in tilapia (*Oreochromis niloticus*) by intraperitoneal and bath immersion administration. *Vaccine* 22, 3769–3773.

Evans, J.J, Pasnik, D.J, Klesius, P.H & Al-Ablani S. (2006). First report of *Streptococcus agalactiae* and *Lactococcus garvieae* from a wild bottlenose dolphin (*Tursiops truncates)*. J. Wildl. Dis. Assoc. 42(3):561–569.

Evans, J.J., Bohnsack, J.F., Klesius, P.H., Whiting, A.A., Garcia, J.C., Shoemaker, C.A. & Takahashi, S. (2008). Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan. J. Med. Microbiol. 57, 1369–1376.

Evans, J.J., Klesius, P.H. & Shoemaker, C.A. (2009). First isolation and characterization of *Lactococcus garvieae* from Brazilian Nile tilapia, *Oreochromis niloticus* (L.), and pintado, *Pseudoplathystoma corruscans* (Spix & Agassiz). *Journal of Fish Diseases* 32: 943– 951.

Facklam, R., Elliott, J., Shewmaker, L. & Reingold, A. (2005). Identification and characterization of sporadic isolates of *Streptococcus iniae* isolated from humans. J Clin Microbiol. 43:933–937.

Fang, H., Ge, R., & Cloning, S. Y. (2004). Characterisation and expression of *Aeromonas hydrophila* major adhesin. *Fish and Shellfish Immunology*, 16, 645–658.

FAO/OIE/WHO. (2006). Report of a Joint FAO/OIE/WHO Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance: Seoul, Republic of Korea, June13–16, 2006. Seoul: WHO.

FAO. (2009). State of food insecurity 2009 Food and Agriculture Organization, Rome.

FAO. (2010). The State of World Fisheries and Aquaculture 2010. Edited by FAO. Rome: FAO.

FAO. (2010). FishStat fishery statistical collections: aquaculture production (1950–2008; released March 2010). Rome, Italy: Food and Agriculture Organization of the United Nations. Ferguson, H., St Johns, Roach, V., Willoughby, S., Parker, C., & Ryan, R. (2000). Caribbean reef fish mortality associated with *S. iniae*. *Veterinary Record*, *147*, 662-664.

FAO. (2011). Fishery and Aquaculture Statistics, FAO, Rome, Italy.

FAO. (2012). The state of world fisheries and aquaculture. FAO Fisheries & Aquaculture Dept, Rome.

FAO. (2013). Food and Agriculture Organization of the United Nations FishStatJ: A Tool for Fishery Statistics Analysis, Version 1.0.1. Available at: http:// www.fao.org/fishery/statistics/software/fishstatj/en

FAO. (2014). Fisheries and Aquaculture topics. The State of World Fisheries and Aquaculture (SOFIA). Topics Fact Sheets. Text by Jean- Francois Pulvenis. In: FAO Fisheries and Aquaculture Department. http://www.fao.org/fishery/

FAO. (2016). The State of World Fisheries and Aquaculture Contributing to Food Security and Nutrition for all. FAO, Rome, Italy.

FAO. (2017). *World aquaculture 2015: A brief overview, by Rohana Subasinghe. FAO Fisheries and Aquaculture Circular No. 1140*. Rome, Italy: Food and Agriculture Organization of the United Nations.

FAO. (2017). "Global Aquaculture Production." Food and Agriculture Organization of the United Nations, Rome. Accessed December 20, 2017. Available online at: http:// www.fao.org/fishery/statistics/global-production/en

FAO. (2018). The state of world fisheries and aquaculture 2018 - Meeting the sustainable development goals. FAO, Rome. 210 pp.

FAO. (2019). Global Aquaculture Production 1950-2016. http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en.

FAO. (2019). FAO yearbook. Fishery and aquaculture statistics 2017. FAO, Rome. 108 pp.

FAO. (2020). Aquaculture growth potential in the Philippines. http://www.fao.org/3/ca8810en/ca8810en.pdf

FAO. (2020a). Fisheries and aquaculture software. FishStatJ - Software for Fishery and Aquaculture Statistical Time Series. In: FAO Fisheries and Aquaculture Department, Rome.

FAO. 2020. Understanding Antimicrobial Resistance in Aquaculture. Asian Fisheries Science. 33.S1:27-32.

FAO. (2021). *Cultured Aquatic Species Information Programme. Oreochromis niloticus. Text by Rakocy, J. E. In: FAO Fisheries Division. Rome. Updated.*

Fashina-Bombata, H. & Megbowon, I. (2012). Pproximate composition and breeding description of an unidentified cichlidof Epe Lagoon, Lagos, Southwest Nigeria commonly called Wesafu. Int. J. Nutr. Metab., 4: 57-63.

Feng, J., Chang, X., Zhang, Y., Zhang, X.Y. and Nie, G. (2019). Effects of *Lactococcus lactis* from *Cyprinus carpio* L. as probiotics on growth performance, innate immune response and disease resistance against *Aeromonas hydrophila*. *Fish Shellfish Immunol* 93, 73– 81.

Ferguson, H. W. (ed.). (2006). *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease* (2nd ed., 366 pp). London, UK: Scotian Press.

Fernandez-Alarcon, M.F., Santana, A.M., Viadanna, P.H.O., Manzini, B., Natori, M.M., Ishikawa, C.M., Dias, D.C., Ranzani-Paiva, M.J.T., Squassoni, G.H., Couto, F.D., Furlan, L.R. & Tachibana, L. (2019). Nile tilapia (Oreochromis niloticus) challenged infection by *Francisella noatunensis* subsp. *orientalis* via an intragastric route protocol. Aquaculture. 510: 380-385.

Fernandez-Bravo, A. & Figueras, M.J. (2020). An update on the genus Aeromonas: Taxonomy, epidemiology, and pathogenicity. Microorganisms. 8, 129.

Fessehaye, Y., Komen, H., Rezk, M.A., Van Arendonk, J.A.M. & Bovenhuis, H. (2007). Effects of inbreeding on survival, body weight and fluctuating asymmetry (FA) in Nile tilapia, *Oreochromis niloticus*. *Aquaculture*, 264, 27– 35.

Featherstone, Z. L. (2014). Investigations into the pathogenesis of aquatic *Streptococcus agalactiae* and *Streptococcus iniae* in Nile tilapia (*Oreochromis niloticus*) (Doctoral Thesis). University of Stirling. Scotland, United Kingdom.

Figueiredo, H.C., Klesius, P.H., Arias, C.R., Evans, J., Shoemaker, C.A., Pereira, D.J. Jr. et al. (2005). Isolation and characterization of strains of *Flavobacterium columnare* from Brazil. Journal of Fish Diseases 28: 199–204.

Figueiredo-Fernandes, A., Ferreira-Cardoso, J.V., Garcia-Santos, S., Monteiro, S.M., Carrola, J., Matos, P., Fontaınhas-Fernandes, A. (2007). Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. Pesqui Vet Bras 27:103–109.

Figueras, M., Soler, L., Chacon, M., Guarro, J., & Martínez-Murcia, A. (2000). Extended method for discrimination of *Aeromonas* spp. by 16S rDNA-RFLP analysis. *International Journal of Systematic and Evolutionary Microbiology*, 50, 2069–2073.

Figueras, M. J. (2005). Clinical relevance of *Aeromonas*. *Reviews in Medical Microbiology*, 16, 145–153

Figueras, M. J., Alperi, A., Saavedra, M. J., Ko, W. C., Gonzalo, N., Navarro, M., & Martínez-Murcia, A. (2009). Clinical relevance of the recently described *Aeromonas aquariorum*. *Journal of Clinical Microbiology*. 47, 3742–3746.

Figueras, M. J., Beaz-Hidalgo, R., Collado, L., & Martínez-Murcia, A. J. (2011). Point of view on the recomendations for new bacterial species description and their impact on the genus *Aeromonas* and *Arcobacter*. *Bergey's International Society for Microbial Systematics*, 2, 1–16.

Figueras, M.J. & Beaz-Hidalgo, R. (2015). *Aeromonas* infections in humans. In Aeromonas; Graf, J., Ed.; Caister Academic Press: Norfolk, UK. pp. 65–108.

Filho, C. I., E. E. Muller, L. G. Pretto-Giordano & A. F. R. L. Bracarense. (2009). Histological findings of experimental *Streptococcus agalactiae* infection in Nile tilapias (*Oreochromis niloticus*). *Brazilian Journal of Veterinary Pathology* 2: 12– 15.

Fitzsimmons, K. (2001). Polyculture of tilapia and penaeid shrimp. Global Aquaculture Advocate. 4 (3): 43-44.

Fosse, T., Giraud-Morin, C. & Madinier, I. (2003). Phenotypes of beta-lactam resistance in the genus *Aeromonas*. Pathol Biol (Paris) 51:290–296.

Francia, M.V., Zabala J.C., de la Cruz F., & Garcia-Lobo J.M. (1999). The *IntI1* integron integrase preferentially binds single-stranded DNA of the attC site. J Bacteriol 181: 6844–6849.

Frank, S.A. (1996). Models of parasite virulence. Quart. Rev. Biol., 71, pp. 37-78.

Freeman, D.J., Falkiner, F.R. & Keane, C.T. (1989). New method for detecting slime production by coagulase negative staphylococci. Journal of Clinical Pathology, 42, pp. 872-874.

Freiwald, A. & Sauer, S. (2009). Phylogenetic classification and identification of bacteria by mass spectrometry. *Nature Protocols,* 4 (5), pp. 732-742.

Fuentes-Silva, C., Soto-Zarazua, G.M., Torres-Pacheco, .I & Flores-Rangel A. (2013). Male tilapia production techniques: A mini-review. Afr. J. of Biotechnol. 12 (36): 5496-5502.

Fujii, Y., Nomura, T., Kanzawa, H., Kameyama, M., Yamanaka, H., Akita, M., & Okamoto, K. (1998). Purification and characterization of enterotoxin produced by *Aeromonas sobria*. *Microbiology and Immunology*, 42, 703–714.

Gaafar, A. Y., Soliman, M. K., Ellakany, H. F., Affr, N. A., Elbialy, A. K., Mona, S. Z., Younes, A. M., & Abozahra, R. (2015). Comparative pathogenicity of methicillin resistant *Staphylococcus aureus* (MRSA) in Nile tilapia (Oreochromis niloticus) and Tilapia zilli. Life Sciences Journal, 12(3), 186–194.

Galindo, C., Sha, J., Fadl, A., Pillai, L. L., & Chopra, A. (2006). Host immune responses to *Aeromonas* virulence factors. *Current Immunology Reviews*, 2, 13–26.

Galindo, C., & Chopra, A. (2007). *Aeromonas* and *Plesiomonas* species. In: Food Microbiology: Fundamentals and Frontiers, 3rd edn (edited by M.P. Doyle & L.R. Beuchat). Washington, DC: ASM Press. 2007 (pp. 381–400).

Garcia, F., Pilarski, F., Onaka, E.M., de Moraes, F.R. & Martins, M.L. (2007). Hematology of *Piaractus mesopotamicus* fed diets supplemented with vitamins C and E, challenged by *Aeromonas hydrophila*. Aquaculture 271:39–46.

Garcia, L.G., Lemaire S., Kahl, B.C., Becker, K., Proctor, R.A., Denis, O., Tulkens, P.M. & Van Bambeke F. (2013). Antibiotic activity against small-colony variants of *Staphylococcus aureus*: review of in vitro, animal and clinical data. J Antimicrob Chemother 68:1455–1464.

Garcia-Garrote, F., E. Cercenado &E. Bouza. (2000). Evaluation of a new system, VITEK 2, for identification and antimicrobial susceptibility testing of enterococci. *J. Clin. Microbiol.*38:2108-2111.

Gardufio, R., Thornton, J. C., & Kay, W. (1993). *Aeromonas salmonicida* grown *in vivo*. *Infection and Immunity*, 61, 3854–3862.

Garrote, A., Bonet, R., Merino, S., Simon-Pujol, D., & Congregado, F. (1992). Occurrence of a capsule in *Aeromonas salmonicida*. *FEMS Microbiology Letters*, 95, 127–132.

Gavín, R., Rabaan, A., Merino, S., Toma´S, J., Gryllos, I., & Shaw, J. (2002). Lateral flagella of *Aeromonas* species are essential for epithelial cell adherence and biofilm formation. *Molecular Microbiology*. 43, 383–397.

Ghittino, C., Latini, M., Agnetti, F., Panzieri, C., Lauro, L., Cappelloni, R. & Petracca, G. (2003) Emerging pathologies in aquaculture: effects on production and food safety. *Veterinary Research Communication* 27, 471–479.

Giari, L., Manera, M., Simoni, E., Dezfuli, B. (2007). Cellular alterations in different organs of European sea bass *Dicentrarchus labrax* (L.) exposed to cadmium. Chemosphere 67, 1171–1181.

Giordanetto, F., Fowler, P.W., Saqi, M. & Coveney, P.V. (2005). Large scale molecular dynamics simulation of native and mutant dihydropteroate synthase-sulphanilamide complexes suggests the molecular basis for dihydropteroate synthase drug resistance. Phil Trans A Math Phys Eng Sci 363(1833), 2055– 2073.

Godoy, D.T., Carvalho-Castro, G.A., Leal, C.A.G., Pereira, U.P., Leite, R.C. & Figueiredo, H.C.P. (2013). Genetic diversity and new genotyping scheme for fish pathogenic *Streptococcus agalactiae*. Lett. Appl. Microbiol. 57, 476–483.

Gordon, L., Giraud, E., Ganière, J.P., Armand, F., Bouju-Albert, A., De La Cotte, N., et al. (2007). Antimicrobial resistance survey in a river receiving effluents from freshwater fish farms. J Appl Microbiol. 102:1167–76.

Gori, A., Harrison, O.B., Mlia, E., Nishihara, Y., Chan, J.M., Msefula, J., Mallewa, M., Dube, Q. *et al*. (2020). Pan-GWAS of *Streptococcus agalactiae* highlights lineage-specific genes associated with virulence and niche adaptation. *MBio* 11, e00728-e00820.

Gottschalk, M., & Segura, M. (2000). The Pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. *Vet Microbiol* 76: 259–272.

Guerrero, R.D. III, 1983. Tilapia farming in the Philippines: practices, problems and prospects. Paper presented at PCARRD/ICLARM Workshop on

Philippine Tilapia economics, Los Bańos, Laguna, Philippines, August 10–13, 1983, 23 p. Abstr. in ICLARM Conf. Proc., (10):4

Guerrero, R.D. III. (2002). Tilapia Farming in the Asia Pacific Region. *In* R.D.III Guerrero & M.R. Guerrero-del Castillo, eds. *Tilapia farming in the 21st century. Proceedings of the International Forum on Tilapia farming (Tilapia Forum 2002),* pp. 42–52. Los Banos, Laguna, Philippine Fisheries Association Inc. 184 pp.

Guerrero, R. D. & Guerrero, L. A. (2004). Brackish water culture of tilapias in the Philippines: An assessment. In RB Bolivar, GC Mair, K Fitzsimmons eds. New Dimensions on Farmed Tilapia. Proceedings of the 16th International Symposium on Tilapia in Aquaculture. Manila, Philippines. 421-425.

Guerrero, R.D. (2018). The making of Molobicus, the tilapia hybrid. Agriculture Monthly 22(2): 30–31.

Guerrero, R. D., III, (2019). Farmed tilapia production in the Philippines is declining: What has happened and what can be done. *Philippine Journal of Science*, *148*(2), xi–xv.

Guo, C. M., Chen, R. R., Kalhoro, D. H., Wang, Z. F., Liu, G. J., Lu, C. P., et al. (2014). Identification of genes preferentially expressed by highly virulent piscine *Streptococcus agalactiae* upon interaction with macrophages. *PLoS ONE* 9:e87980.

Gupta, M.V. & Acosta, B.O. (2004). A review of global tilapia farming practices. Aquaculture Asia 9, 7–12.

Gustafson, C. E., Thomas, C. J., & Trust, J. T. (1992). Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein gene. *Applied and Environmental Microbiology*, 58, 3816–3825.

Gutekunst, H., Eikmanns, B.J. and Reinscheid, D.J. (2004). The novel fibrinogen-binding protein FbsB promotes *Streptococcus agalactiae* invasion into epithelial cells. *Infect Immun* 72, 3495– 3504. doi:10.1128/IAI.72.6.3495-3504.2004.

Guz, L. & Kozinska, A. (2004). Antibiotic susceptibility of *Aeromonas hydrophila* and *A. sobria* isolated from farmed carp (*Cyprinus carpio*). Bull Vet Inst Pulawy 48:391–395

Haas, S., Hain, N., Raoufi, M., Handschuh-Wang, S., Wang, T., Jiang, X., Schonherr, H. 2015. *Biomacromolecules*, **16**, 832– 841;

Hamilton, A., D. L. Popham, D. J. Carl, X. Lauth, V. Nizet, and A. L. Jones. (2006). Penicillin-binding protein 1a promotes resistance of group B streptococcus to antimicrobial peptides. *Infect. Immun.*74**:**6179-6187.

Han, J.E., Kim, J.H., Choresca, C.H., Shin, S.P., Jun, J.W., Chai, J.Y. & Park, S.C. (2012). Prevalence of *tet* gene and complete genome sequencing of *tet* gene-encoded plasmid (pAHH01) isolated from *Aeromonas* species in South Korea. J Appl Microbiol 112(4), 631– 638.

Hanson, B.R., Runft, D.L., Streeter, C., Kumar, A., Carion, T.W. & Neely, M.N. (2012). Functional analysis of the CpsA protein of *Streptococcus agalactiae*. *J. Bacteriol.* 194, 1668– 1678.

Hassan, M.A.M., Noureldin, E., Mahmoud, M.A., & Fita, N.A. (2017). Molecular identification and epizootiology of *Aeromonas veronii* infection among farmed *Oreochromis niloticus* in Eastern Province, KSA. Egypt. J. Aquat. Res. 2017, 43, 161–167.

Hatha, M., Vivekanandhan, A.A. & Joice, G.J. (2005). Antibiotic resistance pattern of motile aeromonads from farm raised freshwater fish. Int J Food Microbiol. 98:131–4.

Hayat, M., Mohd Yusoff, M. S., Samad, M. J., Abdul Razak, I. S., Md Yasin, I. S., Thompson, K. D., & Hasni, K. (2021). Efficacy of Feed-Based Formalin-Killed Vaccine of *Streptococcus iniae* stimulates the Gut-Associated Lymphoid Tissues and Immune Response of Red Hybrid Tilapia. *Vaccines*, *9*(1), 51. MDPI AG.

Henderson, R., Navarro-Garcia, F., Desvaux, M., Fernandez, R., & Ala'Aldeen, D. (2004). Type V protein secretion pathway: the autotransporter story. *Microbiology and Molecular Biology Reviews*, 68, 692–744.

Hernandez, E., Figueroa, J. & Iregui, C. (2009). *Streptococcus* on a red tilapia, *Oreochromis* sp., farm: a case study. *Journal of Fish Diseases* 32, 247–252.

Heuer, O.E., Kruse,H.,Grave, K., Collignon, P.,Karunasagar, I., and Angulo, F.J.(2009).Human health consequences of use of antimicrobial agents in aquaculture. Clin. Infect. Dis. 49, 1248–1253.doi: 10.1086/605667

Heuzenroeder, M.W., Wong, C.Y.F., & Flower, R.L.P. (1999). Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: correlation with virulence in a suckling mouse model. FEMS Microbiol Lett 174(1):131–136.

Hickmann-Brenner, F.W., Mac Donald, K.L., Steigerwalt, A.G., Fanning, G.R., Brener, D.J., Farmer III, J.J. (1987). *Aeromonas veronii*, a new ornithine decarboxylase positive species that may cause diarrhea. Journal Clinical Microbiology. 25, 900-906.

Hiney, M., & Olivier, G. (1999). Furunculosis *(Aeromonas salmonicida),* in Fish Diseases and Disorders III: Viral, Bacterial and Fungal Infections, P. T. K. Woo and D. W. Bruno, Eds. CAB Publishing, Oxford, UK. (pp. 341– 425).

Hirono, I., Tange, N., & Aoki, T. (1997). Iron-regulated haemolysin gene from *Edwardsiella tarda*. *Molecular Microbiology*, 24, 851–856.

Hishamunda, N., Bueno, P.B., Ridler, N. & Yap, W.G. (2009). Analysis of aquaculture development in Southeast Asia: a policy perspective. FAO Fisheries and Aquaculture Technical Paper. No. 509. Rome, FAO. 69p.

Hoai, T.D., Trang, T.T., Tuyen, N.V., Giang, N.T.H. & Van, K.V. (2019). *Aeromonas veronii* caused disease and mortality in channel catfish in Vietnam. Aquaculture. 513, Article 734425.

Hoel, S., Vadstein, O. & Jakobsen, A.N. (2017). Species distribution and prevalence of putative virulence factors in Mesophilic *Aeromonas* spp. isolated from fresh retail sushi, Front. Microbiol. 8 1–11, https://doi.org/10.3389/fmicb.2017.00931

Holland, R. D., Rafii, F., Heinze, T. M., Sutherland, J. B., Voorhees, K. J., & Lay, J. O. Jr (2000). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric detection of bacterial biomarker proteins isolated from contaminated water, lettuce and cotton cloth. *Rapid Communications in Mass Spectrometry*, *14*, 911–917.

Holt, J.G., Krieg, N.R., Sheath, P.A., Staley, J.T., & William, S.T. (1994). *Bergey's manual of determination bacteriology* (pp. 532-538). Philadelphia: Williams & Wilkins.

Hossain, M. J., Waldbieser, G. C., Sun, D., Capps, N. K., Hemstreet, W. B., Carlisle, K., et al. (2013). Implication of lateral genetic transfer in the

emergence of *Aeromonas hydrophila* isolates of epidemic outbreaks in channel catfish. *PLoS ONE* 8:e80943. doi: 10.1371/journal.pone.0080943

Hossain, M. J., D. Sun, D. J. McGarey, S. Wrenn, L. M. Alexander, M. E. Martino, Y. Xing, J. S. Terhune, & M. R. Liles. (2014). An Asian origin of virulent *Aeromonas hydrophila* responsible for disease epidemics in United States farmed catfish. *Molecular Biosystems* 5(3):e00848-14.

Hossain, S., De Silva B.C.J., Wimalasena S.H.M.P., Pathirana H.N.K.S., Dahanayake P.S., & Heo G. (2018). Distribution and Antimicrobial Resistance Genes and Class 1 Integron Gene Cassette Arrays in Motile *Aeromonas* spp. Isolated from Goldfish *(Carassius auratus*). Microbial Drug Resist. Vol. 24, No. 8.

Hossain, S., & Heo, G. J. (2021). Ornamental fish: a potential source of pathogenic and multidrug-resistant motile *Aeromonas* spp. *Letters in Applied Microbiology*, 72(1), 2– 12.

Howard, S. P., Garland, W. J., Green, M. J., & Buckley, J. T. (1987). Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. *Journal of Bacteriology*, 169, 2869–2871.

Huang, X., Feng, Y., Xiong, G., Zhong, L., Liu, S., Fang, P., Chen, H., Wang, K., Geng, Y., Ouyang, P., et al. (2020). A cross-sectional study of Bayesian belief network modeling: Risk factors for hepatopancreatic necrosis syndrome of the Chinese mitten crab (*Eriocheir sinensis*) in China. Aquaculture. 524, 735293.

Huddleston, J. R., Brokaw, J. M., Zak, J. C., and Jeter, R. M. (2013). Natural transformation as a mechanism of horizontal gene transfer among environmental *Aeromonas* species. *Syst. Appl. Microbiol.* 36, 224–234. doi: 10.1016/j.syapm.2013.01.004

Hughes, R.B., Smith, A.C. (2013). Capsule stain protocols. ASM Microbelibrary. http://www.microbelibrary.org/component/resource/laboratory-test/3041-capsule-stain-protocols

Huovinen, P. (2001). Resistance to trimethoprim-sulfamethoxazole. *Clin. Infect. Dis.*32**:**1608-1614.

Hussein, M. A., Hassan, W. H. & Mahmoud, M. A. (2013). Pathogenicity of *Achlya proliferoides* and *Saprolegnia diclina* (Saprolegniaceae) associated with

saprolegniosis outbreaks in cultured Nile Tilapia (*Oreochromis niloticus*). *World Journal of Fish and Marine Sciences* 5: 188– 193.

Huys, G., Cnockaert, M. & Swings, J. (2005) *Aeromonas culicicola* Pidiyar et al. 2002 is a later subjective synonym of *Aeromonas veronii* Hickman-Brenner et al. 1987. *Systematic and Applied Microbiology* 28, 604– 609.

Igbinosa, I.H., Beshiru, A., Odjadjare, E.E., Ateba, C.N. & Igbinosa, E.O. (2017). Pathogenic potentials of *Aeromonas* species isolated from aquaculture and abattoir environments, Microb. Pathog. 107 185–192,

Imperi, M., Pataracchia, M., Alfarone, G., Baldassarri, L., Orefici, G. & Creti, R. (2010). A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of *Streptococcus agalactiae*. *Journal of Microbiological Methods* 80, 212– 214.

Ingham, A. B., & Pemberton, J. M. (1995). A lipase of *Aeromonas hydrophila* showing nonhemolytic phospholipase C activity. *Current Microbiology*, 31, 28–33.

Ip, M., Cheuk, E.S.C., Tsui, M.H.Y., Kong, F., Leung, T.N. & Gilbert, G.L. (2006). Identification of a Streptococcus agalactiae serotype III subtype 4 clone in association with adult invasive disease in Hong Kong. J. Clin. Microbiol. 44, 4252–4254.

Iregui, C., Barato, P., Rey, A., Vasquez, G., & Verján, N. (2014). Epidemiology *of Streptococcus agalactiae* and Streptococcosis in tilapia fish. In iConcept Press Ltd (ed.), Epidemiology: Theory, Research and Practice. 1st edn. Chapter 10. Hong Kong: iConcept Press Ltd, pp.18.

Iregui, C. A., Comas, J., Vasquez, G. M., & Verjan, N. (2016). Experimental early pathogenesis of *Streptococcus agalactiae* infection in red tilapia *Oreochromis* spp. *Journal of Fish Diseases*, 39(2), 205– 215.

Ituma, O.E. & Ukah, N.J. (2017). Training skill needs of secondary school agricultural science graduate in fish farming in Ebonyi state, Nigeria. *Int. J. Bus. Manag. Soc. Res.* 3: 186-191. https://doi.org/10.18801/ijbmsr.030217.21.

Jackson, C.R., Fedorka-Cray, P.J. & Barrett, J.B. (2004). Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J Clin Microbiol* 42, 3558– 3565.

Jackson, K. A., Edwards-Jones, V., Sutton, C. W., & Fox, A. J. (2005). Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. *Journal of Microbiol Methods*, *62*, 273–284.

Jacobs L. & Chenia H. (2007). Characterization of integrons and tetracycline resistance determinants in Aeromonas spp. isolated from South African aquaculture systems. International Journal of Food Microbiology 114, 295–306.

Jaeger, K., Ransac, S., Dijkstra, B., Colson, C., Heuvel, M., & Misset, O. (1994). Bacterial lipases. *FEMS Microbiology Reviews*, 15, 29–63.

Janda, J. M. (1985). Biochemical and exoenzymatic properties of *Aeromonas* species. *Diagnostic Microbiology and Infectious Disease*, 3, 223–232.

Janda, J. M. (1991). Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. *Clinical Microbiology Reviews*, 4(4), 397–410.

Janda, J. M., & Abbott, S. (1996). Human pathogens, In B. Austin, M. Altwegg, P. J. Gosling, and S. Joseph (ed.). The genus *Aeromonas*. John Wiley & Sons, Chichester, England. (pp. 151–173).

Janda, J.M. (2001). *Aeromonas and Plesiomonas.* In: Sussman M, editor. Molecular Medical Microbiology. San Diego: Academic Press.

Janda, J.M., and Abbott, S.L. (2007). *16S rRNA* gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* 45: 2761– 2764.

Janda, J. M., & Abbott, S. L. (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, 23(1), 35–73.

Jantrakajorn S., Maisak H. & Wongtavatchai J. (2014). Comprehensive investigation of streptococcosis outbreaks in cultured Nile tilapia, *Oreochromis niloticus*, and red tilapia, *Oreochromis* sp., of Thailand. *Journal of the World Aquaculture Society* 45, 392– 402.

Jeeva, S., Lekshmi, N.P., Brindha, J.R. & Vasudevan, A. (2013). Studies on antibiotic susceptibility of *Aeromonas hydrophila* isolated from goldfish (*Carassius auratus*). Int J Curr Microbiol App Sci 2:7–13.

Jia, B., Armstrong, B., Stryhn, H., Gardner, I.A., Chang, H. & St-Hilaire, S. (2018). A case study of time-series regression modeling: risk factors for pond-level mortality of farmed grass carp (*Ctenopharyngodon idella*) on a southern Chinese farm Aquaculture. 484: 58-64.

Jiang, H., Chen, M., Li, T., Liu, H., Gong, Y. & Li, M. (2016). Molecular Characterization of *Streptococcus agalactiae* Causing Community-and Hospital-Acquired Infections in Shanghai, China. Frontiers in Microbiology. Volume 7, 1308.

Johns, B.E., Purdy, K.J., Tucker, N.P. & Maddocks, S.E. (2015). Phenotypic and Genotypic Characteristics of Small Colony Variants and Their Role in Chronic Infection. Microbiology Insights. 15:8, 15-23.

Jolley, K.A., Bliss, C.M., Bennett, J.S., Bratcher, H.B., Brehony, C., Colles, F.M., Wimalarathna, H., Harrison, O.B., Sheppard, S.K., Cody, A.J., et al. (2012). Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. Microbiology. 158:1005–1015.

Jones, A.L., Needham, R.H., Clancy, A., Knoll, K.M., & Rubens, C.E. (2003). Penicillin-binding proteins in *Streptococcus agalactiae*: a novel mechanism for evasion of immune clearance. *Mol Microbiol* 47: 247– 256.

Kadlec, K., von Czapiewski, E., Kaspar, H., Wallmann, J., Michael, G.B., Steinacker, U. & Schwarz, S. (2011). Molecular basis of sulfonamide and trimethoprim resistance in fish pathogenic *Aeromonas* isolates. Appl Environ Microbiol 77:7147–7150.

Kaktcham, P.M., Piame, L.T., Sileu, G.M.S., Kouam, E.M.F., Temgoua, J.B., Ngoufack, F.Z. and de Lourdes Pérez-Chabela, M. (2019). Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* 3MT isolated from freshwater Nile Tilapia: isolation, safety traits, bacteriocin characterisation and application for biopreservation in fish pâté. *Arch Microbiol* 201, 1249–1258.

Kalimuddin S, Chen SL, Lim CTK, et al, Singapore Group, B. S. C. 2015 epidemic of severe *Streptococcus agalactiae* sequence type 283 infections in Singapore associated with the consumption of raw freshwater fish: a detailed analysis of clinical, epidemiological, and bacterial sequencing data. Clin Infect Dis. 2017; 64: S145– S152.

Kanai, K., & Wakabayashi, H. (1984). Purification and some properties of protease from *Aeromonas hydrophila*. *Bulletin of the Japanese Society of Scientific Fisheries*. 50, 1367–1374.

Kannika, K., Pisuttharachai, D., Srisapoome, P., Wongtavatchai, J., Kondo, H., Hirono, I., Unajak, S. & Areechon, N. (2017). Molecular serotyping, virulence gene profiling and pathogenicity of *Streptococcus agalactiae* isolated from tilapia farms in Thailand by multiplex PCR. J Appl Microbiol. 122(6): 1497-1507.

Karunasagar, I., Pai, R., Malathi, G.R. (1994). Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture, p. 128

Kashindye, B.B., Nsinda, P., Kayanda, R., Ngupula, G.W., Mashafi, C.A. & Ezekiel, C.N. (2015). Environmental impacts of cage culture in Lake Victoria: the case of Shirati Bay-Sota, Tanzania. SpringerPlus 4: 475.

Kashiwagi, S., Sugimoto, N., Ohta, S., & Kusuda, R. (1977). Chemotherapeutical studies on sodium nifurstyrenate against *Streptococcus* infection in cultured yellowtail. *Fish Pathology*, *12*, 11-14.

Kautsky, N., Rönnbäck, P., Tedengren, M., & Troell, M. (2000). Ecosystem perspectives on management of disease in shrimp pond farming. *Aquaculture* 191: 145– 161.

Kawamura, Y., Itoh, Y., Mishima, N., Ohkusu, K. & Kasai, H. & Ezaki, T. (2005) High genetic similarity of *Streptococcus agalactiae* and *Streptococcus difficilis*: *S. difficilis* Eldar *et al*. 1995 is a later synonym of *S. agalactiae* Lehmann and Neumann 1896 (Approved Lists 1980). *International Journal of Systematic and Evolutionary Microbiology* 55, 961– 965.

Kayansamruaj, P., Soontara, C., Unajak, S., Dong, H.T., Rodkhum, C., Kondo, H., Hiruno, I. & Areechon, N. (2019). Comparative genomics inferred two distinct populations of piscine pathogenic *Streptococcus agalactiae,* Serotype Ia ST7 and Serotype III ST 283, in Thailand and Vietnam. Genomics. 111: 1657-1667.

Kayansamruaj, P., Areechon, N., & Unajak, S. (2020). Development of fish vaccine in Southeast Asia: A challenge for sustainability of SE Asia aquaculture. Fish & Shellfish Immunology, 103, 73– 87.

Keawcharoen, J., Techangamsuwan, S., Ponpornpisit, A., Lombardini, E.D., Patchimasiri, T., & Pirarat, N. (2015). Genetic characterization of a betanodavirus isolated from a clinical disease outbreak in farm-raised tilapia *Oreochromis niloticus* (L.) in Thailand. *Journal of Fish Diseases* 38: 49– 54.

Kenzel, S.L. & Henneke, P. (2014) Interaction of *Streptococcus agalactiae* and cellular innate immunity in colonization and disease. *Front Immunol* 5, 519.

Khan, A. A., & Cerniglia, C. E. (1997). Rapid and sensitive method for the detection of *Aeromonas caviae* and *Aeromonas trota* by polymerase chain reaction. *Letters in Applied Microbiology*, 24, 233–239.

Khor, W.C., Puah, S.M., Tan, J.A.M.A., Puthucheary, S., & Chua, K.H. (2015). Phenotypic and Genetic Diversity of *Aeromonas* Species Isolated from Fresh Water Lakes in Malaysia. PLoS ONE 10(12): e0145933.

Kibria, G., Nugegoda, D., Fairclough, R. & Lam, P. (1996). Aspects of phosphorus pollution from aquaculture. NAGA, The ICLARM Quarterly 19: 20-24.

Kibria, G., Nugegoda, D., Fairclough, R. & Lam, P. (1998). Can nitrogen pollution from aquaculture be reduced? NAGA, The ICLARM Quarterly 21:17-25.

Kihara, M. & Sakata, T. (1997). Fermentation of dietary carbohydrates to short-chain fatty acids by gut microbes and its influence on intestinal morphology of a detritivorous teleost tilapia (*Oreochromis niloticus*). *Comp. Biochem. Physiol. A*, 118, 1201– 1207.

Kim, M.S., Choi, S.H., Lee, E.H., Nam, Y.K., Kim, S.K. & Kim, K.H. (2007). α-Enolase, a plasmin(ogen) binding and cell wall associating protein from a fish pathogenic *Streptococcus iniae* strain. Aquaculture. 265, pp. 55-60.

Kirov, S. M., Jacobs, I., Hayward, L. J., & Hapin, R. H. (1995). Electron microscopic examination of factors influencing the expression of filamentous surface structures on clinical and environmental isolates of *Aeromonas veronii* biotype sobria. *Microbiology and Immunology*, 39, 329–338.

Kirov, S. M., Tassell, B. C., Annalese, B. T., Donovan, L. A. O., Rabaan, A. A., Shaw, G. & Shaw, J. G. (2002). Lateral flagella and swarming motility in *Aeromonas* species. *Journal of Bacteriology,* 184(2), 547-555.

Kitao, T., Aoki, T., & Iwata, K. (1979). Epidemiological study on streptococcicosis of cultured yellowtail (*Seriola quinquiradiata*) 1. Distribution of *Streptococcus* sp. in sea water and muds around yellowtail farm. *Bulletin of the Japanese Society of Scientific Fisheries*, *45*, 567-572.

Kitao, T., Aoki, T. & Sakoh R. (1981). Epizootics caused by β-haemolytic *Streptococcus* species in cultured freshwater fish. *Fish Pathol*. 15: 301–307.

Kitao, H. (1982). Erythromycin: The application to streptococcal infection in yellowtails. *Fish Pathology*, *17*, 77-85.

Kitao, T. (1993) Streptococcal infection. In: *Bacterial Diseases of Fish* (ed. by V. Inglis, R.J. Roberts & N.R. Bromage), pp. 196– 210. Blackwell Scientific Publications, Oxford.

Klesius, P.H., Shoemaker, C.A., & Evans, J.J. (2000). Vaccination: A health management practice for preventing diseases caused by streptococcus in tilapia and other cultured fish. In K. Fitzsimmons & J. Carvalho Filho (Eds.), *Tilapia Aquaculture in the 21st Century, Fifth International Symposium on Tilapia Aquaculture* (pp. 558-564). Rio de Janiero, Brazil.

Klesius, P., Evans, J., Shoemaker, C., Yeh, H., Goodwin, A., Adams, A. & Thompson, K. (2006). Rapid detection and identification of *Streptococcus iniae* using a monoclonal antibody-based indirect fluorescent antibody technique. Aquaculture. 258:180–186.

Klingklib, C. & Suanyuk, N. (2017). *Streptococcus agalactiae* serotype Ib, an emerging pathogen affecting climbing perch (*Anabas testudineus*) and Gunther's walking catfish (*Clarias macrocephalus*) polycultured in Southern Thailand. Thai J Vet Med. 47(2): 183-197.

Knochel, S. (1990). Growth characteristics of motile *Aeromonas* spp. isolated from different environments. *International Journal of Food Microbiology*, 10, 235 244.

Koh, T. H., Kurup, A., & Chen, J. (2004). *Streptococcus iniae* Discitis in Singapore. *Emerging Infectious Diseases*, *10*(9), 1694-1696. https://doi.org/10.3201/eid1009.040029

Kolar, M., Urbanek, K. & Latal, T. (2001). Antibiotic selective pressure and development of bacterial resistance. *Int J Antimicrob Agents* 17, 357– 363.

Kolar, S.L., Kyme, P., Tseng, C.W., Soliman, A., Kaplan, A., Liang, J., Nizet, V., Jiang, D. *et al*. (2015) Group B *Streptococcus* Evades host immunity by degrading hyaluronan. *Cell Host Microbe* 18, 694– 704.

Kostrzewa, M. & Nagy, E. (2016). How MALDI-TOF mass spectrometry can aid diagnosis of hard-to-identify pathogenic bacteria. Expert Rev Mol Diagn 16(5):509–511.

Kotob, M. H., Menanteau-Ledouble, S., Kumar, G., Abdelzaher, M., & El-Matbouli, M. (2017). The impact of co-infections on fish: A review. Veterinary Research, 47(1), 98.

Kozińska, A. (2007). Dominant pathogenic species of mesophilic aeromonads isolated from diseased and healthy fish cultured in Poland. *Journal of Fish Diseases*, 30, 293–301.

Krzyminska, S., Mokracka, J., Koczura, R., Cwiertnia, A. & Kaznowski, A. (2012). *Aeromonas* spp.-mediated cell-contact cytotoxicity is associated with the presence of type III secretion system. Antonie van Leeuwenhoek 101(2): 243–251.

Kulkarni, A., Caipang, C. M., Brinchmann, M. F., Korsnes, K., & Kiron, V. (2009). Loopmediated isothermal amplification, an assay for the detection of atypical furunculosis caused by *Aeromonas salmonicida* in Atlantic cod, *Gadus morhua*. *Journal of Rapid Methods and Automation in Microbiology*, 17, 476–489.

Kupfer, M., Kuhnert, P., Korczak, B.M., Peduzzi, R. & Demarta, A. (2006). Genetic relationships of *Aeromonas* strains inferred from *16S rRNA*, *gyrB* and *rpoB* gene sequences. Int J Syst Evol Microbiol 56:2743–2751. doi:10.1099/ijs.0.63650-0.

LaFrentz, B.R., Garcia, J.C., Waldbieser, G.C., Evenhuis, J.P., Loch, T.P., Liles, M.R., Wong, F.S. & Chang, S.F. (2018). Identification of Four Distinct Phylogenetic Groups in *Flavobacterium columnare* with Fish Host Associations. Front. Microbiol. Volume 9:452.

Lahav, D., M. Eyngor, A. Hurvitz, C. Ghittino, A. Lublin, & A. Eldar. (2004). *Streptococcus iniae* type II infections in rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* 62: 177– 180.

Laganà, P., Caruso, G., Minutoli, E., Zaccone, R., & Delia, S. (2011). Susceptibility to antibiotics of *Vibrio* spp. and *Photobacterium damsela* ssp. Piscicida strains isolated from Italian aquaculture farms. *New Microbiol.* 34, 53–63.

Laith, A.R. & Najiah, M. (2013). *Aeromonas hydrophila*: antimicrobial susceptibility and histopathology of isolates from diseased catfish, *Clarias gariepinus* (Burchell). J Aquac Res Dev 5:215.

Lamy, B., Laurent, F., Verdier, I., Decousser, J.W., Lecaillon, E., Marchandin, H., …Kodjo, A. (2010). Accuracy of 6 commercial systems for identifying clinical *Aeromonas* isolates Diagn. Microbiol. Infect. Dis., 67, pp. 9-14

Lamy, B., Laurent, F. & Kodjo, A. (2010). Validation of a partial *rpoB* gene sequence as a tool for phylogenetic identification of aeromonads isolated from environmental sources. Can J Microbiol 56:217–228. doi:10.1139/W10-006.

Lamy, B., Kodjo, A., Laurent, F., & Col, B. V. H. S. G. (2011). Identification of *Aeromonas* isolates by matrix-assisted laser desorption ionization time of-flight mass spectrometry. *Diagnostic Microbiology and Infectious Disease*, *71*, 1–5. https://doi.org/10.1016/j.diagmicrobio.2011.04.014

Lancefield, R.C. (1933). A serological differentiation of human and other groups of haemolytic streptococci. *Journal of Experimental Medicine* 59, 571– 591.

Landwehr-Kenzel, S., and Henneke, P. (2014). Interaction of *Streptococcus agalactiae* and cellular innate immunity in colonization and disease. *Front. Immunol.* 5:519. doi: 10.3389/fimmu.2014.00519

Lang, S., & Palmer, M. (2003) Characterization of *Streptococcus agalactiae* CAMP factor as a pore-forming toxin. *J Biol Chem* 278: 38167– 38173.

Lannes-Costa, P.S., deOliveira, J.S.S., daSilva Santos, G. & Nagao, P.E. (2021). A current review of pathogenicity determinants *Streptococcus* sp. Journal of Applied Microbiology, 131, 1600-1620.

Lau, S.K., Woo, P.C., Luk, W.K., Fung, A.M., Hui, W.T., Fong, A.H., Chow, C.W., Wong, S.S., Yuen, K.Y. (2006). Clinical isolates of *Streptococcus iniae* from Asia are more mucoid and beta-haemolytic than those from North America. Diagn Microbiol Infect Dis. 54:177–181.

Lazur, A. M., C. E. Cichra, & C. Watson. (2013). The use of lime in fishponds. University of Florida, Institute of Food and Agricultural Sciences Extension FA38, Gainesville.

Le Roux, F., & Blokesch, M. (2018). Eco-evolutionary dynamics linked to horizontal gene transfer in Vibrios. Annu. Rev. Microbiol. 72, 89–110. doi: 10.1146/annurev-micro-090817-062148

Leal, C.A.G., Tavares, G.C. & Figueiredo, H.C.P. (2014). Outbreaks and genetic diversity of *Francisella noatunensis* subsp *orientalis* isolated from farm-raised Nile tilapia (*Oreochromis niloticus*) in Brazil. *Genetics and Molecular Research* 13, 5704– 5712.

Leal, C.A.G. & Figueiredo, H.C.P. (2018). Estreptococose clínica em tilápia: passado e presente. Panorama da Aquicultura, 169.

Lee, S., Ka, J.-O., & Song, H.-G. (2012). Growth promotion of *Xanthium italicum* by application of rhizobacterial isolates of *Bacillus aryabhattai* in microcosm soil. The Journal of Microbiology, 50, 45– 49.

Lee, C., Cho, J.C., Lee, S.H., Lee, D.G., & Kim, S.J. (2002). Distribution of *Aeromonas* spp. as identified by 16S rDNA restriction fragment length polymorphism analysis in a trout farm. *Journal of Applied Microbiology*, 93(6),976–85.

Legario, F.S., Choresca, C.H. Jr., Turnbull, J.F. & Crumlish, M. (2020). Isolation and molecular characterization of streptococcal species recovered from clinical infections in farmed Nile tilapia (*Oreochromis niloticus*) in the Philippines. J Fish Dis. 00:1–12. https://doi.org/10.1111/jfd.13247

Lehane, L., & Rawlin, G. (2000). Topically acquired bacterial zoonoses from fish: a review. *Medical Journal of Australia*, 173(5), 256–259.

Lemos, C. H. P., Ribeiro, C. V. M., de Oliveira, C. P. B., Couto, R. D., & Copatti, C. E. (2018). Effects of interaction between pH and stocking density on the growth, haematological and biochemical responses of Nile tilapia juveniles. *Aquaculture*, 495, 62– 67.

Li, Y. & Cai, S.H. (2011). Identification and pathogenicity of *Aeromonas sobria* on tail-rot disease in juvenile tilapia *Oreochromis niloticus*. Current Microbiology 62, 623–627.

Li, Y., Liu, L., Huang, P.R., Fang, W., Luo, Z.P., Peng, H.L., Wang Y.X. & Li, A.X. (2014). Chronic streptococcosis in Nile tilapia, *Oreochromis niloticus* (L.), caused by *Streptococcus agalactiae*. J Fish Dis. 37(8):757-63.

Li, L., Wang, R., Liang, W., Gan, X., Huang, T., Huang, Y., Li, J., Shi, Y., Chen, M. & Luo, H. (2013). Rare serotype occurrence and PFGE genotypic diversity of *Streptococcus agalactiae* isolated from tilapia in China. Vet. Microbiol. 167, 719–724.

Li, L., Wang R., Liang W., Huang T., Huang Y., Luo F., Lei A., Chen M. & Gan X. (2015). Development of live attenuated *Streptococcus agalactiae* vaccine for tilapia via continuous passage *in vitro*. Fish Shellfish Immunol. 45:955–963.

Li, M., Wang, X., Qi, C., Li, E., Du, Z., Qin, J. G., & Chen, L. (2018). Metabolic response of Nile tilapia (*Oreochromis niloticus*) to acute and chronic hypoxia stress. *Aquaculture*, 495, 187– 195. https://doi.org/10.1016/j.aquaculture.2018.05.031.

Li, S. and Jedrzejas, M.J. (2001). Hyaluronan binding and degradation by *Streptococcus agalactiae* hyaluronate lyase. *J Biol Chem* 276, 41407–41416. doi:10.1074/jbc.M106634200.

Li, Y., & Cai, S. (2011). Identification and pathogenicity of *Aeromonas sobria* on tail-rot disease in juvenile tilapia *Oreochromis niloticus*. *Current Microbiology*, 62, 623–627.

Liang, Y., Melack, J.M. & Wang, J. (1981). Primary production and fish yields in Chinese ponds and lakes. Trans.Am.Fish.Soc., 110:346–50.

Liao, P.-C., Tsai, Y.-L., Chen, Y.-C., Wang, P.-C., Liu, S.-C., & Chen, S.-C. (2020). Analysis of streptococcal infection and correlation with climatic factors in cultured tilapia *Oreochromis* spp, Taiwan. *Applied Sciences*, 10(11), 2076– 3417.

Lijon, M.B., Khatun, M.M., Islam, A., Khatun, M.M. & Islam, M.A. (2015). Detection of multidrug resistance *Aeromonas hydrophila* in farm raised fresh water prawns. J Adv Vet Anim Res. 2:469–74.

Lim, C. and Webster, C. D (Editors). (2006). Tilapia: Biology, Culture and Nutrition. Food Products Press, The Haworth Press Inc. Binghamtom, NY.

Lin, F.P., Lan, R., Sintchenko, V., Gilbert, G.L., Kong, F. & Coiera, E. (2011). Computational bacterial genome-wide analysis of phylogenetic profiles reveals potential virulence genes of *Streptococcus agalactiae*. *PLoS ONE* 6, e17964.

Lio-Po, G. D., Albright, L. J., & Alapide-Tendencia E. V. (1992). *Aeromonas hydrophila* in the epizootic ulcerative syndrome (EUS) of snakehead, *Ophicephalus striatus*, and catfish, *Clarias batrachus*: quantitative estimation in natural infection and experimental induction of dermo-muscular necrotic lesion. In M. Shariff, R. P. Subasinghe, & J. R. Arthur (Eds.), Diseases in Asian Aquaculture, I: proceedings of the first Symposium on Diseases in Asian Aquaculture : 26-29 November 1990, Bali, Indonesia (pp. 461–474). Makati, Metro Manila, Philippines: Fish Health Section, Asian Fisheries Society.

Lio-Po, G. D., Albright, L. J., Michael, C. & Leano, E. M. (1998). Experimental induction of lesions in snakeheads (*Ophicephalus striatus*) and catfish, *Clarias batrachus* with *Aeromonas hydrophila*, *Aquaspirillum* sp., *Pseudomonas* sp. and *Streptococcus* sp. J. Appl. Ichthyol., Vol.14, No.1 and 2, pp. 75-79.

Lio-Po, G.D. & Lim, L.H.S. (2002). Infectious diseases of warmwater fishes in fresh water. Woo P.T.K., Bruno D.W., Lim L.H.S. (Eds.). Diseases and disorders of finfish in cage culture. CABI Publishing, Oxon, U.K.

Liu, C., Feng, J., Zhang, D., Xie, Y., Li, A., Wang, J. & Su Y. (2018). Clustering Analysis of Antibiograms and Antibiogram Types of *Streptococcus agalactiae* Strain from Tilapia in China. Microb Drug Resist. 24 (9): 1431-1439.

Liu, D. (2015). *Aeromonas* vol. 2–3, Elsevier Ltd. https://doi.org/10.1016/B978-0-12-397169-2.00061-5.

Liu, G., Zhu, J., Chen, K., Gao, T., Yao, H., Liu, Y., … Lu, C. (2016). Development of *Streptococcus agalactiae* vaccines for tilapia. *Diseases of Aquatic Organisms*, 122, 163– 170.

Liu, G. Y. et al. (2004). Sword and shield: Linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. *Proc. Natl Acad. Sci. USA* 101, 14491–14496

Liu, G.Y. & Nizetn, V. (2006). The group B streptococcal β-haemolysin/cytolysin *The Comprehensive Sourcebook of Bacterial Protein Toxins*, 3rd edn. (Alouf JE Popoff NR, eds), pp. 9737–9745. Elsevier, Oxford.

Liu, G.Y., Doran, K.S., Lawrence, T., Turkson, N., Puliti, M., Tissi, L. & Nezit, V. (2004). Sword and shield: linked group B streptococcal beta-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense Proc Natl Acad Sci USA, 101, pp. 14491-14496

Liu, J., Li, Z., Li, X. & Wang, Y. (2013). On-farm feed management practices for Nile tilapia (*Oreochromis niloticus*) in southern China. In Hasan MR and New MB (eds) On-farm feeding and feed management in aquaculture. FAO Fisheries and Aquaculture Technical Paper No. 583. pp. 71–99. FAO, Rome.

Liu, W., Zhang, Y., Ma, J., et al. (2020). Determination of a novel parvovirus pathogen associated with massive mortality in adult tilapia. PLoS Pathogens. 6: e1008765

Llobera, A.T., & Gacutan, R.Q. (1987). *Aeromonas hydrophila* associated with ulcerative disease epizootic in Laguna de Bay, Phillippines. Aquaculture, 67: 273-278.

Locke, J.B., Colvin, K.M., Datta, A.K., Patel, S.K., Naidu, N.N., Neely, M.N., Nizet, V. & Buchanan, J.T. (2007a). *Streptococcus iniae* capsule impairs phagocytic clearance and contributes to virulence in fish. *Journal of Bacteriology* 189, 1279– 1287.

Locke, J.B., Colvin, K.M., Varki, N., Vicknair, M.R., Nizet, V., & Buchanan, J.T. (2007b). *Streptococcus iniae* beta-hemolysin streptolysin S is a virulence factor in fish infection. *Dis Aquat Organ* 76: 17– 26.

Locke, J.B., Aziz, R.K., Vicknair, M.R., Nizet, V. & Buchanan, J.T. (2008). *Streptococcus iniae* M-like protein contributes to virulence in fish and is a target for live attenuated vaccine development. *PLoS ONE* 3, e2824.

Lopez, N.A. (2006). Sustainable development and trends in the Philippine aquaculture. In Proceedings of the International Workshop on Innovative Technologies for Eco-Friendly Fish Farm Management and Production of Safe Aquaculture Foods, Denpasar, Indonesia.

Lowry, T., & Smith, S. A. (2007). Aquatic zoonoses associated with food, bait, ornamental, and tropical fish. *Journal of the American Veterinary Medical Association*, 231(6), 876–80.

Luistro, M. (2008). Fish kill destroys ₱8.5M worth of tilapia, bangus in Batangas town. GMA News Online.

Lundborg, I.I. & O. Ljungberg, (1977.) Attack of *Caligus* spp. in brackish water floating cage management. Nord.Vet., 29:20–1.

Lusiastuti, A.M., Textor, M., Seeger, H., Akineden, O. & Zschock, M. (2014). The occurrence of *Streptococcus agalactiae* sequence type 261 from fish disease outbreaks of tilapia *Oreochromis niloticus* in Indonesia. Aquaculture Research. 45, 1260-1263

L'abee-Lund, T.M. & Sorum, H. (2001). Class I integrons mediated antibiotic resistance in the fish pathogen *Aeromonas salmonicida* world-wide. Microbial Drug Resistance 7, 263– 272.

Machimbirike, V. I., Jansen, M. D., Senapin, S., Khunrae, P., Rattanarojpong, T., & Dong, H. T. (2019). Viral infections in tilapines: More than just tilapia lake virus. Aquaculture, 503, 508– 518.

Mahboub, H. H., & Shaheen, A. A. (2021). Mycological and histopathological identification of potential fish pathogens in Nile tilapia. Aquaculture, 530, 735849.

Maiden, M.C. (2006). Multilocus sequence typing of bacteria. Annu Rev Microbiol. 60:561–588.

Magarinos, B., Bonet, R., Romalde.J.L, Martinez, M., Congregado, F., & Toranzo, A. (1996). Influence of the capsular layer on the virulence of *Pasteurella piscicida* for fish. *Microbial Pathogenesis*, 21, 289–297.

Magid, A., & M. M. Babiker. (1975). Oxygen consumption and respiratory behaviour of three Nile fishes. Hydrobiologia 46: 359–367.

Maisey, H.C., Quach, D., Hensler, M.E., Liu, G.Y., Gallo, R.L., Nizet, V. & Doran, K.S. (2008). A Group B streptococcal pilus protein promotes phagocyte resistance and systemic virulence. *Faseb J* 22: 1715– 1724.

Mamaril Sr., A. C. (2001). Translocation of the Clupeid *Sardinella Tawilis* to another lake in the Philippines: a proposal and ecological considerations. In Santiago, C. B., M. L. Cuvin-Aralar & Z. U. Basiao (eds), Conservation and

Ecological Management of Philippine Lakes in Relation to Fisheries and Aquaculture. SEAFDEC, PCAMRD and BFAR, Quezon City: 133–147.

Mandrell, R. E., Harden, L. A., Bates, A., Miller, W. G., Haddon, W. F., & Fagerquist, C. K. (2005). Speciation of *Campylobacter coli, C. jejuni, C. helveticus, C. lari, C. sputorum,* and *C. upsaliensis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environment Microbiology*, *71*, 6292–6307.

Manrique, W. G., da Silva Claudiano, G., Petrillo, T. R., Pardi de Castro, M., Pereira Figueiredo, M. A., de Andrade Belo, M. A., … de Moraes, F. R. (2014). Response of splenic melanomacrophage centers of *Oreochromis niloticus* (Linnaeus, 1758) to inflammatory stimuli by BCG and foreign bodies. *Journal of Applied Ichthyology*, 30, 1001– 1006.

Marchandin, H., Teyssier, C., Siméon De Buochberg, M., Jean-Pierre, H., Carriere, C. & Jumas-Bilak, E. (2003). Intra-chromosomal heterogeneity between the four 16S rRNA gene copies in the genus *Veillonella*: implications for phylogeny and taxonomy. Microbiology 149:1493-1501

Marín, M., Cercenado, E., Sánchez-Carrillo, C., Ruiz, A., Gómez González, Á., Rodríguez-Sánchez, B., et al. (2017). Accurate differentiation of *Streptococcus pneumoniae* from other species within the Streptococcus mitis group by peak analysis using MALDI-TOF MS. *Front. Microbiol.* 8:698. doi: 10.3389/fmicb.2017.00698

Marte, C.L., Cruz, P. & Flores, E.E.C. (2000). Recent Developments in Freshwater and Marine Cage Aquaculture in the Philippines. In: Liao IC, Lin CK, editors. Proceedings of the First International Symposium on Cage Aquaculture in Asia; 1999 November 2-6; Manila, Philippines: World Aquaculture Society – Southeast Asian Chapter, Bangkok. p. 75-88.

Martin-Carnahan, A., & Joseph, S. (2005). *Aeromonadaceae.* In: Brenner, D.J; Krieg, N.R; Staley, J.T; Garrity, G.M. (eds). Bergeys Manual of Systematic Bacteriology, The Proteobacteria, Part B. Vol.2, 2nd ed., Springer-Verlag, New York, USA. (pp. 556–578).

Martínez, M., Simon-Pujol, D., Congregado, F., Merino, S., Rubires, X., & Tomas, J. (1995). The presence of capsular polysaccharide in mesophilic

Aeromonas hydrophila serotypes O:11 and O:34. *FEMS Microbiology Letters*, 128, 69–74.

Martinez-Murcia, A.J., Benlloch, S. & Collins, M.D. (1992). Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. Int J Syst Bacteriol 42:412–421. doi:10.1099/00207713-42-3-412.

Martínez-Murcia, A. J., Anton, A. I., & Rodriguez-Valera, F. (1999). Patterns of sequence variation in two regions of the 16S rRNA multigene family of *Escherichia coli*. *International Journal of Systematic Bacteriology*, 49, 601–610.

Martínez-Murcia, A. J., Soler, L., Saavedra, M. J., Chacón, M. R., Guarro, J., & Stackebrandt, E. & Figueras, M. J. (2005). Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*. *International Microbiology*, 8*,* 259–269.

Martínez-Murcia, A.J., Figueras, M.J., Saavedra, M.J. & Stackebrandt, E. (2007). The recently proposed species *Aeromonas sharmana* sp. nov., isolate GPTSA-6T, is not a member of the genus *Aeromonas*. Int Microbiol 10:61-64

Martinez-Murcia, A.J., Monera, A., Saavedra, M.J., Oncina, R., Lopez-Alvarez, M., Lara, E. & Figueras, M.J. (2011). Multilocus phylogenetic analysis of the genus *Aeromonas*. Syst Appl Microbiol 34:189–199. doi:10.1016/j.syapm.2010.11.014.

Martino, M.E., Fasolato, L., Montemurro, F., Rosteghin, M., Manfrin, A., Patarnello, T., Novelli, E. & Cardazzo, B. (2011). Determination of microbial diversity of *Aeromonas* strains on the basis of multilocus sequence typing, phenotype, and presence of putative virulence genes. Appl Environ Microbiol 77(14), 4986– 5000.

Mata, A.I., Blanco, M.M., Dominguez, L., Fernandez-Garayzabal, J.F. & Gibello, A. (2004). Development of a PCR assay for *Streptococcus iniae* based on the lactate oxidase (*lctO*) gene with potential diagnostic value. Veterinary Microbiology 101, 109-116.

McCarter, L. L. (1995). Genetic and molecular characterisation of the polar flagellum of *Vibrio parahaemolyticus*.*Journal of Bacteriology*, 178, 1310–1319.

Meek, R.W., Vyas, H., & Piddock, L.J.V. (2015). Nonmedical uses of antibiotics: time to restrict their use? *PLoS Biol.* 13:e1002266. doi: 10.1371/journal.pbio. 1002266

Mello, L.V., De Groot, B.L., Li, S., and Jedrzejas, M.J. (2002). Structure and flexibility of *Streptococcus agalactiae* hyaluronate lyase complex with its substrate. Insights into the mechanism of progressive degradation of hyaluronan. *J Biol Chem* 277: 36678– 36688.

Mengistu, S. B., Mulder, H. A., Benzie, J. A. H., & Komen, H. (2020). A systematic literature review of the major factors causing yield gap by affecting growth, feed conversion ratio and survival in Nile tilapia (*Oreochromis niloticus*). *Reviews in Aquaculture*, 12(2), 524– 541. https://doi.org/10.1111/raq.12331.

Merino, S., Aguilar, A., Rubires, X., Simon-Pujol, S., Congregado, F., & Tomás, J. M. (1996). The role of the capsular polysaccharide of *Aeromonas salmonicida* in the adherence and invasion of fish cell lines. *FEMS Microbiology Letters*, 142, 185–189.

Merino, S., Aguilar, A., Rubires, X., Abitiu, N., Regué, M., & Tomas, J. M. (1997). The role of the capsular polysaccharide of *Aeromonas hydrophila* serogroup O:34 in the adherence to and invasion of fish cell lines. *Research in Microbiology*, 148, 625–631.

Merino, S., Aguilar, A., Nogueras, M. M., Regue, M., Swift, S., & Tomas, J. M. (1999). Cloning, sequencing, and role in virulence of two phospholipases (A1 and C) from mesophilic *Aeromonas* sp. serogroup O:34. *Infection and Immunity*, 67, 4008–4013.

Merino, S., Bouamama, L., Knirel, Y., Senchenkova, S., Regué, M., & Tomás, M. (2012). *Aeromonas* surface glucan tttached through the O-antigen ligase represents a new way to obtain UDP-glucose. *PLoS ONE* 7(5): e35707.

Mian, G.F., Godoy, D.T., Leal, C.A., Yuhara, T.Y., Costa, G.M., & Figueiredo, H.C. (2009). Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. *Vet Microbiol* 136: 180– 183.

Miao, W. & Wang, W. (2020). Trends of Aquaculture Production and Trade: Carp, Tilapia, and Shrimp. Asian Fisheries Science. 33.SI: 1-10.

Milani, C.J., Aziz, R.K., Locke, J.B., Dahesh, S., Nizet, V. & Buchanan, J.T. (2010). The novel polysaccharide deacetylase homologue Pdi contributes to virulence of the aquatic pathogen *Streptococcus iniae*. *Microbiology* 156: 543– 554.

Minami, T., Nakamura, M., Ikeda, Y., & Ozaki, H. (1979). A betahemolytic *Streptococcus* isolated from cultured yellowtail. *Fish Pathology*, *14*, 33-38.

Mindanews. (2017). South Cotabato wants moratorium on fish cage ops in Lake Sebu.

Mires, D. (1995). The tilapias. pp. 133–152. *In*: Production of Aquatic Animals: Fishes (eds Nash, C. E., and A. J. Novotony). Elsevier, New York.

Mishra, A., Nam, G., Gim, G., Lee, H., Jo, A., & Kim, H. (2018). Current challenges of streptococcus infection and effective molecular, cellular, and environmental control methods in aquaculture. Molecules and Cells, 41(6), 495– 505.

Mitchell, S. O., Baxter, E. J., Holland, C., & Rodger, H. D. (2012). Development of a novel histopathological gill scoring protocol for assessment of gill health during a longitudinal study in marine farmed Atlantic salmon (*Salmo salar*). Aquaculture International, 20, 813– 825.

Miyazaki, T. & Kaige, N. (1985). A histopathological study on motile aeromonad disease of Crucian carp. Fish Pathol. 21, 181–185. doi: 10.3147/jsfp.21.181.

Mjoun, K., Rosentrater, K. & Brown, M.L. (2010). TILAPIA: Environmental Biology and Nutritional Requirements. In Fact Sheets. Paper 164. http://openprairie.sdstate.edu/extension_fact/164.

Mohammed, H. H., & Peatman, E. (2018). Winter kill in intensively stocked channel catfish (*Ictalurus punctatus*): Coinfection with *Aeromonas veronii*, *Streptococcus parauberis* and *Shewanella putrefaciens*. *Journal of Fish Diseases*, 41, 1339– 1347. https://doi.org/10.1111/jfd.12827

Monir, M.S., Yusoff, S.M., Mohamad, A. & Ina-Salwany, M.Y. (2020). Vaccination of tilapia against Motile Aeromonas Septicemia: A review. J. Aquat. Anim. Health 2020, 32, 65–76.

Montoya-Mejía, M., Rodríguez-González, H. & Nolasco-Soria, H. (2016). Circadian cycle of digestive enzyme production at fasting and feeding conditions in Nile tilapia, *Oreochromis niloticus* (Actinopterygii: Perciformes: Cichlidae). Acta Ichthyologica et Piscatoria ; Szczecin 46.3: 163-170.

Morandi, A., Zhaxybayeva, O., Gogarten, J.P. & Graf, J. 2005. Evolutionary and diagnostic implications of intragenomic heterogeneity in the 16S rRNA gene in *Aeromonas* strains. J Bacteriol 187:6561–6564. doi:10.1128/JB.187.18.6561-6564.2005.

Munang'andu, H. M., Paul, J., & Evensen, Ø. (2016). An Overview of Vaccination Strategies and Antigen Delivery Systems for *Streptococcus agalactiae* Vaccines in Nile Tilapia (*Oreochromis niloticus*). *Vaccines*, *4*(4), 48.

Murray, A. G. & Peeler, E. J. 2005. A framework for understanding the potential for emerging diseases in aquaculture. *Preventive Veterinary Medicine* 67, 223– 235.

Musa, N., Wei, L.S., Shaharom, F. & Wee, W. (2008). Surveillance of bacteria species in diseased freshwater ornamental fish from aquarium shop. World Appl Sci J 3:903–905.

Musa, N., Wei, L.S., Musa, N., Hamdan, R.H., Leong, L.K., Wee, W., Amal, M.N., Kutty, B.M. & Abdullah, S.Z. (2009). Streptococcosis in red hybrid tilapia (*Oreochromis niloticus)* commercial farms in Malaysia. *Aquac. Res.* 40(5):630–632.

Muyot, F.B., Caunan, P.J., Mutia, M.T.M. (2018). Comparative advantage of using extruded floating feed for tilapia (*Oreochromis niloticus*) and milkfish (*Chanos chanos*) cage culture in Taal Lake. Transactions of the National Academy of Science and Technology Philippines 4(1): 52.

Muzquiz, J., Royo, F., Ortega, C., De Blas, I., Ruiz, I. & Alonso, J. (1999). Pathogenicity of streptococcosis in rainbow trout (*Oncorhynchus mykiss*): dependence on age of diseased fish. Bull Eur Ass Fish Pathol. 19:114–119.

Mzula, A., Wambura, P. N., Mdegela, R. H., & Shirima, G. M. (2019). Phenotypic and molecular detection of Aeromonads infection in farmed Nile Tilapia in Southern highland and NorthernTanzania. *Heliyon*, 5, e02220.

Nakatsugawa, T. (1983). A streptococcal disease of cultured flounder. *Fish Pathology* 17: 281– 285.

Naude, T.W. (1975). The occurrence and diagnosis of certain trout diseases. *Fish Farmer*, *15*, 15-20.

Nawaz, M., Sung, K., Khan, S.A., Khan, A.A. and Steele, R. (2006). Biochemical and molecular characterization of tetracycline-resistant *Aeromonas veronii* isolates from catfish. *Appl Environ Microbiol* 72, 6461– 6466.

Nawaz, M., Khan, S.A., Khan, A.A., Sung, K., Tran, Q., Kerdahi, K. & Steele, R. (2010). Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiol 27, 327– 331.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H. & Troell, M. (2003). Effects of Aquaculture on World Fish Supplies. Issues in Ecology (8). 16 p.

Ndi O.L., & Barton M.D. (2011). Incidence of class 1 integron and other antibiotic resistance determinants in *Aeromonas* spp. from rainbow trout farms in Australia. J. Fish. Dis., 34 (8), pp. 589-599.

Neely, M. N., Pfeifer, J. D. & Caparon, M. (2002). Streptococcus-zebrafish model of bacterial pathogenesis. Infection and Immunity 70:3904–3914.

Netto L.N., Leal C.A.G. & Figueiredo H.C.P. (2011). *Streptococcus dysgalactiae* as an agent of septicaemia in Nile tilapia, *Oreochromis niloticus* (L.). *Journal of Fish Diseases* 34, 251– 254.

Newaj-Fyzul, A., Mutani, A., Ramsubhag, A. & Adesiyun, A. (2008). Prevalence of bacterial pathogens and their anti-microbial resistance in Tilapia and their pond water in Trinidad. Zoonoses Public Health 55:206–213. doi:10.1111/j.1863-2378.2007.01098.x

Nguyen, H.T., Kanai, K. & Yoshikoshi, K. (2002). Ecological investigation of *Streptococcus iniae* isolated in cultured Japanese flounder, *Paralicthys olivaceus* using selective isolation procedure. *Aquaculture* 205, 7– 17.

Nguyen, N.H. (2016). Genetic improvement for important farmed aquaculture species with a reference to carp, tilapia and prawns in Asia: achievements, lessons and challenges. *Fish and Fisheries* 17, 483– 506.

Nguyen, V.V., Dong, H.T., Senapin, S., Pirarat, N. & Rodkhum, C. (2016). *Francisella noatunensis* subsp. *orientalis*, an emerging bacterial pathogen affecting cultured red tilapia (*Oreochromis* sp.) in Thailand. Aquaculture Research 47: 3697–3702.

Nho, S.W., Hikima, J.-i., Park, S.B., Jang, H. B. & Cha, I.S. (2013). Comparative genomic characterization of three *Streptococcus parauberis* strains in fish pathogen, as assessed by wide-genome analyses. *PLoS One* 8, e80395.

Nielsen, M.E., Høi, L., Schmidt, A.S., Qian, D., Shimada, T., Shen, J.Y. & Larsen, J.L. (2001). Is *Aeromonas hydrophila* the dominant motile Aeromonas species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? Dis Aquat Org 46:23–29.

Noga, E. J. (2010). Fish Diseases (2nd edition) Willey-Blackwell, ISBN 978-0-8138-0697-6, Singapore.

Noraini, O., Sabri, M.Y. & Siti-Zahrah, A. (2013). Efficacy of spray administration of formalin-killed *Streptococcus agalactiae* in hybrid Red Tilapia J. Aquat. Anim. Health. 25: 142e8.

Nümann, W. (1972). The Bodensee: effects of exploitation and eutrophication on the salmonid community. J.Fish.Res.Board Can., 29 (6):833–47

Odeyemi, O.A., Asmat, A. & Usup, G. (2012). Antibiotics resistance and putative virulence factors of *Aeromonas hydrophila* isolated from estuary. J Microbiol Biotech Food Sci 1:1339–1357.

Ofori, J. K., Abban, E. K., Karikari, A. Y. & Brummett, R. E. (2010). Production Parameters and Economics of Small-Scale Tilapia Cage Aquaculture in the Volta Lake, Ghana, Journal of Applied Aquaculture, 22:4, 337-351.

Ohnishi, K. & Jo, Y. (1981). Studies of streptococcal infection in pond-cultured fishes. I. Characteristics of a β-hemolytic *Streptococcus* isolated from cultured ayu and amago in 1977–1978. *Fish Pathology* 16, 63– 67.

Oidtmann, B.C., Crane, C.N., Thrush, M.A., Hill, B.J. & Peeler, E.J. (2011). Ranking freshwater fish farms for the risk of pathogen introduction and spread. Preventive Veterinary Medicine, 102 (4), 329-340.

OIE. (2009). Methods for disinfection of aquaculture establishments. In: Manual of diagnostic tests for aquatic animals. Paris, France, pp 31–42.

OIE. (2017). Disease notification report 25278, 23/11/2017. https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=25278.

OIE. (2017). “Tilapia Lake Virus (TiLV), Philippines.” World Organisation for Animal Health (OIE). Immediate Notification. Accessed January 10, 2018.

Available online at: http:// www.oie.int/wahis_2/public/wahid.php/Reviewreport/ Review?page_refer=MapFullEventReport&reportid=25278

Opiyo, M.A., Orina, P. & Charo-Karisa, H. (2017). Fecundity, growth parameters and survival rate of three African catfish (*Clarias gariepinus*) strains under hatchery condition. J Aquacult Eng Fish Res 3:75–81. https://doi.org/10.3153/JAEFR17010.

Ormen O., Granum P.E., Lassen J. & Figueras, M.J. (2005). Lack of agreement between biochemical and genetic identification of *Aeromonas* spp. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica* 113, 203– 207.

Ortega, C., García, I., Irgang, R., Fajardo, R., Tapia-Cammas, D., Acosta, J., & Avendaño-Herrera, R. (2018). First identification and characterization of *Streptococcus iniae* obtained from tilapia (*Oreochromis aureus*) farmed in Mexico. *Journal of Fish Diseases*, 41, 773– 782.

Osman, K.M., Al-Maary, K.S., Mubarak, A.S., Dawoud, T.M., Moussa, I.M.I., Ibrahim, M.D.S. *et* al. (2017). Characterization and susceptibility of streptococci and enterococci isolated from Nile tilapia (*Oreochromis niloticus*) showing septicaemia in aquaculture and wild sites in Egypt. *BMC Veterinary Research* 13: 357.

Owatari, M.S., Fernandes, G., Jesus, A., Cardoso, L., Lehmann, N.B., Martins, M.L., Mouriño, J. L. P. (2020). Can histology and haematology explain inapparent *Streptococcus agalactiae* infections and asymptomatic mortalities on Nile tilapia farms? Research in Veterinary Science, Volume 129, pp. 13-20.

Ottaviani, D., Parlani, C., Citterio, B. et al. (2011). Putative virulence properties of *Aeromonas* strains isolated from food, environmental and clinical sources in Italy: a comparative study, Int. J. Food Microbiol. 144 (3) 538–545, https://doi.org/10.1016/j.ijfoodmicro.2010.11.020.

Pablos, M., Rodríguez-Calleja, J.M., Santos, J.A., Otero, A. & García-López, M.L. (2009). Occurrence of motile *Aeromonas* in municipal drinking water and distribution of genes encoding virulence factors, Int. J. Food Microbiol. 135 (2) 158–164, https://doi.org/10.1016/j.ijfoodmicro.2009.08.020.

Pádua S.B. & Cruz C. (2014) Health challenges in tilapia culture in Brazil. *AQUA Culture Asia Pacific* 10, 37– 39.

Painter, K.L., Hall, A., Ha, K.P. & Edwards, A. M. (2017). The electron Transport Chain Sensitizes *Staphylococcus aureus* and *Enterococcus faecalis* to Oxidative Burst. Infection and Immunity 85 (12) e00659-17.

Pakingking, R., Palma, P. & Usero, R. (2015). Quantitative and qualitative analyses of the bacterial microbiota of tilapia (*Oreochromis niloticus*) cultured in earthen ponds in the Philippines. World Journal of Microbiology & Biotechnology 31: 265– 275.

Pakingking, R., Palma, P., & Usero, R. (2020). *Aeromonas* load and species composition in tilapia (*Oreochromis niloticus*) cultured in earthen ponds in the Philippines. Aquac Res. 51:4736-4747. http://doi.org/10.1111/are.14820

Palanca-Tan, R. (2020). BENEFITS FROM LAGUNA LAKE: Perspective of Small Fisher Households. Journal of Management for Global Sustainability. Vol. 8 (2): 11-35.

Palang, I., Withyachumnarnkul, B., Senapin, S., Sirimanapong, W. & Vanichviriyakit, R. (2020). Brain histopathology in red tilapia *Oreochromis* sp. experimentally infected with *Streptococcus agalactiae* serotype III. Microsc Res Tech. 83:877–888. https://doi.org/10.1002/jemt.23481.

Palma, A.L. (2016). Laguna lake fisheries and fish sufficiency. In: Lake Ecosystem Assessment in the Philippines 2016. Los Baños, Philipp.

Palù Peres, A., Martins Gomes, L., Lemos Miguel, M.A., Teruzkin Balassiano, I., Penna Queiroz, M.L., Correâ Freitas-Almeida, A., et al. (2006). Antimicrobial resistance in food and clinical *Aeromonas* isolates. Food Microbiol. 23:504–9.

Papa, R.D.S., Zafaralla, M.T., & Eckmann, R. (2011). Spatio-temporal variation of the zooplankton community in a tropical caldera lake with intensive aquaculture (Lake Taal, Philippines). Hydrobiologia. 664, 119-133.

Papasergi, S., Brega, S., Mistou, M.Y., Firon, A., Oxaran, V., Dover, R., *et al*. (2011). The GBS PI-2a pilus is required for virulence in mice neonates. *PLoS One* 6: e18747.

Parker, J. L., & Shaw, J. G. (2011). *Aeromonas* spp. Clinical microbiology and disease. The Journal of Infection, 62(2), 109– 118. https://doi.org/10.1016/j.jinf.2010.12.003

Patil, H.J., Benet-Perelberg, A., Naor, A., Smirnov, M., Ofek, T., Nasser, A., et al. (2016). Evidence of increased antibiotic resistance in phylogenetically-diverse *Aeromonas* isolates from semi-intensive fishponds treated with antibiotics. Front Microbiol.; 7:1–12.

Peepim, T., Dong, H.T., Senapin, S., Khunrae, P., & Rattanarojpong, T. (2016). Epr3 is a conserved immunogenic protein among *Aeromonas* species and able to induce antibody response in Nile tilapia. Aquaculture, 464 pp. 399-409.

Pemberton, J. M., Kidd, S. P., & Schmidt, R. (1997). Secreted enzymes of Aeromonas. *FEMS Microbiology Letters*, 152(1), 1–10.

Penczak T., Galicka W., Molinski M., Kusto E. & Zalewski M. (1982). The enrichment of a mesotrophic lake by carbon, phosphorus and nitrogen from the cage aquaculture of rainbow trout, *Salmo gairdneri*. *Journal of Applied Ecology* 19, 371– 393.

Pereira, U.P., Mian, G.F., Oliveira, I.C., Benchetrit, L.C., Costa, G.M. & Figueiredo H.C. (2010). Genotyping of *Streptococcus agalactiae* strains isolated from fish, human and cattle and their virulence potential in Nile tilapia *Oreochromis niloticus* (L.). *Veterinary Microbiology* 140, 186– 192.

Perera, R.P., Johnson, S.K., Collins, M.D., Lewis, D.H. (1994). *Streptococcus iniae* associated with mortality of *Tilapia nilotica* × *T. aurea* hybrids. J. Aquat. Anim. Health, 6, pp. 335-340.

Perera, R. P., S. K. Johnson, & D. H. Lewis. (1997). Epizootiological aspects of *Streptococcus iniae* affecting tilapia in Texas. *Aquaculture* 152: 25– 33.

Perera, R. P., Fiske, R. A. & Johnson, S.K. (1998). Histopathology of hybrid tilapias infected with a biotype of *Streptococcus iniae*. *Journal of Aquatic Animal Health* 10: 294– 299.

Perez, T., Enriquez, E.E., Guerrero III, R.D., Simon, D. & Schiemer, F. (2008). Catchment characteristics, hydrology, limnology and socio-economic features of Lake Taal, Philippines. In Schiemer, F., D. Simon, U. S. Amarasinghe & J. Moreau (eds), Aquatic Ecosystems and Development:

Comparative Asian Perspectives. Biology of Inland Waters Series. Backhuys Publishers, Leiden: 63–80.

Pérez-Sancho, M., Cerdá, I., Fernández-Bravo, A., Domínguez, L., Figueras, M. J., Fernández-Garayzába, J. F., & Vela, A. I. (2018). Limited performance of MALDI-TOF for identification of fish Aeromonas isolates at species level. *Journal of Fish Diseases*, 2018(41), 1485– 1493.

Persson, S., Al-Shuweli, S., Yapici, S., Jensen, J. N., & Olsen, K. E. P. (2015). Identification of clinical aeromonas species by rpoB and gyrB sequencing and development of a multiplex PCR method for detection of *Aeromonas hydrophila, A. caviae, A. veronii,* and *A. media*. *Journal of Clinical Microbiology*, 53(2), 653– 656.

Petersen, A., Andersen, J.S., Kaewmak, T., Somsiri, T. & Dalsgaard, A. (2002.) Impact of integrated fish farming on antimicrobial resistance in a pond environment. Appl Environ Microbiol 68:6036–6042.

Petrell, R. J., Shi, X., Ward, R. K., Naiberg, A. & Savage, C. R. (1997). Determining fish size and swimming speed in cages and tanks using simple video techniques. Aquacultural Engineering 16, 45– 62.

Phan, L.T., Bui, T.M., Nguyen, T.T.T., Gooley, G.J., Ingram, B.A., Nguyen, H.V. *et al.* (2009). Current status of farming practices of striped catfish, *Pansianodon hypophthalmus*, in the Mekong Delta, Vietnam. *Aquaculture* 296: 227– 236.

Phillips, M.J., Muir, J.F., Beveridge, M. & Stewart, J. A. (1983). Cage farm management. Fish Farmer 6, 1416.

Phuektes, P., Browning, G.R., Anderson, G. & Mansell, P.D (2003). Multiplex polymerase chain reaction as a mastitis screening test for assay for *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. J Dairy Res. 70:149-155.

Piamsomboon, P., Jaresitthikunchai, J., Hung, T.Q., Roytrakul, S. & Wongtavatchai, J. (2020). Identification of bacterial pathogens in cultured fish with a custom peptide database constructed by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF-MS). BMC Vet Res. 16(1):52. pmid:32046727.

Pietrocola, G., Schubert, A., Visai, L., Torti, M., Fitzgerald, J.R., Foster, T.J., *et al.* (2004). FbsA, a fibrinogen-binding protein from *Streptococcus agalactiae*, mediates platelet aggregation. *Blood* 105: 1052– 1059.

Piotrowska, M. & Popowska, M. (2014). The prevalence of antibiotic resistance genes among *Aeromonas* species in aquatic environments. Annals of Microbiology, 64, 921– 934. https://doi.org/10.1007/s13213-014-0911-2.

Piotrowska, M., & Popowska, M. (2015). Insight into the mobilome of *Aeromonas* strains. *Front. Microbiol.* 6:494. doi: 10.3389/fmicb.2015.00494

Piva, S., Pietra, M., Serraino, A., Merialdi, G., Magarotto, J. & Giacometti, F. (2019). First description of *Streptococcus lutetiensis* from a diseased cat. *Lett. Appl. Microbiol. 69*, 96–99.

Plou, J., Ferrer, M., Nuero, O.M., Calvo, M.V., Alcalde, M., Reyes, F. & Ballesteros, A. (1998). Analysis of Tween 80 as an esterase/lipase substrate for lipolytic activity assay Biotechnol Tech, 12, pp. 183-186

Plumb, J.A., Schachte, J.H., Gaines, J.L., Peltier, W. & Carroll, B. (1974). *Streptococcus* sp. from marine fishes along the Alabama and Northwest Florida coast of the Gulf of Mexico. *Transactions of the American Fisheries Society* 103, 358– 361.

Plumb, J.A. (1993). *Edwardsiella* septicaemia. In: *Bacterial Diseases of Fish* (ed. by V. Inglis, R.J. Roberts & N.R. Bromage), pp. 61– 79. Blackwell Scientific Publications, Oxford.

Podbielski, A., Zarges, I., Flosdorff, A., & Weber-Heynemann, J. (1996). Molecular characterization of a major serotype M49 group A streptococcal DNase gene (sdaD). *Infection and Immunity*, 64(12), 5349–5356.

Poobalane, S. et al (2010). Production and efficacy of an *Aeromonas hydrophila* recombinant S-layer protein vaccine for fish. Vaccine 28:3540–3547.

Popma, T.J. & Lovshin, L. J. (1996). Worldwide Prospects for Commercial Production of Tilapia. Research and Development Series No. 41. International Centre for Aquaculture and Aquatic Environments. Department of Fisheries and Allied Aquacultures Auburn University, Alabama 36849.

Popma, T., & Masser, M. (1999). Tilapia: Life history and biology. Southern Regional Aquaculture Center,SRAC publication No. 283, Stoneville, Ms, USA.

Popovic, N.T., Teskeredzic, E., Strunjak-Perovic, I. & Coz-Rakovac, R. (2000). *Aeromonas hydrophila* Isolated from wild freshwater fish in Croatia. Vet Res Commun 24:371–377.

Praveen, P.K., Debnath, C., Shekhar, S., Dalai, N. & Ganguly, S. (2016). Incidence of *Aeromonas* spp. infection in fish and chicken meat and its related public health hazards: a review, Vet. World 9 (1) 6–11, https://doi.org/10.14202/vetworld.2016.

Pridgeon, J.W. & Klesius, P.H. (2011a). Virulence of *Aeromonas hydrophila* to channel catfish *Ictaluras punctatus* fingerlings in the presence and absence of bacterial extracellular products. Dis Aquat Org 95:209–215.

Pridgeon, J.W. & Klesius, P.H. (2011b). Development and efficacy of novobiocin and rifampicin-resistant *Aeromonas hydrophila* as novel vaccines in channel catfish and Nile tilapia. Vaccine 29:7896–7904.

Pridgeon, J. W., & Klesius, P. H. (2012). Major bacterial diseases in aquaculture and their vaccine development. *CAB Review* 7: 048.

Pridgeon, J. W., & Klesius, P. H. (2013). Major bacterial diseases in aquaculture and their vaccine development. *Anim. Sci. Rev.* 2012:141.

Pridgeon, J.W. & Klesius, P.H. (2013) Development of live attenuated *Streptococcus agalactiae* as potential vaccines by selecting for resistance to sparfloxacin. *Vaccine*, 31, 2705– 2712.

Pritzlaff, C.A., Chang, J.C., Kuo, S. P., Tamura, G.S., Rubens, C.E. & Nizet, V. (2001). Genetic basis for the beta-haemolytic/cytolytic activity of group B Streptococcus. Mol Microbiol. 39 (2): 236-247.

Proctor, R.A., von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M. & Peters, G. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infection. Nature Reviews/Microbiology. Vol 4, 295-305.

Prabu, E., Rajagopalsamy, C., Ahilan, B., Jeevagan, I. J. M. A., & Renuhadevi, M. (2019). Tilapia–An excellent candidate species for world aquaculture: A review. *Annual Research & Review in Biology*, 31(3), 1– 14.

Pradeep, P.J., Suebsing, R., Sirithammajak, S., Kampeera, J., Turner, W., Jeffs, A., Kiatpathomchai, W. & Withyachumanarnkul, B. (2017). Vertical transmission and concurrent infection of multiple bacterial pathogens in naturally infected red tilapia (*Oreochromis* spp.) Aquac. Res., 48, 2706-2717.

Pulido, E., Iregui, C., Figueroa, J. & Klesius, P. (2004). Estreptococosis en tilapias (*Oreochromis* spp.) cultivadas en Colombia. Rev. AquaT, 20 (2004), pp. 97-106.

Puthucheary, S.D., Puah, S.M. & Chua, K.H. (2012). Molecular characterization of clinical isolates of *Aeromonas* species from Malaysia. PLoS ONE 7: e30205

Rabaan, A., & Shaw., J. G. (2002). Lateral flagella and swarming motility in *Aeromonas* species. *Journal of Bacteriology*, 184, 547–555.

Rahmatullah, M., Ariff, M., Kahieshesfandiari, M., Daud, H. M., Zamri-Saad, M., Sabri, M. Y., … Ina-Salwany, M. Y. (2017). Isolation and pathogenicity of *Streptococcus iniae* in cultured red hybrid tilapia in Malaysia. *Journal of Aquatic Animal Health*, 29(4), 208– 213.

Raja, R.A. & Jithendran K.P. (2015). *Aquaculture Diseases Diagnosis and Health Management*, pp. 247– 255. Advances in Marine and Brackish Aquaculture, Springer India.

Rajagopal, L., Vo, A., Silvestroni, A. & Rubens, C.E. (2006). Regulation of cytotoxin expression by converging eukaryotic-type and two-component signalling mechanisms in *Streptococcus agalactiae*. *Mol Microbiol* 62: 941–957.

Ran, C., Qin, C., Xie, M., Zhang, J., Li, J., Xie, Y., ... Zhou, Z. (2018). *Aeromonas veronii* and aerolysin are important for the pathogenesis of motile aeromonad septicaemia in cyprinid fish. Environ Microbiol 20: 3442– 3456.

Rangrez, A.Y., Dayananda, K.M., Atanur, S., Joshi, R., Patole, M.S. & Shouche, Y.S. (2006). Detection of conjugation related type four secretion machinery in *Aeromonas culicicola*. PLoS One 1, e115.

Rasheed, V. & Plumb, J. (1984). Pathogenicity of a non- haemolytic group B *Streptococcus* sp. in Gulf killifish, *Fundulus grandis* Baird & Girard. *Aquaculture* 37, 97– 105.

Rasmussen-Ivey, C.R., Figueras, M.J., McGarey, D., & Liles, M.R. (2016). Virulence factors of *Aeromonas hydrophila*: in the wake of reclassification. Front Microbiol 7, 1– 10.

Ravelo, C., Magariños, B., Romalde, J.L. & Toranzo, A.E. (2001). Conventional versus miniaturized systems for phenotypic characterization of *Lactococcus garvieae* strains. *Bulletin of the European Association of Fish Pathologists* 21, 136– 144.

Ray, A.K., Ghosh, K. & Ringø, E. (2012). Enzyme-producing bacteria isolated from fish gut: a review. *Aquacult. Nutr.*, **18**, 465– 492.

Redner, B. D., & Stickney, R. R. (1979). Acclimation to ammonia by Tilapia aurea. Trans. Am. Fish. Soc. 108: 383–388.

Regecova, I., Pipova, M., Jevinova, P., Maruskova, K., Kmet, V., & Popelka, P. (2014). Species identification and antimicrobial resistance of coagulase-negative staphylococci isolated from the meat of sea fish. *Journal of Food Science*, *79*, M898–M902. https://doi.org/10.1111/17503841.12429

Reigh, R. C., Robinson, E. H. & Brown, P. B. (1991). Effects of dietary magnesium on growth and tissue magnesium content of blue tilapia (*Oreochromis aureus*). J. World Aquacult. Soc. 22:192–200.

Reimschuessel, R. & Ferguson, H.W. (2006). Kidney. In: Systemic pathology of fish – A text and atlas of normal tissues in teleosts and their response in disease (Ed. Ferguson H. W.): 91-118. Scotland Press.

Reputola A. (2018). Retrofitting fishponds amid extreme climate change. Agriculture Monthly 22(8): 16–17.

Rhodes, G., Huys, G., Swings, J., McGann, P., Hyney, M., Smith, P. et al. (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant *TetA.* Appl Environ Microbiol. 66, 3883-3890.

Rivero, O., Anguita, J., Mateos, D., Paniagua, C., & Naharro, G. (1991). Cloning and characterization of an extracellular temperature-labile serine protease gene from *Aeromonas hydrophila*. *FEMS Microbiology Letters*, 65, 1–7.

Roberts, R.J. & Sommerville, C. (1982). Diseases of tilapias. R.S.V. Pullin, R.H. Lowe-McConnell (Eds.), The biology and Culture of tilapias, ICLARM, Manila, Phillippines, pp. 247-263.

Robinson, J.A. & Meyer, F.P. (1966) Streptococcal fish pathogen. *Journal of Bacteriology* 92, 512.

Rodkhum, C., Kayansamruaj, P. & Pirarat, N. (2011). Effect of water temperature on susceptibility to *Streptococcus agalactiae* serotype Ia infection in Nile tilapia (*Oreochromis niloticus*). Thai J. Vet. Med. 41, 309–314.

Rogers, C. & Basurco, B. (2009). Antimicrobial agents in aquaculture: Practice, needs and issues. CIHEAM. vol. 59, pp. 41–59.

Roges, E. M., Gonçalves, V. D., Cardoso, M. D., Festivo, M. L., Siciliano, S., Berto, L. H., Pereira, V., Rodrigues, D., & de Aquino, M. (2020). Virulence-Associated Genes and Antimicrobial Resistance of *Aeromonas hydrophila* Isolates from Animal, Food, and Human Sources in Brazil. *BioMed research international*, *2020*, 1052607. https://doi.org/10.1155/2020/1052607

Rojas, A., Wadsworth, S. (2007). A review of cage aquaculture: Latin America and the Caribbean. In: H Matthias, S Doris, JR Arthur (eds) Cage Aquaculture. Regional Reviews and Global Overview, pp. 73– 100. FAO, Rome.

Romana-Eguia, M.R.R. & Eguia, R.V. (1999). Growth of five Asian red tilapia strains in saline environments. Aquacult 173:161–170.

Romana-Eguia, M.R.R., Ikeda, M., Basiao, Z.U. & Taniguchi, N. (2005). Genetic changes during mass selection for growth in Nile tilapia assessed by microsatellites. Aquaculture Research 36: 69-78.

Romana-Eguia, M.R.R., Eguia, R.V. & Pakingking Jr., R.V. (2020). Tilapia Culture The Basics. Aquaculture Extension Manual No. 66. SEAFDEC-AQD. ISSN 0115-5369.

Romero, J., Feijoo, C.G., & Navarrete, P. (2012). Antibiotics in aquaculture – use, abuse and alternatives, health and environment in aquaculture. E.D. Carvalho, R.J. Silva, G.S. David (Eds.), Health and environment in aquaculture, InTech (2012), pp. 159-198.

Roode, M.C. (1977). *Streptococcus* sp. infection in rainbow trout. *Fish Farmer*, *18*, 6-8.

Romero, B., Morosini, M.I., Loza, E., Rodríguez-Baños, M., Navas, E., Cantón, R. & Campo, R.D. (2011). Reidentification of *Streptoccoccus bovis* isolates causing bacteremia according to the new taxonomy criteria: still an issue? *J Clin Microbiol* 49, 3228– 3233.

Roriz, G.D., Delphino, M.K.D.V.C., Gardner, I.A., Gonçalves, V.S.P. (2017). Characterization of Tilapia farming in net cages at a tropical reservoir in Brazil. Aqua. Rep., 6: 43-48.

Ross, L. G. (2000). Environmental physiology and energetics. pp. 89–128. In: M. C. M. Beveridge and B. J. McAndrew (eds.) Tilapias: Biology and Exploitation, Fish and Fisheries Series 25, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Rossjohn, J., Feil, S. C., McKinstry, W. J., Tsernoglou, D., van der Goot, G., Buckley, J. T., & Parker M.W. (1988). Aerolysin–a paradigm for membrane insertion of beta-sheet protein toxins? J Struct Biol 121: 92– 100.

Russo, R., Mitchell, H., & Yanong, R. P. E. (2006). Characterization of *Streptococcus iniae* isolated from ornamental cyprinid fishes and development of challenge models. *Aquaculture*, 256, 105– 110.

Rychert, J., Creely, D., Mayo-Smith, L. M., Calderwood, S. B., Ivers, L. C., Ryan, E. T., . . . Harris, J. B. (2015). Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of *Vibrio cholerae*. *Journal of Clinical Microbiology*, *53*, 329–331. https://doi.org/10.1128/JCM.02666-14

Saavedra, M.J., Guedes-Novais S., Alves, A., Rema, P., Tacão, M., Correia, A. & Martínez-Murcia, A.J. (2004). Resistance to β-lactam antibiotics in *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*). *International Microbiology* 7, 201– 211.

Saavedra, M.J., Figueras, M.J. & Martínez-Murcia, A.J. (2006). Updated phylogeny of the genus *Aeromonas*. Int J Syst Evol Microbiol 56:2481-2487.

Saikot F.K., Zaman, R. & Khalequzzaman, M. (2013). Pathogenecity test of *Aeromonas* isolated from motile Aeromonas septicemia (MAS) infected Nile tilapia on some freshwater fish. Sci Int 1:325–329.

Sakai, D. (1985). Significance of extracellular protease for growth of a heterotrophic bacterium, *Aeromonas salmonicida*. *Applied and Environmental Microbiology*, 50, 1031–1037.

Sakai, M., Atsuta, S. A. & Kobayashi, M. (1986). A streptococcal disease of cultured Jacopever, *Sebastes schlegeli. Suisanzoshuku (Aquiculture)* 34, 171–177.

Salayo, N.D., Perez, M.L., Garces, L.R. & Pido, M.D. (2012). Mariculture development and livelihood diversification in the Philippines. Marine Policy 36(4): 869–888.

Samal, S.K., Das, B.K. & Pal, B.B. (2014). Isolation, biochemical characterization, antibiotic susceptibility study of *Aeromonas hydrophila* isolated from freshwater fish. Int J Curr Microbiol App Sci 3:259–267.

Santos, J.A., González, C.J., Otero, A. and García-López, M.L. (1999). Haemolytic activity and siderophore production in different *Aeromonas* species isolated from fish. *Applied and Environmental Microbiology* 65, 5612– 5614.

Santos, P.G., Santos, P.A., Bello, A.R. & Freitas-Almeida, A.C. (2010). Association of *Aeromonas caviae* polar and lateral flagella with biofilm formation. *Letters in Applied Microbiology* 52, 49– 55.

Sarig, S. (1969). Winter storage of tilapia. FAO Fish Culture Bulletin. 2: 8–9.

Sawant, A.A., Pillai, S.R. & Jayarao, B.M. (2002). Evaluation of five selective media for isolation of catalase negative gram-positive cocci from raw milk. J. Dairy Sci., 85, pp. 1127-1132.

Salvador, R., Muller, E.E., Freitas, J.C., Leonhadt, J.H., Giordano, L.G.P., & Dias, J.A. (2005). Isolation and characterization of *Streptococcus* spp. group B in Nile Tilapia (*Oreochromis niloticus*) reared in hapas nets and earth nurseries in the northen region of Parana State, Brazil. Ciencia Rural. *Santa Maria*, *35*, 1374-1378.

Scarano, C., Spanu, C., Ziino, G., Pedonese, F., Dalmasso, A., Spanu, V., et al. (2014). Antibiotic resistance of *Vibrio* species isolated from *Sparus aurata* reared in Italian mariculture. *New Microbiol.* 37, 329–337.

Schelkle, B., Shinn, A.P., Peeler, E., Cable, J. . (2009). Treatment of gyrodactylid infections in fish. Dis. Aquat. Organ., 86: 65-75.

Schelkle, B., Doetjes, R., Cable, J. (2011). The salt myth revealed: treatment of gyrodactylid infections on ornamental guppies, *Poecilia reticulate* Aquaculture. 311: 74-79.

Schubert, A., Zakikhany, K., Schreiner, M., Frank, R., Spellerberg, B., Eikmanns, B.J., & Reinscheid, D.J. (2002). A fibrinogen receptor from group B Streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. *Mol Microbiol* 46: 557– 569.

Scoaris, D. O., Colacite, J., Nakamura, C. V., Ueda-Nakamura, T., Filho, B. A. A., & Filho, B. P. D. (2008). Virulence and antibiotic susceptibility of *Aeromonas* spp. isolated from drinking water. *Antonie van Leeuwenhoek*, 93, 111–122.

Segner, H., Sundh, H., Buchmann, K., et al. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiology and Biochemistry* 38, 85– 105.

Seiler, H., Schmidt, V., Wenning, M. & Scherer S. (2012). *Bacillus kochii* sp. nov., isolated from foods and a pharmaceutical manufacturing site. . Int J Syst Evol Microbiol 62, 1092–1098.

Sen, K., & Rodgers M. (2004). Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: A PCR identification. Journal of Applied Microbiology, 97(5), 1077– 1086. https://doi.org/10.1111/j.1365-2672.2004.02398.x

Senderovich Y., Ken-Dror S., Vainblat I., Blau D., Izhaki I., & Halpern M. (2012). A molecular study on the prevalence and virulence potential of *Aeromonas* spp. recovered from patients suffering from diarrhea in Israel. PloS One. 7: e30070. pmid: 22355306

Serrano, P.H. (2005). *Responsible Use of Antibiotics in Aquaculture*, Vol.469. Rome: Food and Agriculture Organization.

Seward, R. J., Ehrenstein, B., Grundmann, H. J., & Towner, K. J. (1997). Direct comparison of two commercially available computer programs for analysing DNA fingerprinting gels. *Journal of Medical Microbiology*, 46, 314– 320. https://doi.org/10.1099/00222615-46-4-314.

Sha, J., Kozlova, E.V. & Chopra, A.K. (2002). Role of various enterotoxins in Aeromonas hydrophila-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. Infect Immun 70, 1924–1935.

Sha, J., Pillai, L., Fadl, A. A., Galindo, C. L., Erova, T. E., & Chopra., A. K. (2005). The type III secretion system and cytotoxic enterotoxin alter the virulence of *Aeromonas hydrophila*. *Infection and Immunity*, 73(10), 6446–6457.

Sha J., Erova T.E., Alyea R.A., Wang S., Olano J.P., Pancholi V. & Chopra A.K. (2009). Surface-expressed enolase contributes to the pathogenesis of clinical isolate SSU of *Aeromonas hydrophila*. Journal of Bacteriology, 191:3095–3107.

Shah, S.Q.A., Karatas, S., Nilsen, H., Steinum, T.M., Colquhoun, D.J. & Sørum, H. (2012). Characterization and expression of the *gyrA* gene from quinolone resistant *Yersinia ruckeri* strains isolated from Atlantic salmon (*Salmo salar L*.) in Norway. Aquaculture 350–353:37–41.

Shahi, N., S. K. Mallik, M. Sahoo, & Das, P. (2013). Characteristics and pathogenicity of a virulent *Aeromonas hydrophila* associated with ulcerative syndrome in farmed Rainbow Trout, *Oncorhynchus mykiss* (Walbaum). *Israeli Journal of Aquaculture* 65: 926– 936.

Shameena, S. S., Kumar, K., Kumar, S., Kumar, S., & Rathore, G. (2020). Virulence characteristics of *Aeromonas veronii* biovars isolated from infected freshwater goldfish (*Carassius auratus*). Aquaculture. 518, 734819.

Sheyin, A.N. & Solomon, K.J. (2017). EndoMicrobial Fauna of Tilapia spp. (*Oreochromis niloticus*) found in a flowing canal at Eden Garden and Park Utako, Abuja. Journal of Fisheries and Livestock Production 5(1):1-8.

Shelton, W.L. (2002). Tilapia culture in the 21st century. In: Proceedings of the International Forum on Tilapia Farming in the 21st Century (Tilapia Forum 2002), (ed. by R.D.I. Guerrero & M.R. Guerrero-Del Castillo), pp. 1–20. Philippines Fisheries Association Inc., Los Banos, Laguna, Philippines.

Shin, H.B., Yoon, J., Lee, Y., Kim, M.S., & Lee, K. (2015). Comparison of MALDI-TOF MS, housekeeping gene sequencing, and 16S rRNA gene

sequencing for identification of *Aeromonas* clinical isolates. Yonsei Med J 56(2), 550– 555.

Shiomitsu, K., Kusuda, R., Osuga, H., & Munekiyo, M. (1980). Studies on chemotherapy of fish disease with erythromycin. *Fish Pathology*, *15*, 17-23.

Shlapobersky, M., Sinyakov, M.S., Katzenellenbogen, M., Sarid, R., Don, J. & Avtalion, R.R. (2010). Viral encephalitis of tilapia larvae: primary characterization of a novel herpes like virus. Virology 399: 239–247.

Shoemaker, C. & Klesius, P. (1997). Streptococcal Disease Problems and Control: A Review, International Symposium on Talipia in Aquaculture. [Cited 20 Dec 2017.

Shoemaker, C.A., Klesius, P.H., & Evans, J.J. (2001). Prevalence of *S. iniae* in tilapia, hybrid striped bass and channel catfish on commercial fish farm in United States. *American Journal of Veterinary Research*, *62*, 174-177.

Silva, B. C., J. L. P. Mouriño, F. N. Vieira, A. Jatobá, W. Q. Seiffert, and M. L. Martins. (2012). Haemorrhagic septicaemia in the hybrid surubim (*Pseudoplatystoma corruscans* × *Pseudoplatystoma fasciatum*) caused by *Aeromonas hydrophila*. *Aquaculture Research* 43: 908– 916.

Silver, A.C., Kikuchi, Y., Fadl, A.A., Sha, J., Chopra, A.K., & Graf, J. (2007). Interaction between innate immune cells and a bacterial type III secretion system in mutualistic and pathogenic associations. Proceedings of the National Academy of Sciences of the United States of America, 104, 9481–9486

Singh, D. V, & Sanyal, S. C. (1992). Enterotoxicity of clinical and environmental isolates of *Aeromonas* spp. *Journal of Medical Microbiology*, 36(4), 269–272.

Sisti, M., Albano, A. & Brandi, G. (1998). Bactericidal effect of chlorine on motile *Aeromonas* spp. in drinking water supplies and influence of temperature on disinfection efficacy. Lett Appl Microbiol 26:347–351.

Skwor, T., Shinko, J., Augustyniak, A., Gee, C., & Andraso, G. (2014) *Aeromonas hydrophila* and *Aeromonas veronii* predominate among potentially pathogenic ciprofloxacin-and tetracycline- resistant *Aeromonas* isolates from lake Erie. Appl Environ Microb 80: 841– 848.

Smith, H.L. & Goodner. K. (1958). Detection of bacterial gelatinases by gelatin-agar plate method. J. Bacteriol., 76, pp. 662-665

Smith, H. (1995). Virulence mechanisms of bacterial pathogens, In J. A. Roth, C. A. Bolin, K. A. Brogden, F. C. Minion, and M. J. Wannemiehler (eds.), The state and future of studies on bacterial pathogenicity, ASM Press, Washington, D. C. (pp. 335–357).

Smyrli, M., Prapas, A., Rigos, G., Kokkari, C., Pavlidis, M. & Katharios, P. (2017). *Aeromonas veronii* infection associated with high morbidity and mortality in farmed European seabass *Dicentrarchus labrax* in the Aegean Sea, Greece Fish Pathol., 52 (2), pp. 68-81.

Snieszko, S. F. (1973). Recent advances in scientific knowledge and developments pertaining to diseases of fishes. Advances in Veterinary Science and Comparative Medicine, 17: 291–314.

Soares, S., Murray, A.G., Crumlish, M., Turnbull, J.F. & Green, D.M. (2012). Evaluating abnormal mortality as an indicator of disease presence in the Atlantic salmon industry using the receiver operating characteristic (ROC). Aquaculture. 370:136-143.

Soler, L., Yáñez, M. A., Chacon, M. R., Aguilera-Arreola, M. G., Catalán, V., Figueras, M. J., & Martínez-Murcia, A. J. (2004). Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes. *International Journal of Systematic and Evolutionary Microbiology*, 54, 1511–1519.

Soliman, M. K., Ellakany, H. F., Gaafar, A. Y., Elbialy, A. K., Zaki, M. S., & Younes, A. M. (2014). Epidemiology and antimicrobial activity of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from Nile Tilapia during an outbreak in Egypt. Life Science Journal, 11, 1245– 1252.

Sørum, H. (2008) Antibiotic resistance associated with veterinary drug use in fish farms. In: Lie Ø (ed) Improving Farmed Fish Quality and Safety. WoodHead Publishing Limited, Cambridge, pp 157–182.

Soto, E., Hawke, J.P., Fernandez, D. & Morales, J.A. (2009). *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica. *Journal of Fish Diseases* 32, 713– 722.

Soto, E., Griffin, M., Arauz, M., Riofrio, A., Martinez, A. & Cabrejos, M.E. (2012). *Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile tilapia. Journal of Aquatic Animal Health 24:81–90.

Soto-Rodriguez S.A., Cabanillas-Ramos J., Alcaraz U., Gomez-Gil B. & Romalde J.L. (2013). Identification and virulence of *Aeromonas dhakensis*, *Pseudomonas mosselii* and *Microbacterium paraoxydans* isolated from Nile tilapia, *Oreochromis niloticus*, cultivated in Mexico. *Journal of Applied Microbiology* 115, 654– 662.

Spratt, B.G. (2012). The 2011 Garrod Lecture: From penicillin-binding proteins to molecular epidemiology. J Antimicrob Chemother. 67: 1578-1588.

Sreedharan, K., Philip, R. & Singh, I.S. (2013). Characterization and virulence potential of phenotypically diverse *Aeromonas veronii* isolates recovered from moribund freshwater ornamental fishes of Kerala, India. Antonie Leeuwenhoek 103, 53–67.

Steckert, L. D., Cardos, L., Jerônimo, G. T., Padua, S. B., & Martins, M. L. (2018). Investigation of farmed Nile tilapia health through histopathology. *Aquaculture*, 486, 161– 169. https://doi.org/10.1016/j.aquaculture.2017.12.021

Stratev, D. & Vashin, I. (2014). *Aeromonas hydrophila* sensitivity to disinfectants. J FisheriesSciences.com 8:324–330.

Stratev, D., Daskalov, H., Vashin, I. (2015). Characterisation and determination of antimicrobial resistance of β-haemolytic *Aeromonas* spp. isolated from common carp (*Cyprinus carpio* L.). Rev Méd Vét. (166):54–61.

Stratev, D. & Odeyemi, O.A. (2016). Antimicrobial resistance of *Aeromonas hydrophila* isolated from different food sources: a mini-review. J Infect Public Health 9:535–544.

Stratev, D., & Odeyemi, O.A. (2017). An overview of motile Aeromonas septicaemia management. Aquaculture International 25: 1095– 1105.

Strmac, M. & Braunbeck, T. (2002). Cytological and biochemical effects of a mixture of 20 pollutants on isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Ecotoxicol. Environ. Saf. 53, 293–304.

Stuiver, M., Leeuwis, C., van der Ploeg, J.D. (2004). The power of experience: farmers' knowledge and sustainable innovations in agriculture. In:

Wiskerske JSC, Van der Ploeg JD (eds) Seeds of transition: essays on novelty production, niches, and regimes in agriculture. Royal van Gorcum, Assen, p 93–117.

Suanyuk, N., Kong, F., Ko, D., Gilbert, G.L. & Supamattaya, K. (2008). Occurrence of rare genotypes of *Streptococcus agalactiae* in cultured red tilapia *Oreochromis* sp. and Nile tilapia *O. niloticus* in Thailand-relationship to human isolates? Aquaculture 284, 35–40.

Suarez, G., Sierra, J. C., & Sha, A. J. (2008). Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila. Microbial Pathogenesis*, 44, 344–361.

Subashkumar, R., Thayumanavan, T., Vivekanandhan, G., & Lakshmanaperumalsamy, P. (2006). Occurrence of *Aeromonas hydrophila* in acute gasteroenteritis among children. *Indian Journal of Medical Research*, 123, 61–66.

Subasinghe, R.P., Bondad-Reantaso, M.G., & McGladdery, S.E. (2001). Aquaculture development, health and wealth. In *Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium.* R.P. Subasinghe, P. Bueno, M.J. Phillips, C. Hough, S.E. McGladdery, and J.R. Arthur (eds). Bangkok: NACA, pp. 167– 191.

Subasinghe, R. (2009). Disease control in aquaculture and the responsible use of veterinary drugs and vaccines: the issues, prospects and challenges. In: C Rodgers, B Basurco (eds) The Use of Veterinary Drugs and Vaccines in Mediterranean Aquaculture. *Options Méditerranéennes, Série A* 86: 5–103.

Subasinghe, R. (2017). Regional Review on Status and Trends in Aquaculture Development in Asia-Pacific-2015. FAO Fisheries and Aquaculture Circular (C1135/5), I.

Subramaniam, K., Gotesman, M., Smith, C.E., Steckler, N.K., Kelley, K.L., Groff, J.M. et al. (2016). Megalocytivirus infection in cultured Nile tilapia Oreochromis niloticus. Diseases of Aquatic Organisms 119: 253–258.

Suebsing, R., Kampeera, J., Tookdee, B., Withyachumnarnkul, B., Turner, W. and Kiatpathomchai, W. (2013). Evaluation of colorimetric loop-mediated isothermal amplification assay for visual detection of *Streptococcus*

agalactiae and *Streptococcus iniae* in tilapia. Lett Appl Microbiol 57, 317–324.

Suebsing, R., Pradeep, P. J., Jitrakorn, S., Sirithammajak, S., Kampeera, J., Turner, W. A., … Kiatpathomchai, W. (2016). Detection of natural infection of infectious spleen and kidney necrosis virus in farmed tilapia by hydroxynapthol blue-loop-mediated isothermal amplification assay. *Journal of Applied Microbiology*, 121(1), 55– 67. https://doi.org/10.1111/jam.13165

Sugiyama, A., & Kusuda, R. (1981). Studies on the characters of *Staphylococcus epidermidis* isolated from diseased fishes. *Fish Pathology*, *16*, 35-41.

Sun, D., Jeannot, K., Xiao, Y., & Knapp, C. W. (2019). Editorial: Horizontal gene transfer mediated bacterial antibiotic resistance. *Frontiers in Microbiology*, **10**, 01933.

Surachetpong, W., Janetanakit, T., Nonthabenjawan, N., Tattiyapong, P., Sirikanchana, K. & Amonsin, A. (2017). Outbreaks of tilapia lake virus infection, Thailand, 2015–2016. *Emerging Infectious Diseases* 23: 1031–1033

Surai, P.F. (2015). Silymarin as a natural antioxidant: an overview of the current evidence and perspectives. Antioxidants. 4: 204– 247. doi:10.3390/antiox4010204.

Suwannasang, A., Dangwetngam, M., Issaro, A., Phromkunthong, E. & Suanyuk, N. (2014). Pathological manifestations and immune responses of serotypes Ia and III *Streptococcus agalactiae* infections in Nile tilapia (*Oreochromis niloticus*). Songklanakarin Journal of Science and Technology 36: 499–500.

Swann, L. & White, M.R. (1991). Diagnosis and treatment of "*Aeromonas* hydrophila" infection of fish. Aquaculture Extension, Illinois-Indiana Sea Grant Program.

Takashima, F. & Hibiya, T. (1995). An Atlas of Fish Histology: Normal and Pathological Features, 2nd ed. Kodansha, Tokyo.

Talagrand-Reboul, E., Latif-Eugenín, F., Beaz-Hidalgo, R., Colston, S., Figueras, M. J., Graf, J., … Lamy, B. (2018). Genome-driven evaluation and redesign of PCR tools for improving the detection of virulence-associated genes in aeromonads. *PLoS One*, 13(8), 1– 17.

Tamayo-Zafaralla, M., Santos, R. A. V., Orozco, R. P., & Elegado, G. C. P. (2002). The ecological status of Lake Laguna de Bay, Philippines. Aquatic Ecosystem Health & Management, 5(2): 127–138.

Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W.I., Takasu, H., Wada, S., et al. (2010). Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* 45, 386–391. doi: 10.1021/ es102725n

Tattiyapong, P., Dachavichitlead, W. & Surachetpong W. (2017). Experimental infection of tilapia lake virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.). *Veterinary Microbiology* 207: 170– 177.

Tayamen, M.M., Reyes, R.A., Danting, M.J., Emdoza, A.M., Marquez, E.B., Salguet, A.C., Gonzales, R.C., Abella, T.A. & Vera-Cruz, E.M. (2002) Tilapia broodstock development for saline waters in the Philippines. Naga, The ICLARM Quarterly 25, 32– 36.

Tena, D., Aspiroz, C., Figueras, M., Gonzalez-Praetorius, A., Aldea, M., Alperi, A., & Bisquert, J. (2008). Surgical site infection due to *Aeromonas* species. Report of nine cases and literature review. *Scandinavian Journal of Infectious Diseases*, 31, 1–7.

Tendencia, E.A. (2003). Investigation of some components of the greenwater system which makes it effective in the initial control of luminous bacteria. *Aquaculture* 218, 115– 119.

Tendencia E.A., De la Peña, L.R., Fermin, A.C., Lio – Po, G., Choresca, C.H., Inui, Y. (2004). Antibacterial activity of tilapia *Tilapia hornorum* against *Vibrio harveyi*. Aquaculture 232: 145 – 152.

Tengjaroenkul, B., Smith, B. J., Caceci, T. & Smith, S. A. (2000). Distribution of intestinal enzymes activities along the intestinal tract of cultured Nile tilapia, Oreochromis niloticus L. Aquaculture 182(3): 317– 327.

Teunis, P. & Figueras, M.J. (2016). Reassessment of the enteropathogenicity of mesophilic *Aeromonas* species. Front. Microbiol. 7, 1395.

Thiyagarajan, P., Bhavani, A.L., Ebbie, M.G. & Chandra, G. (2014). A study on the control of *Aeromonas hydrophila* infection in the cat fish by medicinal plants. Scholars Acad J Biosci 2:144–150.

Thornton, J., Howard, S. P., & Buckley, J. T. (1988). Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin-cholesterol acyltransferase and other lipases. *Biochimica et Biophysica Acta*, 959, 153–159.

Thrusfield, M.V. (2006). Veterinary Epidemiology Blackwell Publishing, Oxford.

Toranzo, A.E., Magariños, B., & Romalde, J.L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture* 246: 37– 61.

Toledo, J. D., Acosta, B. O., Eguia, M. R. R., Eguia, R. V., & Israel, D. C. (2008). Sustainable tilapia farming: a challenge to rural development. Fish for the People, 6(1), 18-25.

Tomás, J. M. (2012). The main *Aeromonas* pathogenic factors. *ISRN Microbiology*, 2012, 1–22.

Torgersen, Y. & Hastein, T. (1995). Disinfection in aquaculture. Rev Sci Tech Off Int Epiz 14:419–434.

Tran-Duy, A., Schrama, J.W., van Dam, A.A. & Verreth, J.A.J. (2008). Effects of oxygen concentration and body weight on maximum feed intake, growth and hematological parameters of Nile tilapia, Oreochromis niloticus. Aquaculture 275, 152–162.

Tran-Duy, A., van Dam, A.A., Schrama, J.W. (2012). Feed intake, growth and metabolism of Nile tilapia (*Oreochromis niloticus*) in relation to dissolved oxygen concentration. Aquaculture Research 43(5): 730–744.

Tran, N.T.K., Dinh, N.T., Tin, N.H., Roem, A.J., Schrama, J.W., Verreth, J.A.J. (2016). Interaction between dissolved oxygen concentration and diet composition on growth, digestibility and intestinal health of Nile tilapia (*Oreochromis niloticus*). Aquaculture 462: 101–108.

Tran, K., Verdegem, M., Wolkenfelt, B., Chadag, M. and Verreth, J. (2019). The relation between farming practices and tilapia production in small-scale fish farms in Bangladesh. Penang, Malaysia: CGIAR Research Program on Fish Agri-Food Systems. Program Report: FISH-2019-06.

Teng, L.J., Hsueh, P.R., Tsai, J.C., Chen, P.W., Hsu, J.C., Lai, H.C., Lee, C.N. & Ho, S.W. (2002). *groESL* sequence determination, phylogenetic analysis, and species differentiation for viridans group streptococci. J Clin Microbiol. 40:3172–3178.

Tveteras, R., Nystoyl, R. & Jory, D.E. (2020). GOAL 2019: Global finfish production review and forecast. GAA (Global Aquaculture Alliance). 2020. https://www.aquaculturealliance.org/advocate/goal2019-global-finfish-production-reviewand-forecast/

Uddin, M.N., Kabir, K.H., Roy, D., Hasan, M.T., Sarker, M.A. and Dunn, E.S. (2020). Understanding the constraints and its related factors in tilapia (*Oreochromis* sp.) fish culture at farm level: A case from Bangladesh. *Aqua.* 530: 735927.

Uematsu, K., Baba, Y., Kake, Y., Ikenaga, T., Moon, S.-J., Miyai, Y., & Yoshida, M. (2007). Central mechanisms underlying fish swimming. Brain Behavior and Evolution, 69, 142–150.

Ugajin, M. (1981). Studies on *Streptococcus sp* as a causal agent of an epizootic among the cultured ayu (*Plecoglossus altivelis*) in Tochigi Prefecture, Japan, 1980. *Fish Pathology*, *16*, 119-127.

Valenti-Weigand, P., Benkel, P., Rohde, M, & Chhatwal, G.S. (1996). Entry and intracellular survival of group B streptococci in J774 macrophages. Infect Immun 64:2467–2473.

Valladão, G. M. R., Alves, L. O., & Pilarski, F. (2016). Trichodiniasis in Nile tilapia hatcheries: Diagnosis, parasite: host-stage relationship and treatment. Aquaculture, 451, 444– 450.

van der Mee-Marquet, N., Domelier, A.S., Salloum, M., Violette, J., Arnault, L., Gaillard, N., *et al*. (2009). Molecular characterization of temporally and geographically matched *Streptococcus agalactiae* strains isolated from food products and bloodstream infections. *Foodborne Pathog Dis* 6: 1177–1183.

Vandamme, L., Devriese, L.A., Kersters, P.K., & Melin, P. (1997). *S. difficile* is a non-hemolytic group B, type Ib *Streptococcus. International Journal of Systemic Bacteriology*, *47*, 81-85.

Vendrell, D., Balcazar, J. L., Ruiz-Zarzuela, I., De Blas, I., Girones, O. &J. L. Muzquiz, L. (2006). *Lactococcus garvieae* in fish: a review. *Comp. Immunol. Microbiol. Infect. Dis.* 29, 177– 198.

Ventura, M. T. & Grizzle, J. M. (1988). Lesions associated with natural and experimental infections of *Aeromonas hydrophila* in channel catfish, *Ictalurus punctatus* Rafinesque. Journal of Fish Diseases 11:397-407.

de Verdal, H., Benzie, J.A., Komen, H., Quillet, E., Vandeputte, M., Chatain, B. et al. (2018) Improving feed efficiency in fish using selective breeding: a review. Reviews in Aquaculture. 10(4): 833–851.

Vermelho, A.B., Meirelles, M.N.L., Lopes, A., Petinate, S.D.G., Chaia, A.A., et al. (1996). Detection of extracellular proteases from microorganisms on agar plates. Mem Inst Oswaldo Cruz 91: 755–760.

Verner-Jeffreys, D.W., Welch, T.J., Schwarz, T., Pond, M.J., Woodward, M.J., Haig, S.J., et al. (2009). High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. *PLoS ONE* 4:e8388. doi: 10.1371/journal.pone.0008388

Vipond, R., Bricknell, I. R., Durant, E., Bowden, T. J., Ellis, A. E., Smith, M., & MacIntyre, S. (1998). Defined deletion mutants demonstrate that the major secreted toxins are not essential for the virulence of *Aeromonas salmonicida*. *Infection and Immunity*, 66, 1990–1998.

Vista, A., Norris, P., Lupi, F. & Bernsten, R. (2006). Nutrient loading and efficiency of tilapia cage culture in Taal Lake, Philippines Philipp. Agric. Sci., 89 (1): 48-57.

Walker, P.J., Gudkovs, N., Mohan, C.V., Stalin Raj, V., Pradeep, B., Sergeant, E., Chandra Mohan, A.B., Ravibabu, G., Karunasagar, I. & Santiago, T.C. (2011). Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) farms in Andhra Pradesh, India. II. Multiple WSSV genotypes associated with disease outbreaks in ponds seeded with uninfected postlarvae. Aquaculture 319, 18– 24.

Wang, C.-y C., Shie, H.-S., Chen, S.-C., Huang, J.-P., Hsieh, I.-C., Wen, M.-S., … Wu, D. (2007). *Lactococcus garvieae* infections in humans: Possible association with aquaculture outbreaks. *International Journal of Clinical Practice*, 61(1), 68– 73.

Wang, N., Yang, Z., Zang, M., Liu, Y. & Lu, C. (2013). Identification of Omp38 by immunoproteomic analysis and evaluation as a potential vaccine antigen

against *Aeromonas hydrophila* in Chinese breams. Fish Shellfish Immunol 34:74–81.

Wang, T., Lefevre, S., Thanh Huong, D.T., Cong, N.V., Bayley, M., Richards, J.G. et al. (2009). Chapter 8 the effects of hypoxia on growth and digestion. In: Fish Physiology. pp. 361–396. Academic Press, London, UK.

Wang, X., Li, X., Zhao, C., Hu, P., Chen, H., Liu, Z., Liu, G. & Wang, Z. (2012) Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows. *Appl Environ Microbiol* 78, 2386– 2392.

Wang, Y.T., Huang, H.Y., Tsai, M.A., Wang, P.C., Jiang, B.H. & Chen, S.C. (2014) Phosphoglycerate kinase enhanced immunity of the whole cell of *Streptococcus agalactiae* in tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol* 41, 250– 259.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M., & H.Tanaka. (1993). Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *Journal of Biological Chemistry*, 268, 18567–18572.

Wen, X., Chen, F., Lin, Y., Zhu, H., Yuan, F., Kuang, D., Jia, Z. & Yuan, Z. (2020). Microbial indicators and their use for monitoring drinking water quality-A review. Sustain. 12, pp. 1-14.

Wendover, N. (2010). Managing tilapia health in complex culture systems. *Global aquaculture advocate*.

White, J.R., Belmont, M.A. &Metcalfe, C.D. (2006). Pharmaceutical Compounds in Wastewater: Wetland Treatment as a Potential Solution. The Scientific World JOURNAL, vol. 6, pp. 1731-1736.

White, P., Christensen, G.N., Palerud, R., Legovic, T., Rosario, W.R., Lopez, N., Regpala, R.R., Gecek, S. & Hernandez J. (2007). Environmental monitoring and modeling of aquaculture in risk areas of the Philippines (emma) final report - Taal Lake. 39pp.

White, P.G., Shipton, T.A., Bueno, P.B. and Hasan, M.R. (2018). Better management practices for feed production and management of Nile tilapia

and milkfish in the Philippines. FAO Fisheries and Aquaculture Technical Paper No. 614. Rome, FAO. 98 pp.

WHO. (2011). Guidelines for drinking-water quality (4th ed.). World Health Organization. Retrieved from http://www.who.int/guide lines/ en/

Williams, T. L., Andrzejewski, D., Lay, J. O., & Musser, S. M. (2003). Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. *Journal of the American Society for Mass Spectrometry*, *14*, 342–351.

von Wintersdorff, C. J. H., Penders, J., Van Niekerk, J. M., Mills, N. D., Majumder, S., Van Alphen, L. B. Savelkoul, P. H., & Wolffs, P. F. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology*, 7, 173.

Wong, C. Y., Heuzenroeder, M. W., & Flower, R. L. (1998). Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. *Microbiology*, 144, 291–298.

World Health Organization. (1999). Food safety issues associated with products from aquaculture. Report of a Joint FAO/NACA (WHO Study Group). WHO Technical Report Series 883. Geneva, Switzerland.

World SeaFood Market. (2005). A fish called tilapia. <http://www.thefishsite.com/articles/68/a-fish-called-tilapia>.

Wurts, W.A. & Masser, M.P. (2004). Liming Ponds for Aquaculture Southern Regional Aquacultural Centre Publication.

Xu, X.J., Ferguson, M.R., Popov, V.L., Houston, C.W., Peterson, J.W., & Chopra, A.K. (1998). Role of a cytotoxic enterotoxin in *Aeromonas*-mediated infections: development of transposon and isogenic mutants. Infection and Immunity 66, 3501– 3509.

Xu, D.H., Shoemaker, C.A., & Klesius, P.H. (2007). Evaluation of the link between gyrodactylosis and *Streptococcus* of Nile tilapia (*O. niloticus*). L. *Fish Diseases*, *30*, 230-238.

Yamamoto, S., Miyake, K., Koike, Y., Watanabe, M., Machida, Y., Ohta, M., & Iijima, S. (1999). Molecular characterization of type-specific capsular polysaccharide biosynthesis genes of *Streptococcus agalactiae* type Ia. *J Bacteriol* 181: 5176– 5184.

Yamamoto S., Kasai H., Arnold D.L., Jackson R.W., Vivian A., & Harayama S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology. 146: 2385–2394. pmid:11021915

Yambot, A.V. (1998). Isolation of *Aeromonas hydrophila* from *Oreochromis niloticus* during fish disease outbreaks in the Philippines. Asian Fish Sci 10:347–354.

Yang, B., Chen, C., Sun, Y., Cao, L., Zhang, D., Sun, W.,…Qian, A. (2021). Comparative genomic analysis of different virulent strains reveals reason for the increased virulence of *Aeromonas veronii*. J Fish Dis. 44:11–24. https://doi.org/10.1111/jfd.13262

Yáñez, M. A., Catalan, V., Apraiz, D., Figueras, M. J., & Martınez-Murcia, A. J. (2003). Phylogenetic analysis of members of the genus *Aeromonas* based on gyrB gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, 53, 875–883.

Yáñez, J.M., Joshi, R., Yoshida, G. M. (2020). Genomics to accelerate genetic improvement in tilapia. Anim. Genet., 5: 658-674, 10.1111/age.12989.

Yang, Y.I., Lin, C.K., Diana, J.S. (1996). Influence of Nile tilapia (*Oreochromis niloticus*) stocking density in cages on their growth and yield in cages and in ponds containing the cages. Aquaculture 146, Issues 3-4. p. 205-215.

Yanong, R.P.E., & Francis-Floyd, R. (2002). Streptococcal infections of fish. Report from University of Florida. Series from the Department of Fisheries and Aquatic Sciences, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

Yap, W. G. (2001). Culture of tilapia in saline water. In Fishlink 2001, 29-31 May 2001 (11 pp). Iloilo City, Philippines: University of the Philippines Aquaculture Society, Inc.

Yardimci, B. & Aydin, Y. (2011). Pathological findings of experimental *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*). J. Fac. Vet. Med. 58, 47–54.

Yosef, S. (2009). Rich food for poor people: Genetically improved tilapia in the Philippines. In: Millions Fed: Proven Successes in Agricultural

Development. The International Food Policy Research Institute Discussion Paper 925.

Young, J.R., Evans-Kocinski, S., Bush, R.D. & Windsor, P.A. (2015). Improving smallholder farmer biosecurity in the mekong region through change management. *Transbound. Emerg. Dis.* 62, 491– 504.

Yu, H. B., Srinivasa Rao, P. S., Lee, H. C., Vilches, S., Merino, S., Tomas, J. M & Leung, K. Y. (2004). A type III secretion system is required for *Aeromonas hydrophila* AH- 1 pathogenesis. *Infection and Immunity*, 72, 1248–1256.

Zamri-Saad, M., Amal, A.M.N. & Siti-Zahrah, A. (2010). Pathological changes in red tilapias (*Oreochromis* spp.) naturally infected by *Streptococcus agalactiae*. *Journal of Comparative Pathology* 143, 227– 229.

Zamri-Saad M., Amal M.N.A., Siti-Zahrah A. & Zulkafli A.R. (2014). Control and prevention of streptococcosis in cultured tilapia in Malaysia: a review. *Pertanika Journal of Tropical Agricultural Science* 37, 389– 410.

Zelikoff, J.T. (1998). Biomarkers of immunotoxicity in fish e other non-mammalian sentinel species predictive value for mammals. Toxicology 129, 63–71.

Zhang, Y. L., Arakawa, E., & Leung, K. (2002). Novel *Aeromonas hydrophila* PPD134/91 genes involved in O-antigen and capsule biosynthesis. *Infection and Immunity*, 70(5), 2326–2335.

Zhang, Y., Lau, L., Arakawa, E., & Leung, K. (2003). Detection and genetic analysis of group II capsules in *Aeromonas hydrophila*. *Microbiology*, 149, 1051–1060.

Zhang, Q.Y. & Gui, J.F. (2015). Virus genomes and virus-host interactions in aquaculture animals. Science China Life Sciences 58: 156–169.

Zhang, D. X., Kang, Y. H., Song, M. F., Shu, H. P., Guo, S. N., Jia, J. P., Tao, L. T., Zhao, Z. L., Wang, C. F., Wang, G. Q., Qian, A. D., & Shan, X. F. (2019). Identity and virulence properties of *Aeromonas* isolates from healthy Northern snakehead (*Channa argus*) in China. Letters in Applied Microbiology, 69, 100–109.

Zhu, M., Wang, X.R., Li, J., Li, G.Y., Liu, Z.P. & Mo, Z.L. (2016). Identification and virulence properties of *Aeromonas veronii* bv. *sobria* isolates causing an

ulcerative syndrome of loach *Misgurnus anguillicaudatus*. *Journal of Fish Diseases* 39, 777– 781.

Zlotkin, A., Hershko, H. & Eldar, A. (1998). Possible transmission of *Streptococcus iniae* from wild fish to cultured fish. *Applied and Environmental Microbiology* 64, 4065– 4067.

Zlotkin, A., Chilmonczyk, S., Eyngor, M., Hurvitz, A., Ghittino, C. & Eldar A. (2003). Trojan horse effect: phagocyte-mediated *Streptococcus iniae* infection of fish. *Infection and Immunity* 71, 2318– 2325.

APPENDICES

Appendix 1: Survey Questionnaire

(insert code)

Questionnaire
for investigation of bacterial diseases affecting farmed tilapia in the Philippines

Instruction: Please answer the following questions (I) as accurately as possible and to answer factual questions to the best of your knowledge. Tick (✓) the box or supply the answer whichever is applicable. Your information will be kept strictly confidential.

I. Questions	II. Answer/Response	
A. Farm Background Information		
1A. Name of farm	1A ……	
2A. Name of owner	2A ……	
3A. Location of the farm	3A ……	
(address/province+GPS if possible)	……	
	……	
	……	
4A. Date of interview (dd/mm/yy)	4A ……	
5A. Species/Strain of Tilapia	5A ……	
	1. *O. niloticus* (Nile tilapia)	
	2. *O. mossambicus* (Mozambique tilapia)	
	3. *O. aureus* (Blue tilapia)	
	4. Hybrid (specify if possible)	
	_____	
	5. Other: _____	
	……..	
6A. Type of Farming System	6A.	
	1. Intensive (artifical feeding only)	
	2. Semi-Intensive (natural food + art. feeding)	
	3. Extensive (natural food only)	
7A. Size of production (annual in MT)	7A. Annual Production (MT): _____	
8A. Any other fish cultured in the area?	8A.	
	1. Yes	
	Specify: _____	
	----------	
	2. No	
9A. Start of farm operation (dd/mm/yy)	9A ……	
10A. How long has the farm operated?	10A. _____	
11A. Where do you sell your fish?	11A. _____	
12A. What farm records do you keep?	12A.	
	1. Production	

1

	2. Stocking Density
	3. Diseases/Mortalities
	4. Water Quality Parameters
	5. Others (specify): _____
B. Background of person interviewed	
1B. Responsibility	1B
	1. Owner
	2. Manager
	3. Worker
	4. Family member
2B. Person interviewed name	2B …….
3B. Age	3B ……years old
4B. Sex	4B
	1. Male
	2. Female
5B. Address	5B……
	……
	Tel. …….
6B. Have you attended any training or seminar course recently (time 1-2 years)?	6B
	1. Yes
	2. No
If yes, please give details including who provided the training DOF, company ect.	- ……
	- ……
	- ……
	- ……
C. Production Environment and System	
1C. What is the source of water for the farm?	1C
	1. Freshwater
	2. Brackishwater
	3. Marine
2C. What type of farming system?	2C
	1. Earthen ponds
	2. Fish pen
	3. Floating net cages
	4. Shallow fish cages
	5. Concrete tanks
3C. Production Scale	3C
	1. Extensive
	2. Semi-Extensive
	3. Intensive
4C. Proximity to Human Settlement (House/s)	4C
	1. Close (0-5 km)
	2. Mid (6-10 km)

	3. Far (11 or more km)	
5C. Farm Size	5C	
	1. 0-1 ha	
	2. 2-5 ha	
	3. 6 or more ha	
6C. Number of Production per Year	6C	
	1. One	
	2. Two	
	3. Three or more	
7C. Total number of cages/pens/ponds in farm.	7C	
	1. Cage: _____	
	2. Pen: _____	
	3. Pond: _____	
8C. Source of fry.	8C	
	1. Wild	
	2. Own hatchery	
	3. Neighbouring farm/hatchery	
	4. Distant hatchery in the Philippines	
9C. Stocking Density	9C	

10C. Stocking Size	10C	

11C. For brackishwater and marine farms, do you acclimatize the fish?	11C	
	1. Yes	
	2. No	
12C Method of acclimatization	12C	
	1. Abrupt (one salinity level)	
	_____ ppt	
	2. Gradual (series of salinities)	
	----------ppt	
D. Husbandry, feed and water management		
1D. Do you feed the fish?	1D	
	1. Yes	
	2. No	
2D. What kind of feed?	2D	
	1. Commercial Pellet	
	2. Commercial and Farm-made feed	
	3. Farm made	
3D. Do you use fertilisation of pond?	3D	
	1. Yes	

3

a. Animal manure
b. Agricultural products
c. Trash fish
d. Others: _____
2. No

4D. What is the feeding regimen?

4D .....
1. Once a day
2. Twice a day
3. Every other day
4. Other: _____

5D. Do you exchange water on the farm?

5D
1. Yes
Exchange rate: _____
2. No

6D. Do you measure water quality during production period?

6D
1. Yes
a. Frequency: _____
b. Parameters:
A- Daily **B-** once/week **C-** once/month
b.1 Alkalinity _____
b.2 Ammonia _____
b.3 Dissolved Oxygen _____
b.4 Nitrate _____
b.5 Nitrite _____
b.6 pH _____
b.7 Salinity _____
b.8 Temperature _____
b.9 Others: ____

7D. How often do you measure water quality?

7D
1. Daily
2. Twice a week
3. Weekly
4. Once a crop
5. Others:

8D. Do you clean or treat the tanks/ponds/nets

8D

1. Yes
a. Physical
b. Chemical
2. No

What kind of chemical is used?

.....

4

E. Diseases and Health Management

1E. Have you ever observed your fishes get sick , die, behave and grow abnormally?

1E
1. Yes
2. No

2E. How do you know if your fish is sick?

2E
1. Fish stop feeding
2. Fish change colour
3. Fish change behaviour
4. Abnormal shape or size
5. Marks (lesions, tumour, ulcers) in body
6. Damaged fins
7. Swelling eyes or bulging stomach
8. Fish dies
9. Don't know

3E. In what month/s of the year do your fishes usually get sick?

3E

4E. Prevalence (number of individual affected. at a particular time) of disease problems

4E.
1. ≤ 25%
2. 50%
3. 75%
4. 100%

5E. In what culture period diseases occur?

5E
1. First month of culture
2. Middle of culture period
3. Last month of culture period

6E. Does your farm suffer any high mortality problem?

6E
1. Yes
2. No

If **Yes**, how often you had the problem?

7E. Do you report mortality/disease outbreaks to government fisheries authorities?

7E
1. Yes
2. No

5

8E. During disease outbreaks, what have been your largest losses? Normal survival?	8E Losses: _____ % Survival: _____ %
9E. If disease outbreak occurs, what do you do?	9E 1. Nothing 2. Change water 3. Treatment 4. Harvest
10E. What type of treatment/s you use?	10E 1. Traditional : _____ 2. Chemical: _____ 3. Antibiotics: _____ 4. Others: _____
11E. Who give you advice on treatment?	11E 1. BFAR 2. Self 3. Other farmers 4. Private companies/pharmaceuticals
12E. What do you do with diseased fish?	12E 1. Discard 2. Burn 3. Eat 4. Sells
13E. Does disease/mortality outbreaks causes significant losses to income/production?	13E 1. Yes 2. No 3. Don't know
14E. Could you estimate the size of losses?	14E 1. Yes 2. No
15E. What is/are the indications of type of disease problem?	15E 1. Appearance: _____ _____ 2. Behavior: _____ _____
16E. How do you know the scale of the disease problem?	16E 1. By observation 2. By sampling

Appendix 2: Protocols

Appendix 2.1: DNA Extraction (Crude Boiling Method)

1. Centrifuge bacterial suspension (5 ml in TSB, 24 h culture at 28 C) at 3000 rpm for 15 min at 4 C.

2. Decant the supernatant and resuspend bacterial pellet with 1 ml of sodium chloride-Tris-EDTA (STE) buffer (0.1 M NaCl, 10 mM Tris at pH 8, 1 mM EDTA). Transfer this suspension in 1.5 ml Eppendorf tube.

3. Centrifuge the suspension at 13, 000 rpm for 1 min at 4 C.

4. Remove supernatant, resuspend pellet immediately in 100 ul of Tris-EDTA (TE) buffer (10 mM Tris at pH 8, 1 mM EDTA).

5. Heat the suspension at 95 C for 10 min, then immediately place on ice for 3 min.

6. Centrifuge the suspension at 13, 000 rpm for 1 min at 4 C.

7. Collect the supernatant and transfer into new Eppendorf tube leaving behind the cellular debris. Aliquot supernatant into smaller Eppendorf tubes and store at -20 C.

Quantify DNA concentration using Nanodrop.

Appendix 2.2: *16S rRNA* PCR

1. Put on nitrile gloves
2. Label a clean 0.2mL PCR tube
3. Keep all reagents on ice

Table 1. Ingredients and quantities required for a 25µL PCR reaction.

Ingredients	**Vol. for 25µL PCR reaction (µL)**
2x HS MytaQ mastermix	12.5
Milli-Q water	8.5
20F (Forward) (10µm)	1
1500R (Reverse) (10µm)	1
Sample DNA	2

4. Add milli-Q water into the 0.2mL PCR tube. Change tip
5. Add 2 x HS MytaQ mastermix into the 0.2mL PCR tube. Change tip
6. Add the forward primer to the inside wall of the 0.2mL PCR tube. Change tip
7. Add the reverse primer to the inside wall of the 0.2mL PCR tube. Change tip
8. Add the sample DNA to the inside wall of the 0.2mL PCR tube. Change tip
9. *Aeromonas hydrophila* NCIMB 9240 or *Vibrio paraheamolyticus* NCIMB 1902 should be used as a positive reference control for Eubacterial DNA
10. Make up a positive control PCR reaction in a clean-labelled 0.2mL PCR tube. Change tip between each reagent

Table 2. Ingredients and quantities required for a 25µL positive control PCR reaction.

Ingredients	**Vol. for 25µL PCR reaction (µL)**
2x HS MytaQ mastermix	12.5
Milli-Q water	8.5
20F (Forward) (10µm)	1
1500R (Reverse) (10µm)	1
Positive control DNA	2

11. Make up an internal negative control PCR reaction in a clean labelled 0.2mL PCR tube. Change tip between each reagent:

Table 3. Ingredients and quantities required for a 25µL negative control PCR reaction.

Ingredients	**Vol. for 25µL PCR reaction (µL)**
2x HS MytaQ mastermix	12.5
Milli-Q water	10.5
20F (Forward) (10µm)	1
1500R (Reverse) (10µm)	1

12. Ensure that lids are tightly closed on each tube
13. Centrifuge briefly at 6000rpm for 10 seconds in a benchtop centrifuge to ensure contents of the tube are fully mixed

14. Place samples in the middle of the thermocycler plate, as this will have a more uniform temperature.
15. Programme the Biometra Tgradient thermocycler to the following conditions:

Table 4. Thermal cycling conditions.

Step	Function	Temperature	Time	Cycles
1	Initial Denature	95°C	1 min	30 x cycles of step 2, 3 & 4.
2	Denature	94°C	15s	
3	Annealing	56°C	20s	
4	Extension	72°C	1min	
5	Final Extension	72ºC	2 min	

16. Total running time: 1h. 7m
17. The results from the PCR amplification should be visualised in a 1% (w/v) agarose gel loaded with a 1Kb DNA ladder (SP/ M.5).
18. Store PCR products in the fridge/ freezer until needed for further downstream applications.

Appendix 2.3: Species-specific Duplex PCR

1. Include appropriate controls within the PCR protocol: positive controls (*S. agalactiae* NCIMB 701348 and *S. iniae* ATCC 29178), a negative control for PCR mix (Milli-Q water with no DNA) and controls to illustrate primer specificity (100 bp ladder).

2. The oligonucleotide primers to be used for the detection of *S. agalactiae* or *S. iniae* are:

Primer	Direction	Nucleotide Sequence	Amplicon size (bp)	Pathogen
LOX-1	Forward	AAGGGGAAATCGCAAGTGCC	870	*S. iniae*
LOX-2	Reverse	ATATCTGATTGGGCCGTCTAA		
STRA-AgI	Forward	AAGGAAACCTGCCATTTG	270	*S. agalactiae*
STRA-AgII	Reverse	TTAACCTAGTTTCTTTAAAACTAGAA		

3. Perform amplification in a 25 µl reaction mixture using master mix that contain:

Ingredients	Vol. for 25µL PCR reaction (µL)
2x HS MytaQ mastermix	12.5
Milli-Q water	4.5
LOX-1 (Forward) (10 pmol)	1.5
LOX-2 (Reverse) (10 pmol)	1.5
STRA-AgI (10 pmol)	1.5
STRA-AgII (10 pmol)	1.5
DNA sample	2.0

4. The amplification profile is consisted of an initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 25s, with a final extension of 72°C for 10 min.

5. Run PCR products by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE (Tris-acetate-EDTA) buffer with ethidium bromide (0.5 μg ml-1), visualized on a UV transilluminator.

Appendix 2.4: Molecular serotyping of piscine *S. agalactiae* capsule *cps* gene

1. Include appropriate controls within the PCR protocol: positive control (*S. agalactiae* NCIMB 701348), a negative control for PCR mix (Milli-Q water with no DNA) and controls to illustrate primer specificity (100 bp ladder).

2. The oligonucleotide primers to be used for the capsular serotyping of *S. agalactiae* are:

Serotype	Target Gene	Primer	Nucleotide (5' → 3')*	Target Region (bp)
Ia	*cpsL*	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	
	cpsG	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	272
		cpsG-R	ATGCTCTCCAAACTGTTCTTGT	
Ib	cpsG	cpsG-F	ACATGAACAGCAGTTCAACCGT	272
		cpsG-R	ATGCTCTCCAAACTGTTCTTGT	
	cpsJ	cpsJ-Ib-F	GCAATTCTTAACAGAATATTCAGTTG	621
		cpsJ-Ib-R	GCGTTTCTTTATCACATACTCTTG	
	cpsL	cpsL-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	
III	*cpsG*	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	352
		cpsG-2-3-6-R	TCCATCTACATCTTCAATCCAAGC	
	cpsL	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	

3. Perform amplification in a 25 µl reaction mixture using master mix that contain:

Ingredients	Vol. for 25µL PCR reaction (µL)
2x HS MytaQ mastermix	12.5
Milli-Q water	0.5
cpsL-F (10 pmol)	1.0
cpsL-R (10 pmol)	1.0
cpsG-F (10 pmol)	1.0
cpsG-R (10 pmol)	1.0
cpsJ-Ib-F (10 pmol)	1.0
cpsJ-Ib-R (10 pmol)	1.0
cpsG-2-3-6-R (10 pmol)	1.0
DNA sample	5.0

4. The amplification profile is consisted of: 1 cycle of 1 min at 95°C; 15 cycles of 60 s at 95°C, 60 s at 54°C and 2 min at 72°C; 25 cycles of 60 s at 95°C, 60 s at 56°C and 2 min at 72°C; final cycle of 10 min at 72°C.

5. Run PCR products by electrophoresis in a 1.5 % (w/v) agarose gel prepared in 0.5X TAE (Tris-acetate-EDTA) buffer with ethidium bromide (0.5 µg ml-1), visualized on a UV transilluminator.

Appendix 2.5: *rpoD* PCR amplification and sequencing

1. Include appropriate controls within the PCR protocol: positive control (*A. hydrophila* NCIMB 9240), a negative control for PCR mix (Milli-Q water with no DNA) and controls to illustrate primer specificity (100 bp ladder).

2. The oligonucleotide primers to be used for the capsular serotyping of *S. agalactiae* are:

Primer	Target Gene	Sequence (5'-3')	Product size (bp)
70F	*rpoD*	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGG NACNGT	820
70Fs		ACGACTGACCCGGTACGCATGTA	
70R	*rpoD*	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCY TTYTT	
70Rs		ATAGAAATAACCAGACGTAAGTT	

3. Perform amplification in a 25 µl reaction mixture using master mix that contain:

Ingredients	Vol. for 25µL PCR reaction (µL)
2x HS MytaQ mastermix	12.5
Milli-Q water	8.5
70F (10 pmol)	1.0
70R (10 pmol)	1.0
DNA sample	2.0

4. The amplification profile is consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94°C for 60s, annealing at **58-63**°C for 30s and extension at 72°C for 30s, with a final extension of 72°C for 2 min.

Note: The annealing temperature ranged from **58 to 63** °C depending on the G+C content of the template DNA. Higher annealing temperatures were used for higher G-C contents.

5. Run PCR products by electrophoresis in a 1.5 % (w/v) agarose gel prepared in 0.5X TAE (Tris-acetate-EDTA) buffer with ethidium bromide (0.5 μg ml-1), visualized on a UV transilluminator.

6. Then purify the PCR products purified using QIAquick PCR Purification Kit (Qiagen).

7. Then purified PCR porducts will be sent to Eurofins Genomics for sequencing using the sequencing primers 70Fs (5'-ACGACTGACCCGGTACGCATGTA-3') and 70Rs (5'-ATAGAAATAACCAGACGTAAGTT-3') which correspond to the first 23 nucleotides of PCR primers 70F and 70R, respectively.

Appendix 3: Protein spectral profiles

Appendix 3.1

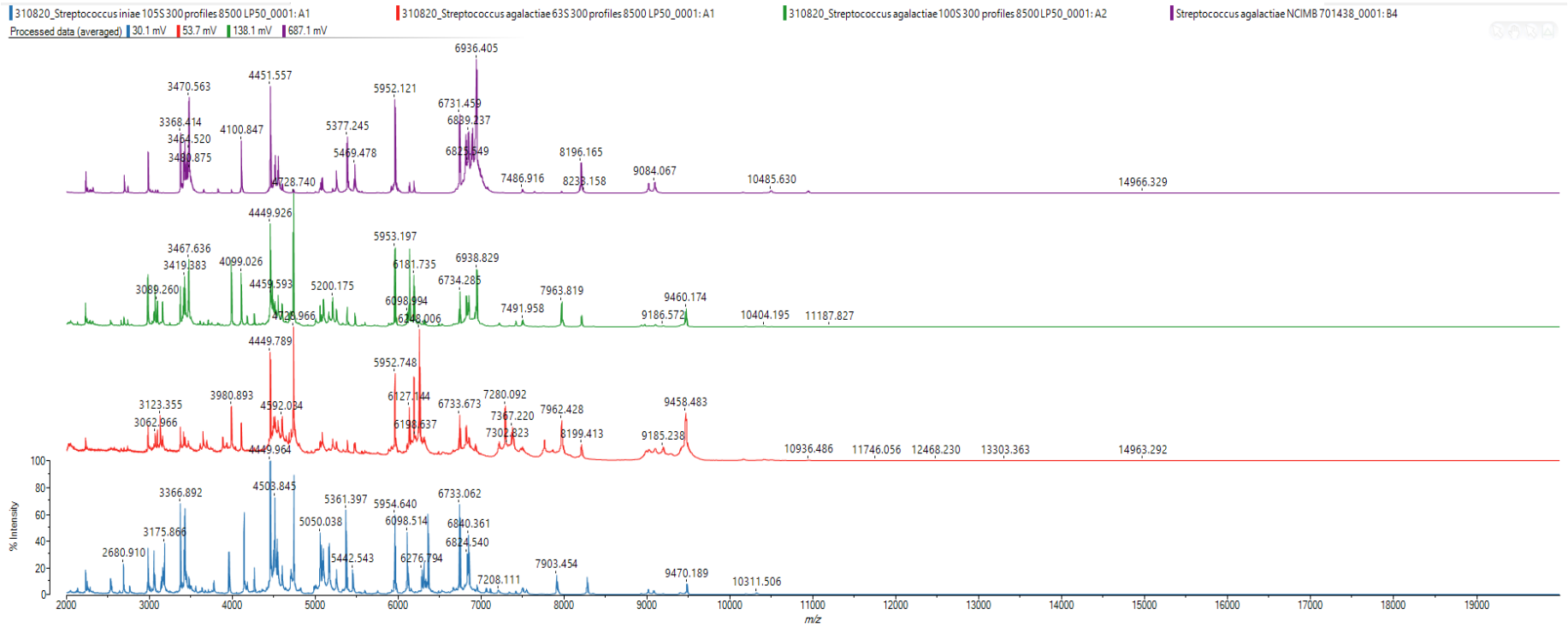

Figure 4.10**.** Depicts the optimal mass spectrum results achieved with isolates undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The four *Streptococci* isolates were grown for 48 hours before subculture and were procured at subculture 2. The blue spectrum exhibits isolate *S. iniae* 105, the red spectrum displays isolate *S. agalactiae* serotype Ia 63, the spectrum in green shows *S. agalactiae* serotype Ib 100 and the violet spectrum depicts *S. agalactiae* NCIMB 701348.

Appendix 3.2

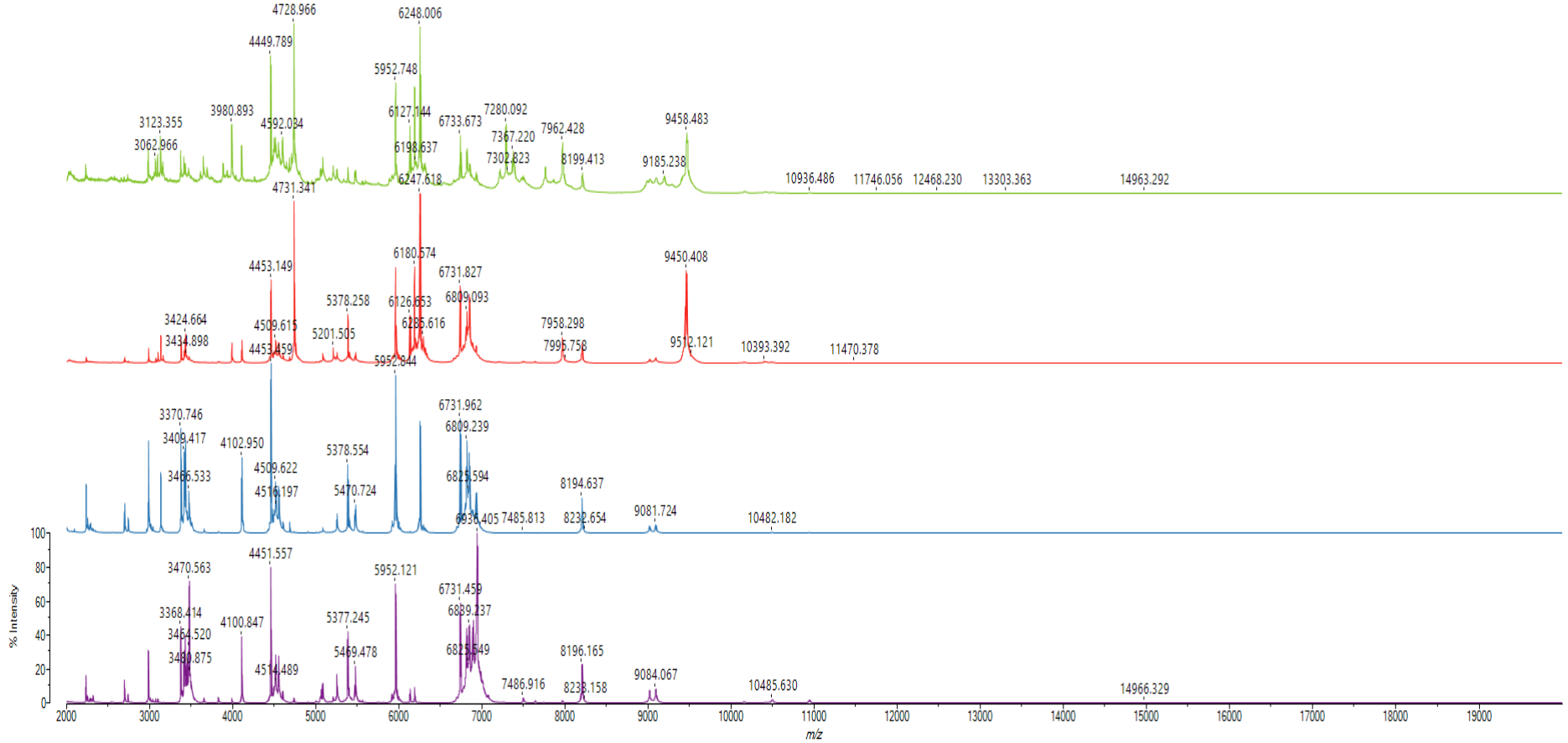

Figure 4.11**.** Depicts the optimal mass spectrum results achieved with isolates undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The four *Streptococci* isolates were grown for 48 hours before subculture and were procured at subculture 2. The violet spectrum exhibits isolate *S. agalactiae* NCIMB 701348, the blue spectrum displays isolate 122, the spectrum in red shows 132 and the yellow green spectrum depicts isolate 63.

Appendix 3.3

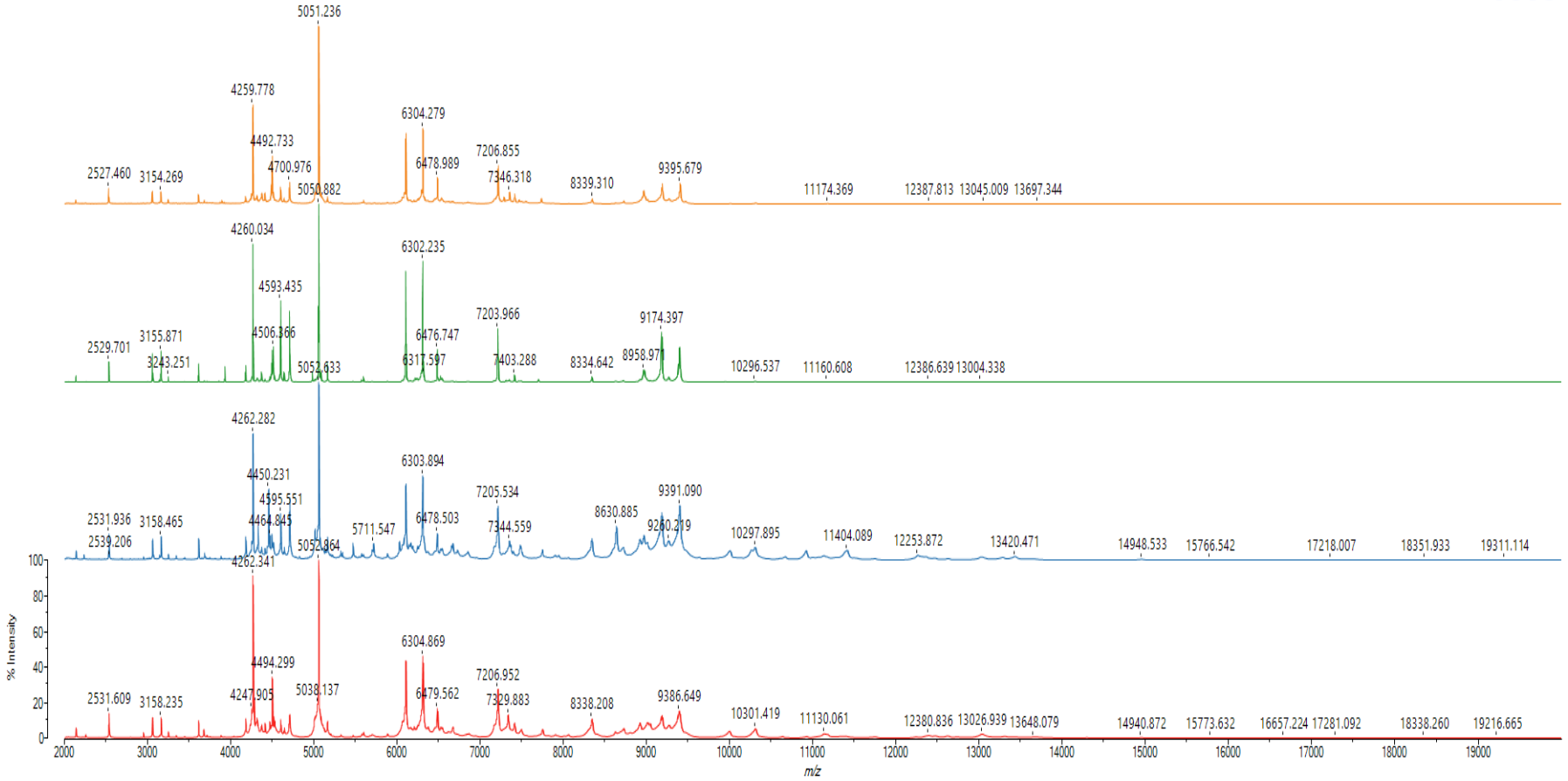

Figure 5.3**.** Depicts the optimal mass spectrum results achieved with isolate undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The four *Aeromonas* isolates were grown for 24 hours before subculture and were procured at subculture 2. The red spectrum displays isolate *A. veronii* ATCC 35624, the spectrum in blue shows isolate *A. hydrophila* NCIMB 9240, the green spectrum depicts isolate 39 and the orange spectrum exhibits isolate 69.

Appendix 3.4

Figure 5.4**.** Depicts the optimal mass spectrum results achieved with isolate undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The five *Aeromonas* isolates were grown for 24 hours before subculture and were procured at subculture 2. The red spectrum displays isolate *A. veronii* ATCC 35624, the spectrum in blue shows isolate 4, the violet spectrum depicts isolate 23, the green spectrum depicts isolate 26 and the orange spectrum exhibits isolate 34.

Appendix 3.5

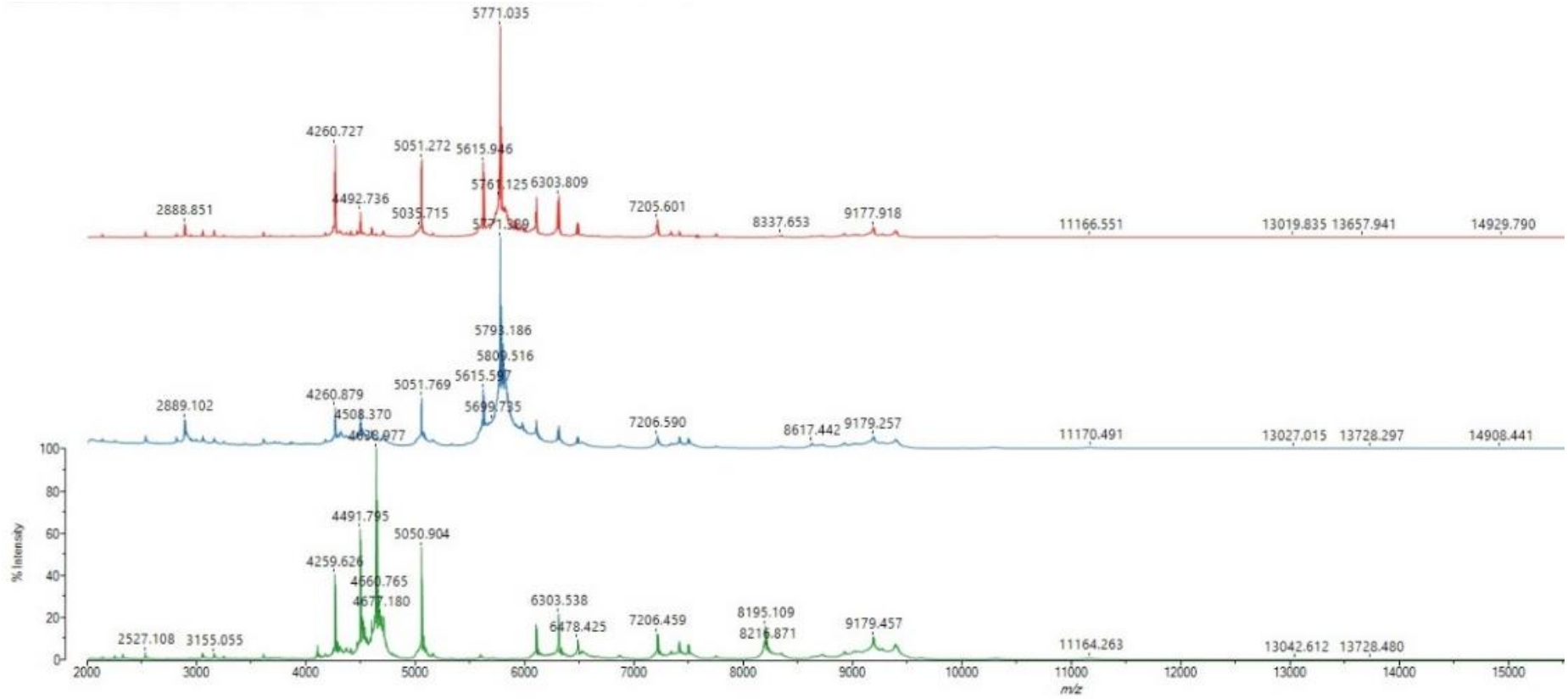

Figure 5.5**.** Depicts the optimal mass spectrum results achieved with isolate undergoing ethanol-formic acid extraction after being grown on TSA at 28° C. The five *Aeromonas* isolates were grown for 24 hours before subculture and were procured at subculture 2. The green spectrum displays isolate 34, the spectrum in blue shows isolate 41, and red green spectrum depicts isolate 67.

Appendix 4. Publication

Received: 8 June 2020 | Revised: 25 July 2020 | Accepted: 27 July 2020

DOI: 10.1111/jfd.13247

ORIGINAL MANUSCRIPT

Journal of Fish Diseases WILEY

Isolation and molecular characterization of streptococcal species recovered from clinical infections in farmed Nile tilapia (*Oreochromis niloticus*) in the Philippines

Francis S. Legario[1,2] | Casiano H. Choresca Jr.[3] | Jimmy F. Turnbull[1] | Margaret Crumlish[1]

[1]Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, UK

[2]Natural Sciences Department, Iloilo Science and Technology University, Iloilo City, Philippines

[3]National Fisheries Research and Development Institute-Fisheries Biotechnology Centre, Science City of Munoz, Philippines

Correspondence
Francis S. Legario, Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Scotland, UK.
Email: franslegario@gmail.com

Funding information
British Council Newton Fund: DOST-Newton PhD Scholarships, Grant/Award Number: 279508540

Abstract

Streptococcosis cause severe losses for global tilapia farming, especially in developing countries. The aim of this study was to identify and characterize streptococci recovered from Nile tilapia farmed in the Philippines. Moribund and apparently healthy fish were sampled from grow-out cages, ponds and hatcheries. Clinical signs observed included exophthalmia, eye opacity, ascites, lethargy, erratic swimming and haemorrhages. Results showed that both *Streptococcus iniae* and *Streptococcus agalactiae* were associated with disease in these sites. Consistent with global reports, including those from South-East Asia, *S. agalactiae* was more widespread than *S. iniae*. Molecular serotyping of the *S. agalactiae* isolates identified the serotype Ia and serotype Ib. Histopathological findings were meningitis, meningoencephalitis and septicaemia. Identical virulence profiles were found for all strains of *S. iniae*, while *S. agalactiae* strains were separated into virulence profile I and profile II. All strains were susceptible to the tested antibiotics and resistant to oxolinic acid. Only *S. agalactiae* serotype Ib showed resistance to sulphamethoxazole–trimethoprim. This is the first study from the Philippines to characterize the streptococci involved in disease outbreaks in tilapia aquaculture. Outputs from this study will promote the development of efficacious disease control strategies in tilapia farming for the Philippines and South-East Asia.

KEYWORDS

molecular typing, serotype Ia, serotype Ib, *Streptococcus agalactiae*, *Streptococcus iniae*, virulence genes

1 | INTRODUCTION

Streptococcosis outbreaks occur in a wide range of farmed fish species, globally (Mishra et al., 2018). Outbreaks in farmed freshwater tilapia continue to threaten global production, contributing to severe economic losses encountered worldwide (Amal & Zamri-Saad, 2011; Li et al., 2015; Liu et al., 2018; Mishra et al., 2018). Numerous incidences of streptococcosis have been reported in intensive tilapia culture systems in South-East Asia and have caused tremendous financial damage since tilapia farming is the most important, if not globally, in the region (Kayansamruaj, Areechon, & Unajak, 2020). While a range of bacterial aetiological agents have been identified from streptococcosis infections in fish, by far the greatest causes of these diseases are the Gram-positive, *Streptococcus agalactiae* and *S. iniae* (Agnew & Barnes, 2007; Mishra et al., 2018; Zhou et al., 2008). Understanding the pathogenesis in aquatic outbreaks is complex, as

Link to pdf online at *Journal of Fish Diseases*
https://doi.org/10.1111/jfd.13247