IMMUNE RESPONSES AND PROTECTION OF FRY NILE TILAPIA (OREOCHROMIS NILOTICUS) IMMUNIZED BY IMMERSION AND ORAL BIVALENT VACCINES AGAINST STREPTOCOCCUS AGALACTIAE AND AEROMONAS VERONII

by

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A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in
Aquaculture and Aquatic Resources Management

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May 2022

AUTHOR'S DECLARATION

I, Andres Ludovic Stephane Quentin, declare that the research work carried out for this thesis was in accordance with the regulations of the Asian Institute of Technology. The work presented in it are my own and has been generated by me as the result of my own original research, and if external sources were used, such sources have been cited. It is original and has not been submitted to any other institution to obtain another degree or qualification. This is a true copy of the thesis including final revisions.

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ACKNOWLEDGEMENTS

ABSTRACT

Fighting bacterial infections inducing mass mortality in fish is critically important in the aquaculture industry in order to sustain its intensification. This research project aims to develop simple and inexpensive heat-killed inactivated bivalent vaccines against group B streptococci (GBS) *Streptococcus agalactiae* and against gram-negative *Aeromonas veronii* for prophylaxis of Nile tilapia (*Oreochromis niloticus*) for which there is currently no vaccine commercially available in Thailand. The objective of the study is to characterize a part of the immune response elicited in Nile tilapia after vaccination. A total of $(\text{n} = 900 \; fry)$ with an average mass of $(1.1\text{g} \pm 0.1\text{g})$ were divided into 3 groups in separate tanks and vaccinated: Control (Ct) (untreated condition, no immunization is expected); IMM+OR: Immersion vaccine in 20L for 4.5h, containing 10 million CFU/mL per bacteria as inactivated culture broth (day 1) + oral booster vaccine fed twice daily (in amount of 3% fish bodyweight) containing 10 billion inactivated CFU/kg of *S.agalactiae* and 1 billion inactivated CFU/kg of *A.veronii* and coated with 100mL of soybean oil per kg of feed (day 21 to day 28). OR+OR: first oral vaccination (day 1 to day 7) with oral booster (day 21 to day 28). Specific immune responses to vaccinations in each group (Ct, IMM+OR, OR+OR) was determined by indirect ELISA assay. As a final step, the fish were transfered into 6 tanks of 100L. Bacterial challenge trials were conducted by immersing duplicates of 50 fish per tank filled with a diluted solution harboring live bacteria (either *S.agalactiae* or *A.veronii*) at 10 million CFU/mL in 19L for 6h, then diluted by adding 19L of extra clean water, and monitored for two weeks. From the results of Elisa assay, it was observed that both IMM+OR and OR+OR could stimulate IgM production against the two pathogens. OR+OR failed to protect (0% of overall survival probability and 51% against *A.veronii* and *S.agalactiae*) and only IMM+OR was found to be trustworthy and effective in eliciting protection and preventing mortalities after artificial infection trials (more than 80% of overall survival probability against both pathogens). This research propose a new effective vaccination approach that is both convenient and inexpensive against *S.agalactiae* and *A.veronii* in the Nile tilapia fry and which could ultimately benefit the small and medium scale fish farms and nurseries.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A.veronii	*Aeromonas veronii*
S.agalactiae	*Streptococcus agalactiae*
Ab	antibodies
Ag	antigenic particles or antigens
CC	clonal complexes
CFU	colony-forming units (CFU)
Ct	control
ECP	extracellular product of the bacterium
ELISA	enzyme-linked immunosorbent assay
FKV	formalin killed vaccine
GALT	gut-associated lymphoid tissue
GBS	group B streptococci
HKV	heat-killed vaccine
HRP	horseradish peroxidase
ICW	inactivated whole-cell vaccine
ICW	inactivated whole-cell antigens
IgM	immunoglobulins M
IHNV	infectious hematopoietic necrosis virus
IM	intramuscular injection
IMM	immersion
IMM+OR	immersion + oral (booster)
IP	intra peritoneal injection
IPNV	infectious pancreatic necrosis virus
ISAV	infectious salmon anemia virus
ISKNV	infectious spleen and kidney necrosis virus
KHV	koi herpesvirus
Nile tilapia	*Oreochromis niloticus*
OR+OR	oral + oral (booster)
PBS	phosphate buffer saline
RPS	relative percentage survival

SAV	salmon anemia virus
SVCV	spring viremia of carp virus
TSA	tryptone soya agar
TSB	tryptic soy broth

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

With an ever-increasing population, the world population will reach 10 billion people by 2050, resulting in a 52 percent rise in global demand for animal proteins. More seafood is required to ensure food security and universal access to fish. Aquaculture may be the answer. The Nile tilapia, *Oreochromis niloticus*, is a freshwater fish that is farmed all over the world. Tilapia farming is a high-growth industry. Despite the impact of Covid-19, freshwater farmed tilapia output has increased from over 3 million tonnes to over 6 million tonnes for the first time in 2021, expanding by 3.3 percent in 2020. (Conference on GOAL 2020)

Figure 1.1

Production of tilapia per country. China crossed the 1.7 million tonne mark in 2019, Indonesia is the second largest tilapia producer followed by Egypt. (Data from FAO, GAA, OECD, PeixeBR).
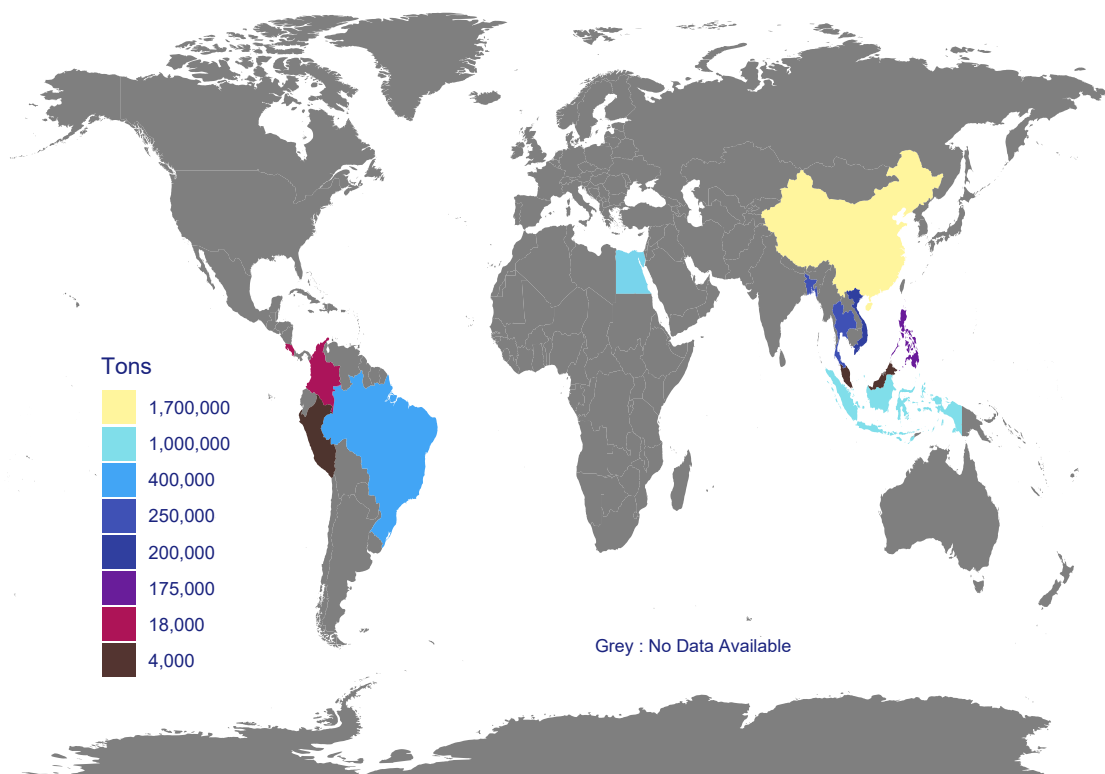
In Thailand freshwater fish culture is of first importance for economy and also for food security. The Nile tilapia is a species of cichlid fish that are native to the northern half of Africa and the Levante area, including Palestine, and Lebanon. Numerous introduced populations exist outside its natural range. It is also commercially known as mango fish, nilotica, or boulti or simply tilapia. Imported in Thailand a few decades ago, Nile tilapia is the most produced fish and represent almost 50 % of the total freshwater fish coming from farms (Figure 1.2). However it is also the most exposed to infections by major zoonotic pathogens and emerging zoonotic pathogens.

Figure 1.2

Proportion of freshwater fish species farmed in Thailand. Adapted from: Dr Nopadon Pirarat (IVVN's third annual conference)
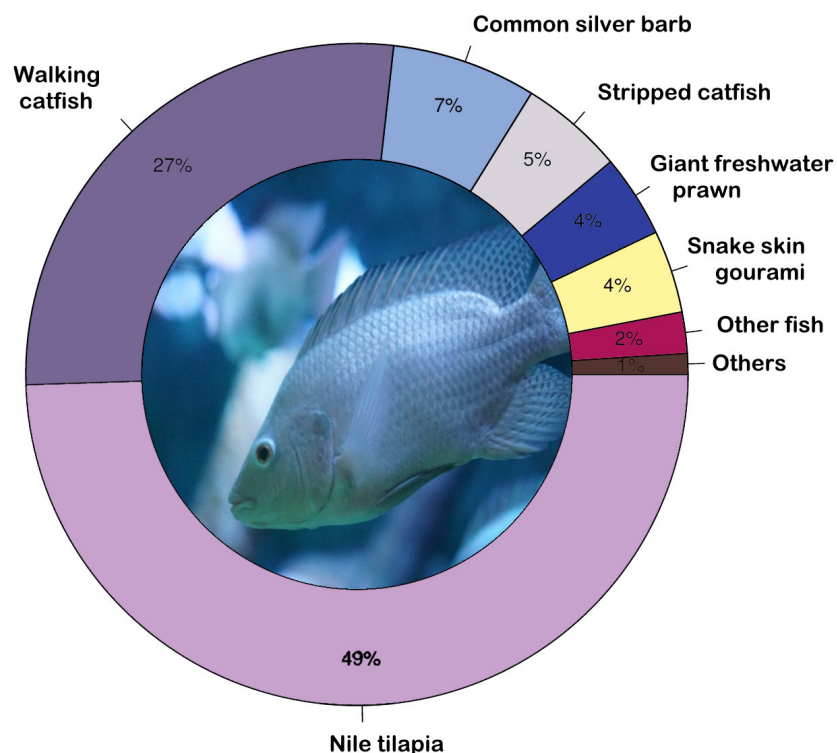
Streptococcus agalactiae and *Aeromonas veronii*, two major bacterial pathogens are the etiological agent of streptococcosis and motile aeromonas septicemia (MAS) in freshwater cultured fish. In order to sustain an intensified and resilient Cichlids' aquaculture,

researchers are studying the immune responses of fish to viral and bacterial infections. Developping and implementing new vaccines can limit the damage from disease outbreaks. Although a lot of effort is put in research and development for the last decades in order to improve disease resistance via pedigree manipulation and use of probiotics, vaccination is still relevant. The benefits of vaccination are still poorly acknowledged and understood among aquaculturists and farmers, moreover a vaccine may not always be accessible depending on the region. (Delphino et al., 2019).

1.2 Statement of the Problem

The problem lies in the increase in aquaculture of freshwater tilapia in regions likely to favor the appearance of aquatic streptococcal and aeromonas diseases caused by *S.agalactiae A.veronii*, especially during so-called intensive cultures, i.e. with a density of fish that exceeds the threshold from which the growth and severity of bacterial diseases are greatly promoted (Shoemaker et al., 2000). The main challenge therefore lies in finding a solution to limit or at least control bacterial diseases in aquaculture ponds and if possible with biocontrol methods which meet several constraints such as:
- Restricting the use of antibiotics or chemical agents promoting selection pressure on bacteria and which would promote the appearance of resistant bacteria to said antibiotic.
- Not using chemicals that pollute fresh water such as disinfectants. -Using biocontrol solutions that are easy to set up and maintain and inexpensive. - Finding appropriate complementary solutions of effective prevention such as vaccination in addition to the good management of the farm in term of biosecurity.

Preventing diseases is possible, prophylaxis methods such as vaccination are indeed quite effective in this matter. The bacteria *Streptococcus agalactiae* and *Aeromonas veronii* are present in Thailand and may continue to create substantial economic losses for the industry (i.e. for the farmers and for the aquaculture corporations). In addition of harming the fish farming industry, group B streptococci (GBS) are also pathogenic to humans and can provoke food illnesses, and less frequently encephalomeningitis if a diseased fish is handled or consumed raw.

On the field, streptococcal illnesses can be treated and avoided in a variety of methods. Strict cleanliness and biosecurity requirements are usually the simplest to execute in the farm, especially in closed and semi-open growth systems. The key to preventing or limiting the advent of illnesses is good husbandry techniques and careful attention to

3

the condition of the water. However, ailments do arise from time to time, necessitating the use of alternative treatments. Only an expert, such as a fish health specialist or a pathologist, will be able to comprehend the issue and choose the best course of action.

Indeed, antibiotic treatments are only a tool that will not exterminate infectious bacteria, but rather contain the infection in the fish body long enough for the fish immune system to control the disease. Choosing adequate antimicrobial treatment require a good knowledge of bacteria strains and species, but also of laws and regulations. Antibiotics susceptibility plate test is necessary to determine which antibiotic must be used. Because of the lack of education, tetracyclines and erythromycine were misused (Ventola, 2015). Tetracyclines are not efficient against *S.agalactiae* and Erythromycine has a limited efficiency with around 60% of isolates being sensitive. It makes sense of using antibiotics if antibiotics are used correctly. For instance there is no resistance to penicillins for *S.agalactiae*, the isolates are 100% sensitive. Da Cunha et al. (2014)'s team found that GBS agalactiae clones infecting humans carry genetic elements that confer to the bacteria a resistance to tetracyclines (tetR). They suggest that this selection is the consequence of the massive use of tetracycline in fish feeds and in animal feed. They also explain that this gene confering the AMR (i.e. Antimicrobial resistance) is fixed in the bacteria and is likely to not disappear with time.

Some fish producers routinely use antibiotics without first conducting an antimicrobial sensitivity test. Farmers cycle antibiotics, changing the antibiotic class and molecule every time, or every season, to improve treatment efficiency. It is not a reliable method, and it may be deemed an antibiotic overuse. The importance of enlisting the help of a fish health professional is that the specialist may offer preventative measures such as vaccination of healthy animals, as well as provide advice on how to enhance the sanitary elements of the aquaculture production unit (Yanong, 2003). For decades, it was common practice to add tetracyclines to fishfeed and to prevent bacterial infections, but not without consequences. Da Cunha et al. (2014)'s research team focused on the epidemiology of GBS explained the probable origin of the recrudescence neonatal human infections by GBS, *S.agalactiae*. They used comparative genome analysis and phylogenetic reconstruction of 229 isolates from a collection obtained from human GBS infections and they hypothesized that the increase of incidence of the disease corresponds to the selection and worldwide dissemination of only a few clones.

4

Fish vaccine prophylaxis allows a specific number of fish in the pond to be protected, reducing the negative repercussions of a bacterial outbreak in the aquaculture basin. However, this is only one approach among multiple that may be used to limit the prevalence of bacterial infections. The goal of the study is to compare immune responses to a newly designed pathogen-killed vaccine administrated by 2 different routes. Our idea is to research on what could be the easiest way to vaccinate Nile tilapia for an hypothetical small and medium scale nursery or fish farm. In a controlled experiment, the effect of two methods of immunization for streptococcus infection and motile aeromonas septicemia on disease specific antibody levels and survival rates in fry tilapia (*Oreochromis niloticus*) will be investigated.

In the first vaccination method, fish $(1.1 \pm 0.1g)$ were randomly assigned to 2 indoor tanks A, B (with a density of 150 fish/tank) and among which 2 of the tanks were supplied with 3% BW of oral vaccine feed, twice daily (noon and afternoon) for $2 * 7$ days with 21 days interval.

In the second method, fish from the same source $(1.1 \pm 0.1g)$ were randomly assigned to 2 indoor tanks C, D (with 150 fish/tank), among which 2 fish tanks were immersed in a vaccine solution for 4.5 hours and fed 21 days later with 3% BW of oral vaccine feed pellets, twice daily (noon and afternoon) for 7 days.

1.3 Hypothesis

1. Natural, inexpensive mucosal soybean-oil coated inactivated bivalent vaccine and bivalent inactivated immersion vaccines can stimulate specific immune responses in Nile tilapia and confer protection against *S.agalactiae* and/or *A.veronii* infection.

Research questions

1. Can a soybean oil-based oral bivalent inactivated vaccine with a booster dose (OR+OR) stimulate IgM responses against both *S.agalactiae* and *A.veronii* in Nile tilapia fry ?

2. Can a bath immersion inactivated bivalent vaccine with soybean oil-based oral bivalent inactivated vaccine booster (IM+OR) stimulate IgM responses against both *S.agalactiae* and *A.veronii* in Nile tilapia fry?

3. Which one of the two methods (OR+OR and IM+OR) is better at protecting fry Nile tilapia from *S.agalactiae* and *A.veronii* infections?

1.4 Objectives of the Research Project

1. To produce mucosal oral bivalent vaccines and bath-immersion bivalent vaccines containing inactivated *S.agalactiae* and *A.veronii*.

2. To investigate immune responses of tilapia fry after immunization with bivalent vaccines.

3. To evaluate protective efficacy of both vaccines in experimental challenge.

1.5 Organization of the Thesis

1.5.1 Conceptual Framework

First, a literature search has allowed for the selection and definition of broad experimental designs, procedures, research hypotheses, and the overall structure of the project. In a second phase, the modification of many protocols has been performed to match our study constraints . A better allocation of resources (materials, animals, and biological materials) has allowed the experimental design to be defined based on the experiment's objectives (which biomarkers or analytes we want to assess and at what time intervals). Thirdly, create an inventory and gather or order the different items required to conduct the research, followed by the set-up of the tank system and laboratory. Once the three steps have been done, the experimental part of the research has started: housing the fish and developing various vaccines, immunizing the fish and studying immune responses,

studying the protective effectiveness of vaccinations by survival challenge and survival analysis.

Figure 1.3

Conceptual framework
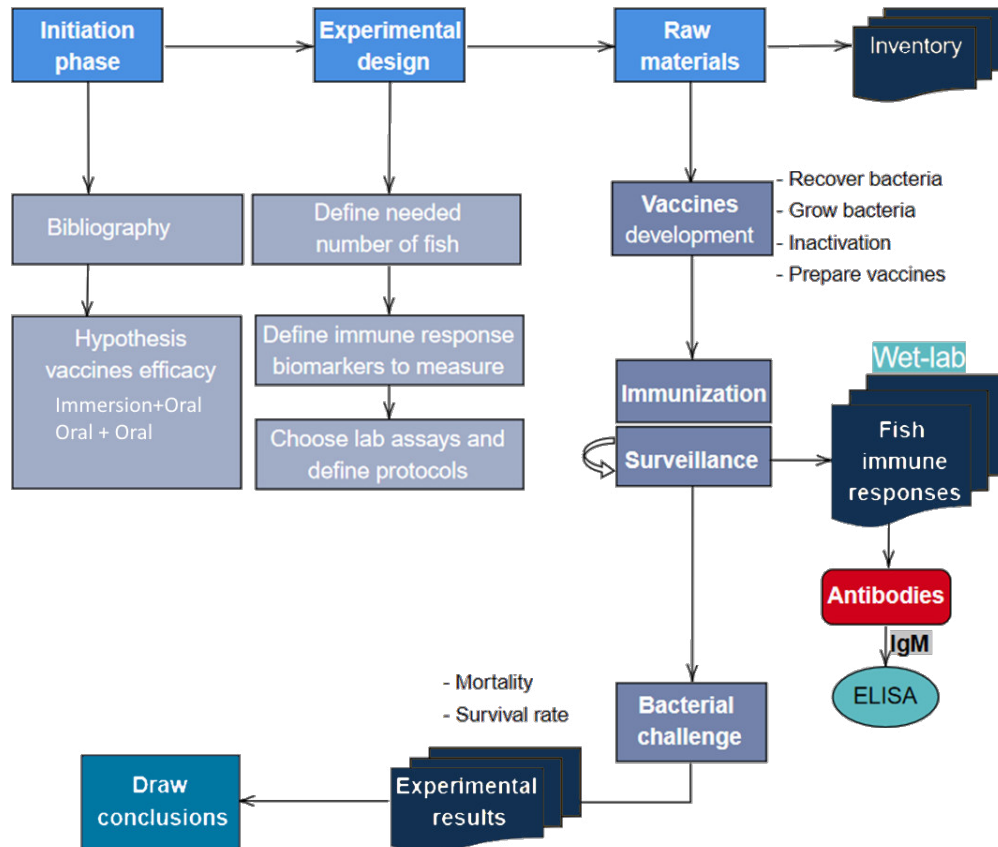
1.5.2 Funding

The research project has been funded by The Asian Institute of Technology Initiation Grant to Dr. Ha Thanh Dong.

CHAPTER 2
RELEVANT LITERATURE

2.1 Importance of Tilapia and Disease Challenges

This section takes stock of tilapia diseases and gives a general overview of the situation with recent issues.

In Thailand freshwater fish culture is of first importance for economy and also for food safety. Nile tilapia is the most produced fish and represent almost 50% of the total freshwater fish coming from farms (Figure 1.2). However it is also the most exposed to infections by major zoonotic pathogens and emerging zoonotic pathogens. In the early 1990s, the number of described cases of *S.agalactiae* infections in farmed fish, but also in fish wildness has increased sharply in all four corners of the world, USA, Israel, Thailand, Brazil, China (Delannoy et al., 2013; Duremdez et al., 2004; Mian et al., 2009; Wilkinson et al., 1973; J. J. Evans et al., 2015; Chen et al., 2012; Mishra et al., 2018; Liu et al., 2013; Barkham et al., 2019; Brouwer & Samkar, 2021; Leal et al., 2019).

Since the middle of the 20th century, the first cases of *S.agalactiae* infections in farmed fish have been reported, particularly in the Notemigonus species crysoleucas (Robinson & Meyer, 1996). To date, infections with *S.agalactiae* have been found in tilapia, trout, mullet *(Mugil cephalus)*, and five-banded amberjack *(Seriola quinqueradiata)* (Eldar et al., 1995; Mian et al., 2009; Pereira et al., 2010; Mishra et al., 2018; FAO, 2021). But also in Barcoo grunter, Golden pompano, Giant Queensland grouper, Ya-fish, Silver pomfret, Snakehead, Carps and Asian seabass.

Depending on the culture systems, the factors favoring the appearance of bacterial strep-tococcus and aeromonas diseases are a degradation of the water quality and in particular water that is too turbid and with too high a salinity (Chang & Plumb, 1996), which has the effect of stressing the animal immune defenses and their ability to defend against pathogens, but also too high temperature which promotes bacterial growth because a higher temperature increases the multiplication rate of the bacterium. On hot sunny days (with a temperature above 37 °C) and during high heat climatic events, in addition to promoting the development of microorganisms, Nile tilapia also have more difficulty

to breathe because the oxygen dissolved will be too low. (Musa et al., 2017; Chown et al., 2004; Abele & Puntarulo, 2004).

At the time of COP26 or 2021 United Nations Climate Change Conference, it is alarming that the different nations cannot agree on a plan to curb the CO2 emissions in the next decades. In the actual scenario, is it very clear that tilapia industry in Thailand will face a huge crisis, starting with a recrudescence of more infectious and virulent pathogens due to increasing pond temperature year round, coupled to economic losses due to high fish mortality in farms because of high temperature climatic events. Tilapia cannot survive in water above 38-40 °C. (Mora et al., 2017; Pandit & Nakamura, 2010).

Figure 2.1

Number of days per year above the lethal threshold in 2100 with +4 degrees celsius (i.e., with air saturated with 100% water) in the worst scenario of global warming. Credits: (Mora et al., 2017)
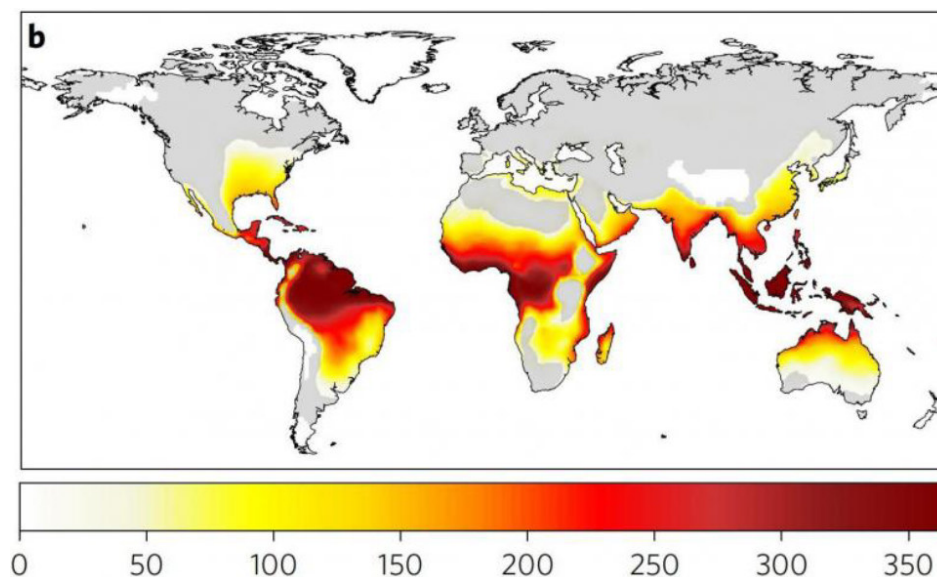
Effective prevention of diseases by the mean of vaccination is the most rationale and valuated response to the problem of increasing bacterial outbreaks in Nile tilapia aquaculture farms. Effective prevention by vaccination makes it possible to protect the population upstream, before the disease spreads and therefore has three rather positive consequences: 1- The protection of at least more than half of the population of vaccinated

fish against all of the different strains/isolates. 2- Slowing down the kinetics of the epidemic 3- Reducing the spread of pathogens in the surrounding environment because the tilapia population acts as a potential vector of the disease to other animals (i.e., reservoir), although it was put in past evidence that tilapia can carry the bacteria *S.agalactiae* without being sick or showing visible clinical signs in some individuals. Most of the animals are susceptible to the pathogens.

2.2 Diseases Caused by Streptococcus agalactiae and Aeromonas veronii

Streptococcus agalactiae, also called Group B Streptococcus (GBS), is a streptococcus widespread in human populations: about a third of the population harbors this bacterium in its digestive, genital or urinary system. This pathogenic bacterium causes mastitis in dairy cows and invasive infections in fish (Kalimuddin & Chen, 2015). Food-borne invasive human infections with *Streptococcus agalactiae* are exceptional in Southeast Asia. Unfortunately sometimes bactrial diseases are not only limited to fish and 238 cases of intoxication were identified in Singapore, associated with the consumption of Yusheng, a raw fish salad eaten during the festivities of the Chinese New Year. (Tan et al., 2016).

Table 2.1

Streptococcus agalactiae and Aeromonas veronii bacterial agents and their detailed information of affected fish species, locations, hosts, and clinical criteria. Adapted from: (Mishra et al., 2018) (Sreedharan et al., 2011)

Species	Fish species	Clinical criteria
Streptococcus agalactiae	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 hemorrhage, opaqueness of cornea, ascites
Aeromonas veronii	Asian seabass, Carassius auratus, Cyprinus carpio, Ctenopharyngodon idella, Nile tilapia, Silurus asotus	Anorexia, ascitic fluid appear yellow, distended abdomen, hemorrhage, lethargy, scale protrusion, sepsis, ulcer syndrome

In the wild, streptococcosis is not a single disease. But many different diseases with their own pathogenesis all caused by *S.agalactiae*. The disease/pathogenesis depend on the isolate and its lineage. Phylogenetically, the species consists of five major lineages called clonal complexes (CC) -$CC1$, $CC10$, $CC17$, $CC19$ and $CC23$. Each isolate or ST has its own infectious profile and its own prevalence and cause a different strepto-coccosis disease (Kwatra et al., 2016). $ST-17$ isolates are the most likely to induce invasive infections (sepsis and meningitis) in newborns (not because of fish consumption), $ST-23$ isolates are likely to be found in adults, newborns, land animals and aquatic animals such as seals and tilapia (Pereira et al., 2010; R. Wang et al., 2017). $ST-7$ isolates are present in humans, and land animals but also in bullfrog and fish such as tilapia (Delannoy et al., 2013; J. J. Evans et al., 2008; Kayansamruaj et al., 2015; J. J. Evans et al., 2009). Similarly, $ST-283$ is a problematic pathogen in Asia, it belongs to $CC283$ and has 3 variants: $ST-491$, $ST-379$, $ST-1311$. $ST-283$ is not new and has been around for decades (Six et al., 2019; Barkham et al., 2018; Brouwer & Samkar, 2021; Samkar et al., 2016; Kalimuddin et al., 2017; Delannoy et al., 2013).

The emergence of *S.agalactiae* $ST - 283$ in South-East Asia dates back to the 1990s. This strain has also been isolated in Brazil, the aquaculture sector in this country having been contaminated during imports of live fish from Singapore (Leal et al., 2019). It is therefore clearly associated with contamination of aquaculture sectors and the emergence of human cases of food origin. Hence, it represents a threat to both food security and food safety. This observation led FAO to launch a call for data and experts to examine and discuss the data and reference documents available on food-borne *S.agalactiae* group B, from a risk assessment perspective (FAO, 2021).

Aeromonas spp. cause a wide spectrum of diseases in men and animals (Delannoy et al., 2013). About 85 % of gastrointestinal disorders in humans are attributed to *Aeromonas veronii biovar sobria*, *Aeromonas hydrophila* and *Aeromonas caviae*. Other human pathogens are *Aeromonas veronii biovar veronii*, *Aeromonas jandaei* and *Aeromonas schu-bertii*, which cause wound infections, meningitis, osteomyelitis, Septic thritis, endocarditis, peritonitis, urinary and eye infections. *Aeromonas spp.* are isolated from fish, meat, meat products, milk, dairy products and vegetables. A particular risk is posed by the ability of Aeromonas to grow in preserved foods in the refrigerator (Stratev & Rusev, 2012).

In fish, *Aeromonas spp.* cause Aeromonas infections or Motile Aeromonas Septicemia (MAS) (Dong et al., 2017; Dong et al., 2015). The bacteria is opportunist and Ruth Francis-Floyd, a professor and extension specialist for aquatic medicine at the university of Florida explain that Aeromonas infections are probably the most common bacterial disease diagnosed in cultured warmwater fish. Usually, mortality rates are low (10% or less) and losses may occur over a period of time (2 to 3 weeks or longer). In these instances, some factor; usually stress, has caused the fish to become more susceptible to the bacteria. Common sources of stress are poor water quality, overcrowding, or rough handling. In many cases, it may not be necessary to treat Aeromonas infections with medicated feeds. For example, if fish are heavily parasitized, they may resist the bacterial disease if the parasites are removed. Similarly, if disease susceptibility is attributed to poor water quality, then correction of the basic husbandry problem could result in a resolution of the bacterial disease outbreak.

Figure 2.2

Clinical symptoms of nile tilapia infected by GBS. (A) Healthy tilapia, showing eye (E), opercular (O), liver (L), spleen (S), intestine (I) and brain (a). (B) Unilateral exophthalmia; (C) Corneal opacity; (D) Abdominal distension; (E) Opercular hemorrhage; (F) Hepatic hemorrhage (arrow); (G) Splenomegaly(arrow); (H) Thinned intestinal wall; (I) Meningeal congestion; (J) High mortality in floating cage tilapia farms in Brazil; (K) Erratic swimming of moribund red tilapia in Malaysia; (L) Ascites in tilapia farms in Brazil. (Credits: (Y. He et al., 2017; FAO, 2021))

2.3 Tilapia Vaccination for Infectious Disease Prevention

Vaccination is a very effective and safe method. It is durable and suitable for the Nile tilapia. Most vaccines available on the market against *S.agalactiae* for aquaculture are of the type inactivated. They contain killed bacteria (i.e. dead bacteria, also refered as whole-cell killed bacteria) that have been grown and inactivated (i.e. terminated) by the use of a disinfectant or by using ultra high temperature. They harbour the best results compared to all other vaccine types. Other vaccines are experimental (Pretto-Giordano et al., 2010).

2.3.1 Principle of Vaccination and Types of Vaccines

A vaccine is a biological preparation which is administered to the fish in order to stimulate its immune system and to develop a protective and relatively durable adaptive immunity against the infectious agent of a disease. The primary immune reaction from the fish immune system remembers the presented threatening antigen from the vaccine so that, upon subsequent contamination, the immunity thus acquired can be activated more quickly and more strongly. The active substance of a vaccine is either an antigenic agent with pathogenicity attenuated by a killed or weakened form of the pathogenic microorganism, or by one of its toxins, or by one of its characteristic components, for example an envelope protein, or a nucleic acid. Several types of vaccines exist depending on the process used to obtain neutralizing antibodies: whole attenuated or inactivated viruses, replicative or non-replicating genetically modified viral vectors (adenovirus, vaccinia), vaccine subunits obtained by genetic recombination, toxoids and nucleic acids: DNA , messenger RNA (Appendix B).

Immune system of Nile tilapia is of two types: innate and adaptive immunity. The organs, cells and molecules in each of the two types are dissimilar to other species of fishes. However there is molecular evidence suggesting that common patterns and similarity of immune system exists throughout jawed vertebrates (Secombes & Wang, 2012). The innate immune system is the first line of defense of the tilapia against pathogens and is a crucial factor in disease resistance. The adaptive immune system appeared in cartilaginous fishes 500 million years ago and only exists in agnaths (vertebrates without jaws) and gnathostomes (jawed vertebrates). Upon infection by a pathogen such as bacterial or viral infection, the immune response from adaptive immunity is delayed in

time, but aims to give to the tilapia long lasting immunity via the production of specific immunoglobulin proteins. It is a key for a successful vaccination (Secombes & Wang, 2012).

Vaccination of Nile tilapia induce a B-type specific immune response (i.e. adaptive) resulting in the production of specific antibodies to vaccine's immunogens (i.e. antigenic particles or antigens (Ag)). The B-type specific response is the result of the capture of antigenic particules contained in vaccine by dendritic cells and their presentation to helper T-cells in fish lymphoid organs. Upon the presentation of the antigen to helper T-cell, B-cells are further activated into plasma B cells and antibodies are continuously produced. Antibodies are immunoglobulins of class M, T and D, their role is to control and neutralize the pathogen until its destruction and clearance. In Nile tilapia, *Streptococcus agalactiae* vaccines train the immune system to produce specific antibodies as demonstrated previously by passive immunization experiments. (Pasnik et al., 2006)

The easiest way to prepare a vaccine is to grow the pathogen, then inactivating it with the desired physico-chemical treatment. Inactivated or inert vaccines, also known as whole-cell vaccines or inactivated whole-cell vaccine (ICW) are vaccines which have lost all infectivity by physicochemical process. They are therefore free from any infectious risk. Whole-cell vaccines are composed of bacterial bodies in their entirety, but can also contain extracellular products (ECP), large proteins (more than 20 kDa, depending on the centrifugation procedure) and concentrated extracellular products. The inactivation is achieved via treatment consisting of disinfectants: formaldehyde, β-propiolactone, hydrogen peroxide, but also by thermal treatments, UVs, radiations, microwaving.

A formalin killed vaccine (FKV) or heat-killed vaccine (HKV) contains killed cells as antigens but can also contain concentrated extracellular products (ECP). The preparation is relatively inexpensive and easy, with very good inactivation results, sterilization by heat is proven safe, even for humans, as the CDC recommends to boil water at least 1 minute before human consumption, for the HKV preparation temperature must be high enough to kill the microorganisms without damaging the antigens as those cannot tolerate more than 60-70 Celsius. For FKVs, it seems that it could be replaced by newer methods with hydrogen peroxide (H2O2) that are more efficient at inactivating pathogens and faster because of reduced manufacture time (Ramos-Espinoza et

al., 2020) and are also causing less environmental pollution. For FKVs or more generally inactivated vaccines, several intra-muscularl or intra-peritoneal injections shots may also be given to maintain immunity throughout fish lifespan or fish culture cycle. The success of the vaccination by FKV or HKV is influenced by the quality of the vaccine preparation and vaccine composition but also the administration scheme and environmental factors. Simply put, the vaccine should contain high quality antigens with diverse antigenic composition of the microorganism, be good enough to stimulate the immune system and protect the animal for a long time after a unique injection (Klesius et al., 2000). Most of the vaccines are efficient in increasing specific antibody titers for a few months to a year which corresponds to the duration of a complete a farm production cycle. (Pasnik, Evans, & Klesius, 2005).

Antigenic composition (bacteria and strains) in FKV or HKV influences the protection offered to the fish against pathogens (Klesius et al., 2000). The protection by vaccines against identical bacterial strain is known as **"homologous protection"** whereas protection confered by vaccines made from a different strain or by a combination of non-homologous strains of the same genus and same species is known as **"heterologous protection"** (Longhi et al., 2012; Ramrez-Paredes et al., 2019; Suwannasang et al., 2017; Shahin et al., 2019). Cross-protection or **cross-immunity** refer to when the vaccine induce a protection against different species of bacteria the same genre (for example, a vacccine from streptococcus of species A would protect against a streptococcus of species B due to the two bacterial species belonging to the genus streptococcus and therefore sharing common morphological and molecular antigenic features). Cross-immunity exists within streptococcus spp. (Q. Wang et al., 2020; Thu Lan et al., 2021), but also within *Aeromonas spp.* (Sukenda, Sumiati, et al., 2017) .

Antigenic composition (intact killed cells and concentrated extract or extracellular products) correspond to the two different antigenic fractions that we can find after a bacterial culture in liquid medium. A bacterial culture centrifugated at appropriate speed will allow to separate the whole bacterial cells in the form of a pellet and the supernatant containing extracellular products extracellular product of the bacterium (ECP). Pasnik, Evans, Panangala, et al. (2005) produced ECP vaccine by taking tryptic soy broth (TSB) *S.agalactiae* cell culture and proceeding to purification by centrifugation leading to the obtention of cell free-fluid (containing the ECP) concentrated in

27 °C for 72–125 h followed by 3% formalin inactivation. Concentrated extracellular fractions have been shown to produce protective immunity in Nile tilapia against *S.agalactiae* (Pasnik, Evans, Panangala, et al., 2005). As explained by (Munang'andu et al., 2016), Immunization using only ECP is not sufficient to protect Nile tilapia against *S.agalactiae* and score low on challenge tests relative percentage survival (RPS) scores. A better method is to combine concentrated ECP with inactivated whole-cell antigens (ICW), and results in improved protection and efficiency than a vaccine with only ICW without ECP (J. Evans, 2007). The possible explanation lies in the chemotactic activity of *S.agalactiae*'s ECPs which is attracting pro-inflammatory macrophages able of antigen uptake and presentation at the injection area in the peritoneal cavity. (Klesius et al., 2007)

Route of administration is important to consider when vaccinating. For injection, depending on the fish anatomy, it is recommended to use a thin needle that can easily penetrate the skin and scales and deliver the solution of antigens. There are multiple possibilities for the location of the vaccine administration on the fish body. Each recipient tissue has its own kinetics for the release antigens. In Nile tilapia, intraperitoneal injection IP (i.e. in the abdominal cavity) and intramuscular injection IM are most common (Klesius et al., 2000). The manipulation can potentially can cause stress caused by the handling and injection of the fish but little to no mortality associated with the vaccination process *per se*. Injection has many advantages such as a longer duration of protection, multiple antigens can be combined in a single vaccine by a unique administration. Every fish in the population receives the vaccine at the correct dose and can include diverse palettes of adjuvants. (WHO, 2021)

Age of the fish at the time of vaccination is an important factor to consider for a successful immunization of a population, the administration of a vaccine by injection is not feasible when the fish is too small (fry), ideally the animal should be above 3-10 grams (fingerling or older). Other methods of vaccination can allow to vaccinate juveniles earlier. During fish development egg and larval stages, and depending on if the fertilization happened or not, the transcription of immune genes is starting and/or increasing such as the heavy chain locus of immunoglobulins at low levels, thus indicating the onset of B-cell development and the preparation to pathogen exposure (Seppola et al., 2009). It is now proven that a portion of the fish innate and adaptive immunity is transfered from

17

the parents to the offsprings. The transfer is passive and comes from the mother. In the carps, maternally derived antibodies are transfered to offsprings and offer protection against their specific pathogens (Swain et al., 2006).

Although maternal immunity doesn't exist or is not known to happen in all fish, it is composed of Vitellogenin-derived proteins and complement proteins, IgM and yolk proteins as well as lysozymes, lectin, cathelicidin (Zhang et al., 2013). Practically, the manipulation of maternal immunity transfer can be used to enhance the survival rate of fish larvae. Vaccinating broodstock was investigated in turbot and the effect of the transfer of maternal immunity did improve immune factors in offsprings and gene expression of immune genes at the early stages of development (Mingming et al., 2004). In Nile tilapia, the increase in resistance of fry offsprings from a vaccinated GIFT mother to a challenge with *Streptococcus iniae* was demonstrated (Nur et al., 2004). Later the same was demonstrated with vaccination of broodstock against A. hydrophila which could protect fry tilapia from infection through maternal immunity (Sukenda, Carman, et al., 2017; Pasaribu et al., 2018). Nisaa et al. (2017) found antibody improvement in fry Nile tilapia after vaccination of broodstock against *S.agalactiae* (Pasaribu et al., 2018). Nisaa et al. (2016) found that vaccinating broodstock tilapia during gonadal development stage 2 is the most profitable for the fry in term of transfer of maternal immunity and the most efficient at protecting the eggs and larvae after hatching against *S.agalactiae*.

Rearing conditions such as low water temperatures interfere negatively with antibody production. It seems that the threshold temperature is around 20-21 °C, below that value the expression of IgM, IL-1β, TNF-α, and IFN-γ genes is blunted and decreased resulting in a lower amount of corresponding mRNAs. Practically, a lower survival was found in vaccinated Nile tilapia after a challenge at 21 °C with *S.agalactiae* (J. Wang et al., 2020). Salinity level is also able to influence the effectiveness of the vaccination, and of the immune response. J. Wang et al. (2018) found that for a salinity superior or equal to 20 ppt, mRNA expression levels of IgM, IL-1β, and IFN-γ are down-regulated, resulting in lowered antibody production and finally in a lower survival upon infection with *S.agalactiae* (J. Wang et al., 2018).

Conservation depends upon vaccine composition and storage facilities, FKV preparations is decaying over time with a base efficiency of 70% on RPS it was shown that the

efficiency lower to 30% after one year when stored in the fridge. Moreover, the choice of adjuvants will influence the immune response of the fish and also the kinetics of the liberation of the antigens into the fish's body.

2.3.2 Current Status of Vaccines for Tilapia

The prophylactic treatments for *S.agalactiae* and *Aeromonas veronii* bacteria that are now available are given in table 2.2 below. In practice, commercial vaccinations against *S.agalactiae* and *A.veronii* bacteria is very popular in Brazil (more than half of the country's tilapia are vaccinated by injection) and Israel. These vaccines are made from microorganisms of different strains and also contain adjuvants, usually a single injection will protect the fish during its lifetime. They arrive as a solution in a multi-liter container that may need to be diluted. The vaccine must then be injected by someone who is capable of doing so and has expertise vaccinating animals without injuring them.

Because tilapia is a low-value species, vaccination may not be cost-effective or desirable in some countries/regions owing to the expense of syringes, handling, or simply the time necessary to vaccinate large numbers of fish. This is why creating a low-cost vaccine that protects against many infections (heterologous, bivalent vaccination) might help to alleviate these limits until more efficient injectable commercial vaccine production can scale up and lower the costs.

Table 2.2

Vaccines against Streptococcus agalactiae and Aeromonas veronii for aquaculture.

Pathogen	Vaccine type	Antigens/targets	Delivery method	Vaccine name	Company
Streptococcus agalactiae Serotype Ia & Serotype III	Inactivated	Whole cell inactivated Streptococcus spp.	IP	Strep Sa	AQUAVAC
Aeromonas veronii	Inactivated Oil-based (Palm oil)	Whole cell inactivated Aeromonas spp.	IP	Autogenous Aeromonas veronii vaccine	PHARMAQ AS

19

There are several ways to vaccinate (Munang'andu et al., 2016), such as immersion of fish in water bodies containing bacterial antigens, intraperitoneal / intramuscular injections of bacterial antigens, and oral vaccination through ingested food which consists of a mixture of food and antigens. These three methods are summarized in the table 1.3 below as shown by (Vinitnantharat et al., 1999).

Table 2.3

Comparison of three vaccination methods in fish. (Vinitnantharat et al., 1999)

	Immersion	Injection	Oral
Application	Easy	Delicate	Very easy
Stress	Light	Moderate	No
Job / labor	Moderate	Intensive	No
Efficiency	Good	Excellent	Passable
Duration	3-12 months	12-24 months	2-4 months

Simple HKV can be used as a prophylaxis treatment for aqua-cultured freshwater fish, including Nile tilapia. This method is cost-efficient and generally give good results in terms of survival and economic benefice (Delphino et al., 2019) but has not yet been developed and evaluated for a combinaison of *S.agalactiae* and *A.veronii*. A combined vaccination with a bivalent vaccine formulation is without doubts the best solution for preventing those two infections at once. Two pathogens, *S.agalactiae* and *A.veronii* are the etiological agent of bacterial infections in freshwater cultured fish as explained previously. Bacteria have cosmopolitan distribution. They induce mass mortality of the infected fish within a few days. The two virulent bacterial isolates used in this research originate from Thailand and were isolated from sick fish.

Previous literature has shown the possibility to develop monovalent and polyvalents vaccines with different modalities of vaccination (Munang'andu et al., 2016). An efficient vaccine must protect at least 70-80% of animals and for a long period but even 50% of RPS is beneficial to the farmers on every aspects (Delphino et al., 2019). Many vaccines were developed for aquaculture but an heterologous vaccine that would protect against *S.agalactiae* and *A.veronii* is yet to come. The first assumption for the

research, is that it might be possible to create a bivalent vaccine version of whole inactivated pathogens.

Infact, planning and executing a vaccination by injection in two doses is labor intensive and time consuming (Table 2.3) but also twice the amount of needles and vaccine volume would be needed. Until now, Nile tilapia are vaccinated with different whole killed cell vaccines of *S.agalactiae* and *A.veronii* alone (Table 2.2). Because we aim to produce simple and inexpensive vaccines for small scale farms we decide to use the immersion and oral methods for the delivery of vaccines. We also decided to select soybean oil as a coating agent for our pellets because it is available everywhere and a quite affordable way to prepare the oral vaccines.

2.3.3 Gaps and Limitations

In summary, as explained previously, there are 3 ways to vaccinate the fish. In practice, the injectable vaccine is preferred. The vaccine is oftentime adjuvanted with oil (vegetal or mineral, or IFA) or with other components in order to prolong the immune stimulation, with a higher production of antibody and for a longer time, thus leading to increased vaccine potency. One to two doses may be needed. It is laborious and expensive. Despite those constrains, Delphino et al. (2019) explained that the vaccination of the fish against *S.agalactiae* is very likely to be profitable in more than 97% of cases in Brazil when the cumulative mortality in the fish farm would be as important as 20% of all tilapia for one production cycle. There are benefits for the farmers on the economic aspect, but also on the environment by reducing the use of antibiotics. It was determined that a vaccine with at least 50% efficacy (i.e. able to protect half of the population of fish, assessed by the use of RPS indicator) would be profitable for brazilian farmers.

Bacterial diseases plague tilapia farmers all over the world, particularly when they are intensive fish farming in hot climates or during extreme heat events. The issue is that there are few vaccines accessible throughout the world, and none are available in Thailand at this time. (Kayansamruaj et al., 2020). Commercial vaccines may be costly, and they typically do not cover all bacterial infections. Furthermore, there is a paucity of knowledge on the immunological response of fish to vaccines, as well as the influence of adjuvants on the immune response and vaccines need to be proven safe for the fish and for human consumption by health authorities.

CHAPTER 3
MATERIALS AND METHODS

3.1 Experimental Fish and Husbandry

Nile tilapia fry are obtained from AIT, the university local hatchery. All animals are at first kept in a single large tank and observed for diseases for two weeks. Then they will be split into 6 tanks and acclimated for at least a week, the remaining fish are kept in the single 1000 L large tank. Five weeks after the vaccination day, all of the remaining animals are transferred to 6 new tanks inside a level-2 biosecurity room. It is a requirement for the challenge trial, thus preventing a leak of pathogenic organism into the surrounding environment.

Figure 3.1

Semi-open 100 liters tank system in use for fish rearing during the experiment. A second similar system is installed in the adjacent biosafety room and is in use for challenge trials.
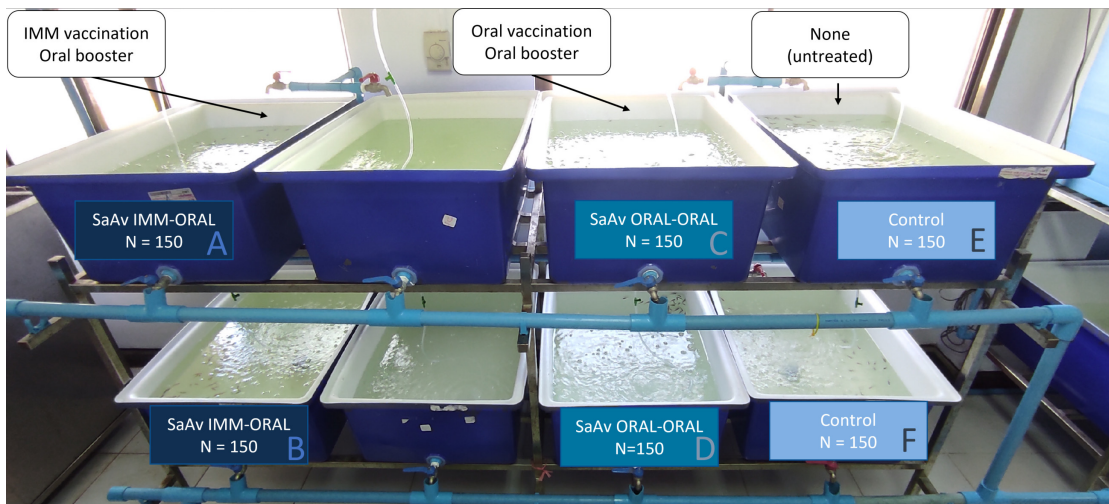
3.2 Bacterial Preparation and Bacterial Culture Method

3.2.1 Strains and Media

Two bacterial strains, *S.agalactiae* and *A.veronii* were recovered from frozen glycerol cryo-conservation solution by culturing them onto rich TSA/TSB culture media for 24h. The two bacterial isolates used in this research originate from Thailand and were isoled from a dead diseased Nile tilapia. TSA stands for tryptic soy agar (TSA), it is the solid countertype of liquid tryptic soy broth (TSB). The culture is done inside of an incubator, at a temperature of 30C.

Table 3.2

Summary of the parameters for the culture of bacteria in the incubator.

Bacteria strain	Growth conditions	:
Aeromonas veronii	Culture Media:	Tryptic soy broth TSB
	Temperature:	30C
	Shaking:	Yes
Streptococcus agalactiae	Culture Media:	Tryptic soy broth TSB
	Temperature:	30C
	Shaking:	Yes

3.2.2 Bacterial Culture Method

A bacterial culture is an active population of bacteria which will develop and multiply at regular interval, 1 becomes 2 which divides to form 4 (2^2) then 2^3 2^4 2^5 2^6 and so on. The culture is stopped at the desired optic density following the previsions obtained previously by the growth curve, we can target at which time we should harvest our bacteria. It is wise to harvest at the mid-log phase or at the end of the exponential growth phase (end-log phase) because the bacteria are multiplying at a high rate. To determine approximately what is the concentration in viable bacteria in our 800mL culture, a sample is taken and serial dilutions are made to the billion and petri plates can be spread with the last 4 dilutions to obtain CFU/mL. Counting of bacterial cells can also be done in haemocytometer.

Cell forming units (CFU/mL) is a semi quantitative measure of the ability of a bacterial solution to create colonies. A bacterial colony on a petri dish can only develop from a single viable bacteria, therefore it does not count the dead cells and the liquid phase of the bacterial solution from which the bacteria originates. In our project, our goal is to produce a solution containing antigenic particles from the bacteria (dead + alive). The concentration of the vaccine is probably a little bit underestimated for that reason.

Figure 3.2

Production of heat killed vaccine solution: A bacterial culture is grown until a specific OD600, then a hot water bath is heated to 56C, the flasks have a cap and don't allow the water to get in. The bain-marie will inactivate the bacteria within half an hour. (A) The two microorganisms employed in the vaccine formulation were from previously infected farms or wild fish. The germs were kept cold and cultured on agar to keep them alive. A single colony is chosen and cultivated in sterile TSB culture media in a Falcon tube. To make an aliquot that will be utilized as a growth starter, the culture must be allowed to develop for at least one night (inoculum). In order to produce an OD600 = 0.01-0.025, a volume of roughly 10-15 mL of O.N (overnight) bacteria is poured from the falcon tube into a Duran flask holding approximately 800 mL of sterile TSB. The future solution to use for the immersion challenge trials, the culture was set on growth O.N. at 140 round per minute and at a temperature 30 Celsius inside a dedicated incubator, in order to yield the maximum amount of bacteria. (B) The cultures (A) are shaken and incubated at 36C for 5-6 hours to prepare the future vaccine solution, and the optical density is determined by taking a sample in triplicate and diluting it by 10, such as 1 volume of culture for 9 volumes of TSB (9+1). The TSB that was utilized to create the blank is the same sterile TSB that was used to culture the two bacteria. Prior to inoculation, a tiny amount of additional TSB should be preserved to do the blank. The same sample is utilized to create serial dilutions without wasting more time, and the three lowest dilutions are used to count the viable colonies by pouring a volume of 100uL on the petri dish using the spread plate technique (which counts also as an additional 10 fold dilution). (C) The colonies are counted the next day on the plates that were prepared in duplicate the day before. For the optical density at which the culture was halted and utilised, the number of viable colonies may be roughly calculated for reach of two microorganisms.

A.

Streak Sa from stock onto TSA	→	Inoculation of a single colony of bacteria Sa in 30mL (overnight)	→	Inoculation of O.N 30mL Sa in 800mL TSB

Streak Av from stock onto TSA	→	Inoculation of a single colony of bacteria Av in 30mL (overnight)	→	Inoculation of O.N 30mL Av in 800mL TSB

B.

Harvest 5-6 hours later and read OD. Take sample for viable bacterial count. **Either use live bacteria or inactivate**	→	Read OD600, Using blank TSB Dilute 10 times to read OD. Sa = 0.170*10 = 1.7	→	Serial dilutions in TSB 8 times 1+9mL. 10^{-1} to 10^{8}	→	Streak 100uL Sa onto TSA 10^{-7}, 10^{-8}, 10^{-9}

Harvest 5-6 hours later and read OD. Take sample for viable bacterial count. **Either use live bacteria or inactivate**	→	Read OD600, Using blank TSB Dilute 10 times to read OD. Av = 0.220*10 = 2.2	→	Serial dilutions in TSB 8 times 1+9mL. 10^{-1} to 10^{8}	→	Streak 100uL Av onto TSA 10^{-7}, 10^{-8}, 10^{-9}

C.

Count colonies between 30-300	→	For Sa OD=1.7 CFU.mL^{-1}=1.18*10^9	→	Inactivate to produce vaccines **or** use live bacteria for challenge

| Count colonies between 30-300	→	For Av OD=2.2 CFU.mL^{-1}=0.217*10^9	→	Inactivate to produce vaccines **or** use live bacteria for challenge

25

3.3 Preparation of Heat-Killed Vaccine Solution (HKVs)

A tiny portion of a few mL is utilized for serial dilution and spread plate viable colony count once the culture in TSB reaches the appropriate OD600 after a few hours. Meanwhile, the Duran flask is immersed in a pre-heated 56C hot water bath for 30 minutes. The heat will inactivate and damage the bacterial cells of *S.agalactiae* and *A.veronii*. A small amount of the heat killed solution can be distributed on a petri plate to confirm that no microorganisms are present and hence that inactivation has been achieved and the Duran flasks of a volume of 1 liter are kept in the refrigerator which will be used to make bath immersion vaccines and oral mucosal feed vaccines.

Figure 3.3

Production of heat killed vaccine solution: A bacterial culture is grown until a specific OD600, then a hot water bath is heated to 56C, the flasks have a cap and don't allow the water to get in. The bain-marie will inactivate the bacteria within half an hour. Each bacteria is cultivated separately on its own in sterile broth. (A) For Streptococcus agalactiae (B) For Aeromonas veronii.
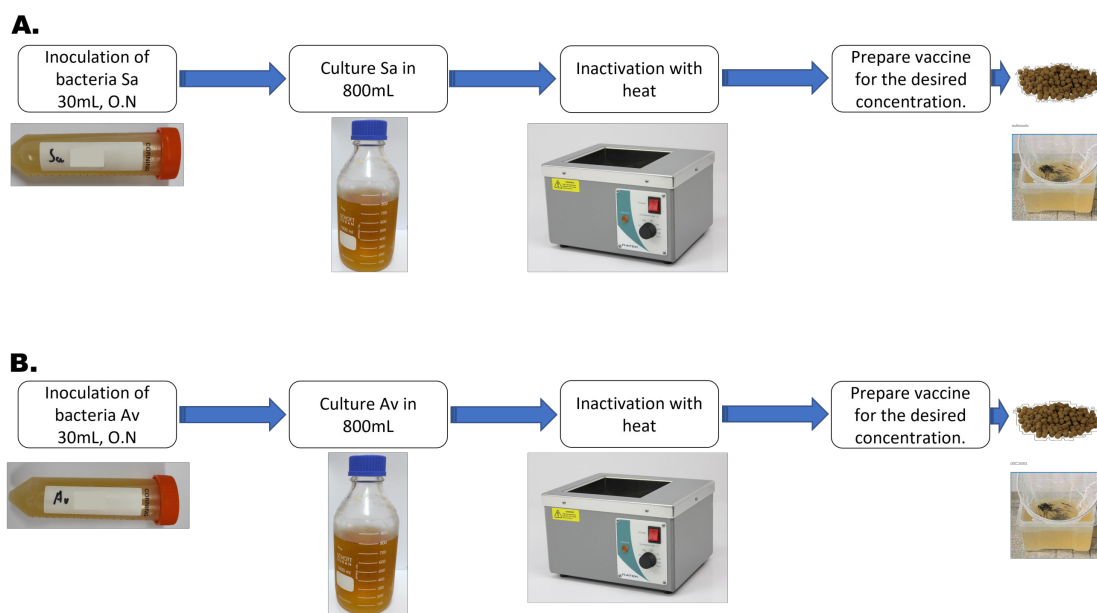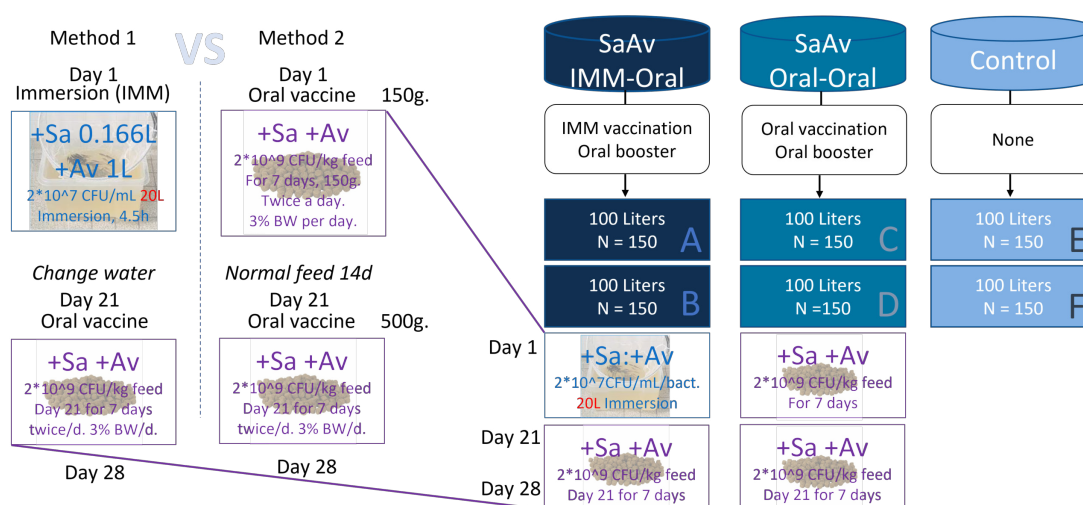
3.4 Vaccination

In the first vaccination method immersion + oral (booster) (IMM+OR), fish from the same source $(1.1 \pm 0.1g)$ were randomly assigned to two indoor tanks A, B (with 150 fish/tank), fish were immersed in a vaccine solution on the first day for 4.5 hours and fed 21 days later (on day 22) with 3% BW of oral vaccine feed pellets coated in oil, twice daily (noon and afternoon) for 7 more days.

In the second vaccination method oral + oral (booster) (OR+OR), fish $(1.1 \pm 0.1g)$ were randomly assigned to 2 indoor tanks C, D (with a density of 150 fish/tank) and among which 2 of the tanks were supplied with 3% BW of oral vaccine feed pellets coated in oil, twice daily (noon and afternoon) for a week, from day 1 to day 8, then normal feed was given for 14 days. On day 22, oral feed booster was given for 7 days until day 29.

Figure 3.4

*Experimental design for vaccination: The round shapes are the 3 treatments (IMM-OR, OR-OR, Control). The composition of the four vaccines is the following : **Tanks A, B IMM+OR:** First vaccine exposure by bath immersion $2 * 10^7 CFU/mL/bacteria$, and a booster by feeding oral bivalent vaccine soybean oil coated pellets for 7 days at day 22. **Tanks C, D OR+OR:** Two weeks of bivalent oral vaccine coated with soybean oil and fed from day 1 to day 8, then from day 22 to day 28 (booster). **Tanks E, F Ct:** No bacterial strain hence no antigens from Sa or Av, therefore no immunization is expected.*

3.4.1 Method 1: Bath Immersion

Bath immersion is a method of vaccination easy to do. First, all the fish from tank A and from tank B are moved into 2 buckets containing 20 liters of water in each. In each bucket, heat-killed vaccine solution (HKVs) will be added until the bucket reach a final concentration for a single bacteria of 10 million of CFU/mL. Therefore the total concentration in Sa and Av is 20 millions of CFU/mL so the bucket contains the 2 types of bacteria. From our inactivated Sa culture containing $1.2 * 10^9$ CFU/mL 166mL is added to 20 liters. And from inactivated *A.veronii* culture concentrated at $0.217 * 10^9$CFU/mL, one liter is added to 20 liters.

Figure 3.5

*Procedure for vaccinating with a bath immersion of the fish into a water containing the diluted vaccine solution. **(A)** The fish to be vaccinated will be immersed in two tanks holding water and vaccination solution. To vaccinate 150 fry, a volume of 20 liters is adequate. For best impact, vaccination should last 4.5 hours, and it was empirically terminated when 2-5 fish perished, most likely owing to stress caused by environmental change. During the immersion vaccination procedure, the tanks are oxygenated. **(B)** A summary of the procedure shows that after 4.5 hours, 3% of the fish perished.*

A.

B.

2*10^7
CFU/mL   4.5 h

Mortality after 4.5 hours = 3 %
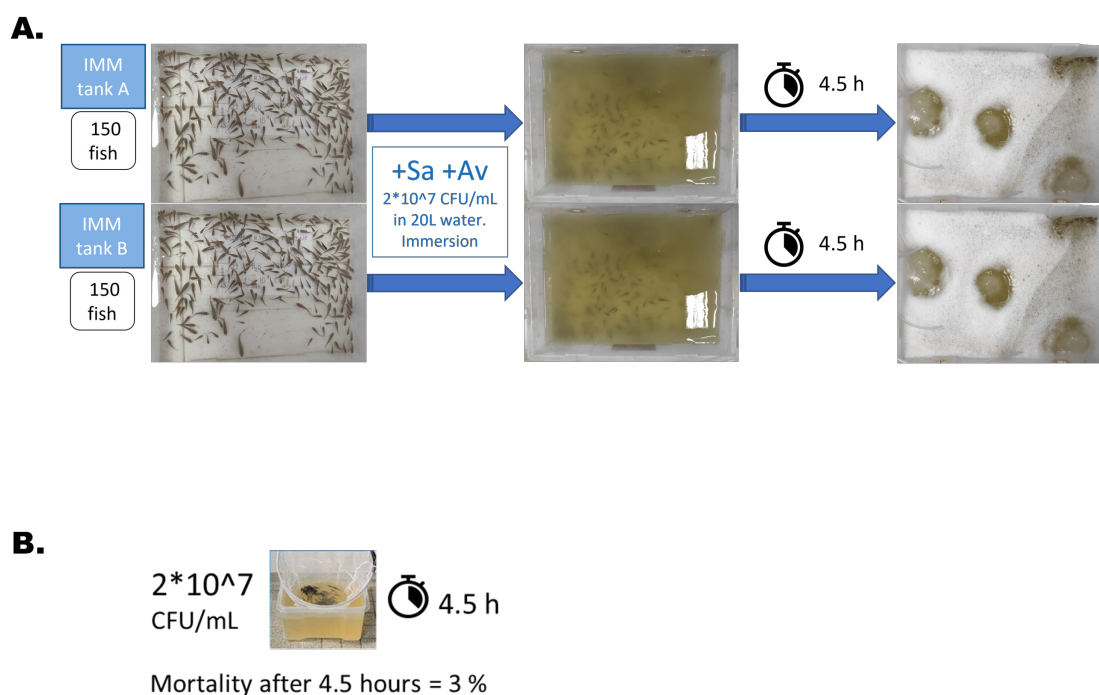
3.4.2 Method 2: Oral Mucosal Feed Vaccine

Feed pellets are mixed with dead bacteria at a concentration of 10^9 CFU of *Aeromonas veronii* per Kg and 10^{10} CFU of *Streptococcus agalactiae* per Kg of feed to make oral feed. Wet from bacteria, the feed is coated with soybean oil by simply mixing it in, then dried overnight in a 36-40C oven (the step is not shown in the following figure). Before using, the feed is kept in the refrigerator.

Figure 3.6

Procedure for the preparation of oral vaccine consisting in inactivated bacterium added to the feed pellets and coated with soybean-oil. (A) The feed pellets are placed inside of a beaker. Bacteria is poured onto the feed and mixed evenly. Then soybean oil is added to the beaker to coat the feed. (B) The pellets inside of the beaker are placed onto a tray on aluminium and placed in oven overnight. The feed is divided into 2 bags, one bag for the first vaccination (from day 1 to day 7), one bag for the booster vaccination (from day 21 to day 28).

29
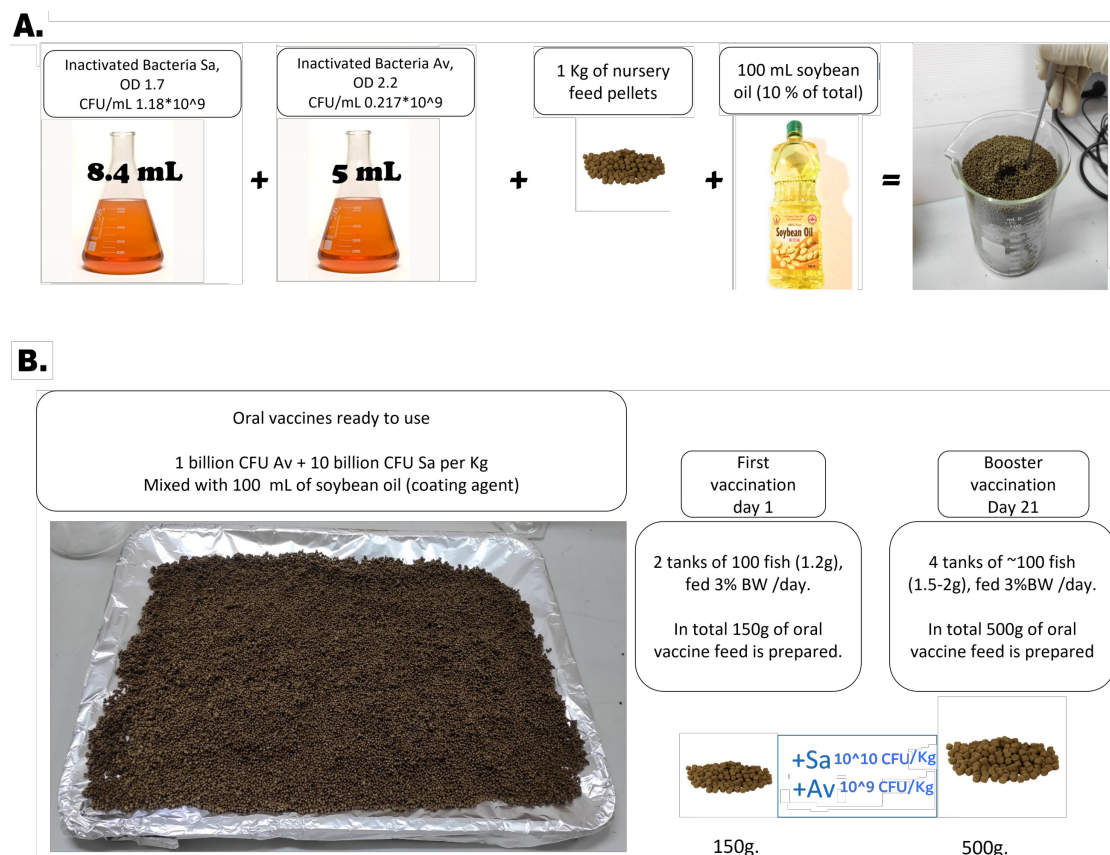
3.5 Preparation of Head and Gut Supernatants Containing IgM Antibodies

This section shows how to isolate the supernants containing IgM soluble proteins so that the effects of vaccination on antigen-specific antibody levels may be studied further. IgM is the predominant class of antibodies in the fish sera but also in tissues and therefore must be extracted. Because the fry is small, it is difficult to do a blood swab and therefore it was decided to use the whole head (which contains headkidney and gills) with high immune system activity, but also the gastrointestinal tract because it is the first line of defense against ingested pathogens

Figure 3.7

Procedure for obtaining the immunoglobulin-M containing supernatant: (A) Clove oil terminates the fish. Animals are dissected, the operculums are removed, and the head and intestines are taken. (B) To avoid fungus contamination, the head is homogenized in 600uL of PBS containing 0.02 percent sodium azide. Inside an eppendorf tube containing 300mL PBS and 0.02 percent sodium azide, gut tissues (digestive system, intestine, spleen, and gallbladder) are homogenized. The supernatant is centrifuged and transferred to a fresh eppendorf tube and kept in a freezer at $-20\,^{\circ}\mathrm{C}$.
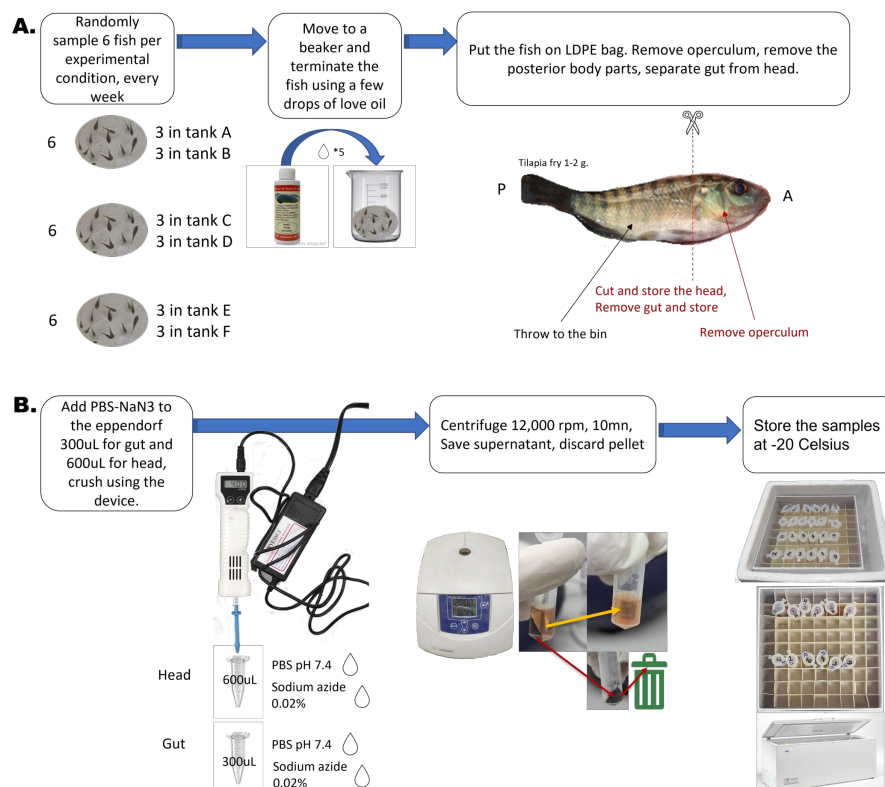
### 3.6 Methodology for ELISA Assays for Specific IgM Levels

ELISA is an enzyme-linked immunosorbent assay "for the presence of antibodies, antigens", proteins and glycoproteins in biological samples. ELISA technique is widely used for rapid diagnostic tests such as the diagnosis of HIV infection, pregnancy tests or the detection of food allergens. The principle of this technique is based on the use of an enzyme conjugated to an antibody which by reacting with a colorless substrate gives a colored reaction product and which is therefore detectable. The colorful product is refered as chromogenic substrate. Different enzymes are used for ELISA tests including alkaline phosphatase, horseradish peroxidase (HRP) or beta-galactosidase. The ELISA assay measure the relative level of antibodies and/or give information on the antibodies (Ab) titer of the fish in IgM. There are few variations of ELISA assays such as direct, sandwich and indirect elisa.

Figure 3.8

General workflow for ELISA assay.
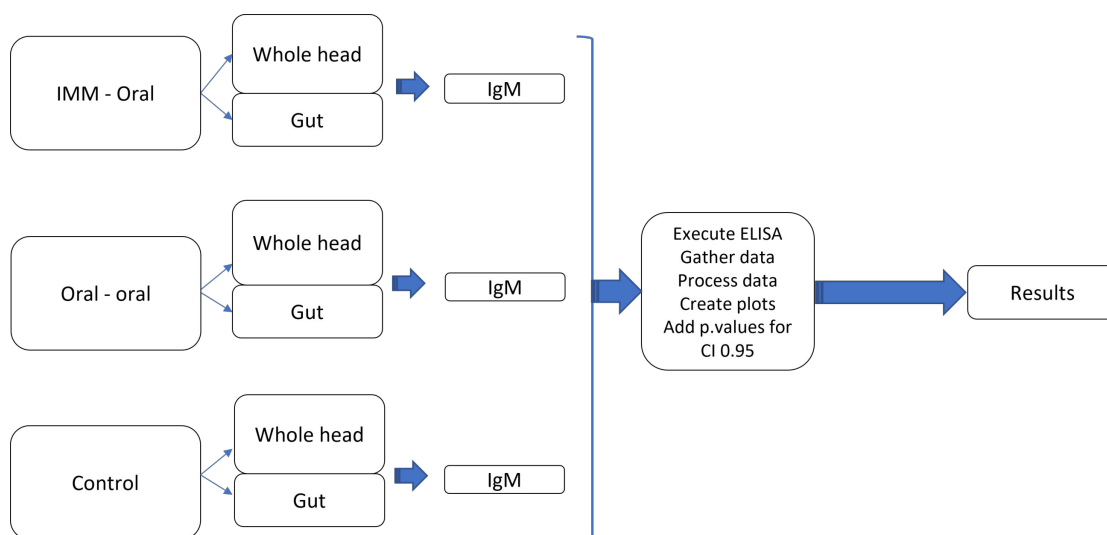
The strength and amount of antibody response in the head and gut samples was determined by indirect ELISA. In the assay, the antigen consisted of heat inactivated whole cell bacteria, either Sa or Av immobilized during 2 hours at room temperature by a direct adsorption to the assay microplate after dilution into carbonate coating buffer, pH 9.6. Detection of the antigen can then be performed by using a matched set of primary
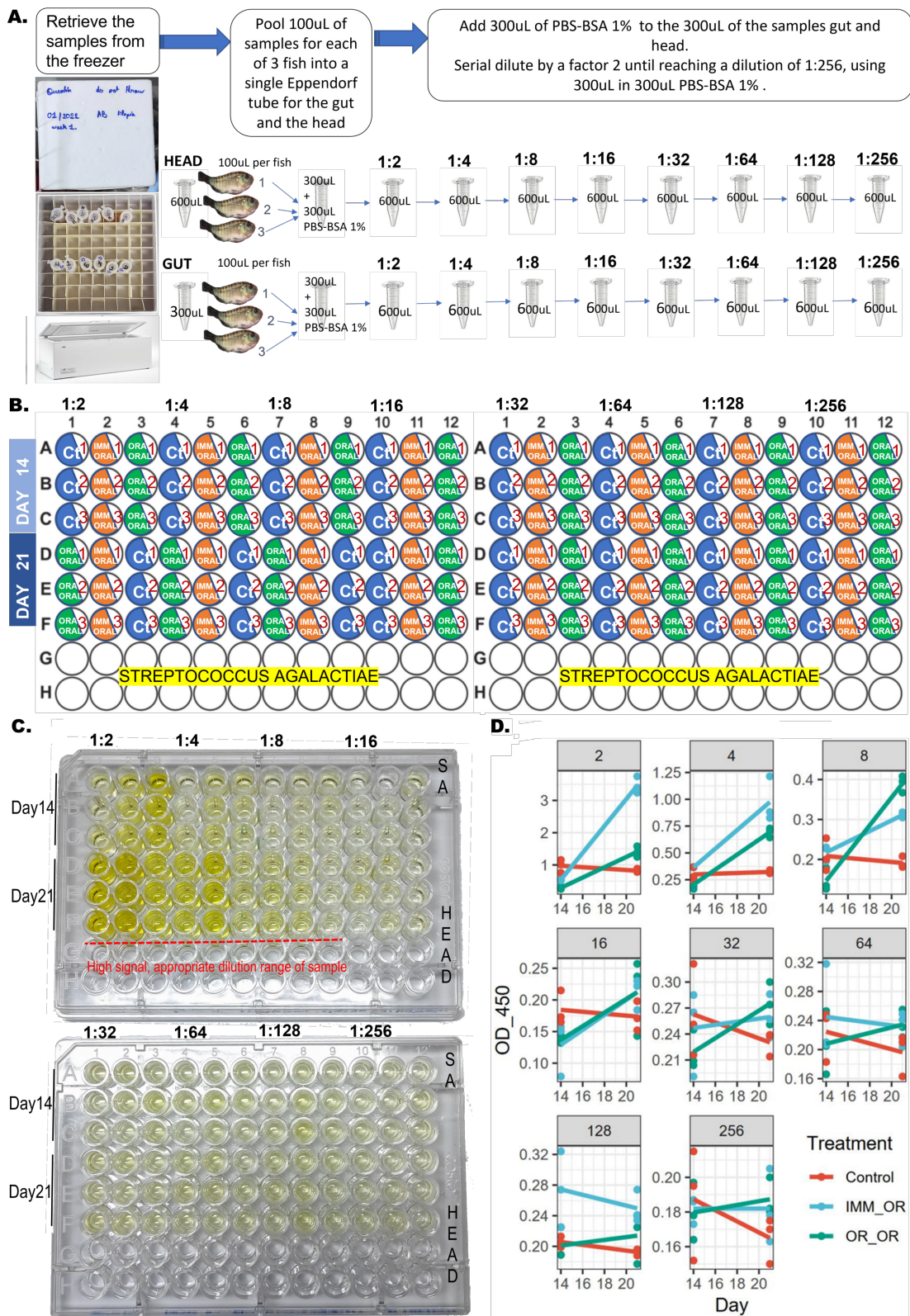
antibodies contained in a serum (head serum or gut serum) and conjugated secondary antibodies consisting of tilapia anti-IgM. Chromatic signal was revealed upon addition of goat anti-mouse antibody horseradish peroxidase and its substrate (TMB). Washing occurred between each steps using PBST (PBS, containing 0.05% Tween-20). Sera, secondary antibody and HRP were diluted in PBS-BSA (1%) at respective dilutions of (1/2,1/4,1/8), (1/50), (1/3000). The absorbance was measured 3 times at 450nm.

3.6.1 Determination of the Optimal Sample Dilution

It is crucial to identify the best sample dilution for each sample type since undiluted samples may result in excessively high OD450 readings. The optimal dilution is one with the greatest difference between the control and vaccinated groups. Within the linear range, with a low non-specific binding signal to signal ratio and a high signal to background noise ratio, the best dilution is found. To determine the proper dilution for the ELISA assay, whole head supernatant or whole gut supernatant samples were collected from three fish from each of the three experimental groups: control, immersion-oral, and oral-oral, and 2-fold serially diluted solutions were utilized for each time period. As a result, the effective dilution for all plates and samples at each timepoint may be established. From the following results we decided to go for dilution 8 which is the most accurate and only this dilution will be represented in the graphs and will be used to draw conclusions and results.

Figure 3.9

Procedure to determine what is the best dilution of the sample for the ELISA assay. (A) Samples are removed from the freezer and 3 fish supernatants ($100uL$ each) per group for the same timepoint are added to an eppendorf containing 300uL of $PBS-BSA1\%$. Then serial dilutions are carried out until a dilution factor of 256 is reached. (B) Platemap of the ELISA showing how the samples are arranged. For the head, days 14 and 21' were selected because the samples are likely to contain streptococcal-specific antibodies. The blue dots represent the control group, the orange dots represent the immersion-oral group and the green dots represent the oral-oral group. (C) Results obtained by indirect ELISA for the above platemaps. It is possible to see that some wells are darker than others, especially for day 21 in the two groups that are not the control. Dilutions by 2, 4, 8 were retained to carry out the rest of the elisa assays because the signal is strong compared to the control. (D) The previous plates were put in a 96 wells microplate reader and the corresponding values were added on an excel sheet and plotted for each dilution (from 2 to 256). The values are consistent at dilutions by 2, 4 and 8 but beyond that the values are no longer reliable, probably because of pipetting errors.

A. Retrieve the samples from the freezer → Pool 100uL of samples for each of 3 fish into a single Eppendorf tube for the gut and the head → Add 300uL of PBS-BSA 1% to the 300uL of the samples gut and head. Serial dilute by a factor 2 until reaching a dilution of 1:256, using 300uL in 300uL PBS-BSA 1%.
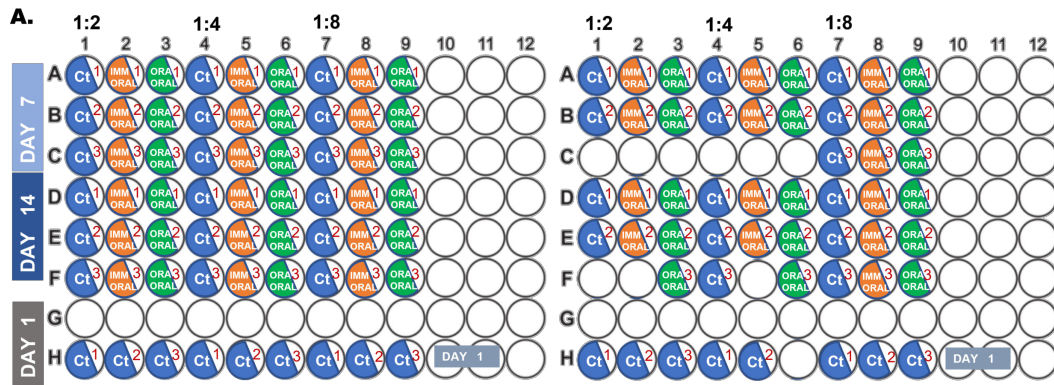
3.6.2 Indirect Elisa Assay for IgM Levels in the Head

The goal of this experiment is to figure out what the approximate relative levels of antigen-specific immunoglobulins M (specific the two bacteria) in the samples would be. By relative, we imply how a group's values compare to those of other groups. Be-
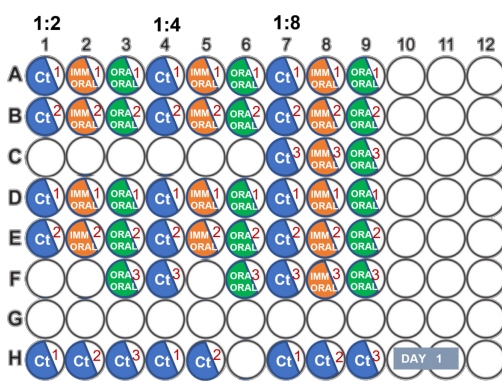
cause each sample comes from a similar origin and each population of fish that has received the same vaccine, these will be subjected to the same indirect ELISA procedure. As a result, it will be feasible to determine which of the three groups (IMM+OR, OR+OR, and Control) will have a value that differs from the others at each timepoint. Therefore, a high value of antigen-specific immunoglobulins in one group compared to another indicates that the sample studied contains a greater amount of IgM. The actual protein titer in the sample can't be determined, but that's not a big deal because we're only looking to see if populations exposed to different vaccinations create varying levels of specific IgM, which is a disease-fighting protein.
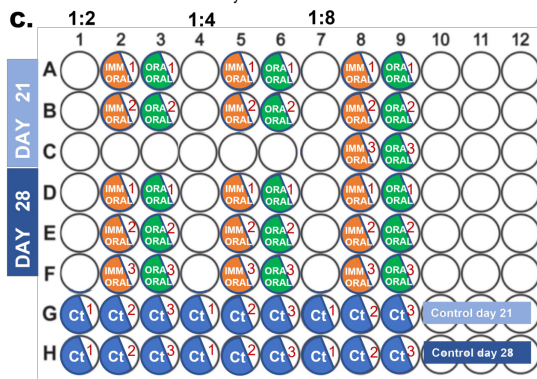
Figure 3.10

(A) Indirect ELISA plates, the left plate was adsorbed with whole dead Streptococcus agalactiae antigens while the right plate was adsorbed with whole dead Aeromonas veronii antigens. The blue pellets represent the samples from the control group, that is to say the non-vaccinated fish. The orange dots represent the group initially vaccinated by immersion with an oral vaccine booster on day 21 for 7 days. The green pellets represent the group vaccinated orally for 7 days and then a second time on day 21 for 7 days. The white circles mean the absence of serum or antigen coating. Each sample is deposited in 3 different wells (triplicate) from up to bottom which is indicated by the small red number on the upper right corner of the pellet. Plates contains fish sample from the day 1, 7, and 14 post-vaccination. The samples were diluted by $1:2$; $1:4$; $1:8$. Some wells were discarded because of pipetting errors or improper preparation. (B) Associated result after stopping the chromatic enzymatic reaction with acid. The plaque changes from blue to yellow depending on the analyte/antibody concentration. The plates are read in a microplate reader in order to obtain the absorbance values at $450nm$. (C) Similar to (A), with plates containing fish sample from the day 21, and 28 post-vaccination(s). (D) Similar to (B), photos of platemaps from day 21 and 28 post-vaccination(s).

A.

STREPTOCOCCUS AGALACTIAE (head)

AEROMONAS VERONII (head)

B.

Oral vaccination gives the highest IgM values

Immersion, very high IgM levels

Control, unvaccinated, negligible IgM levels

C.
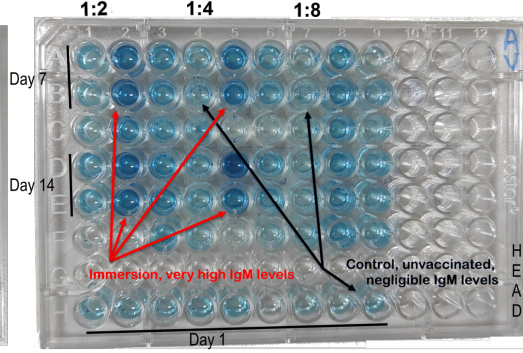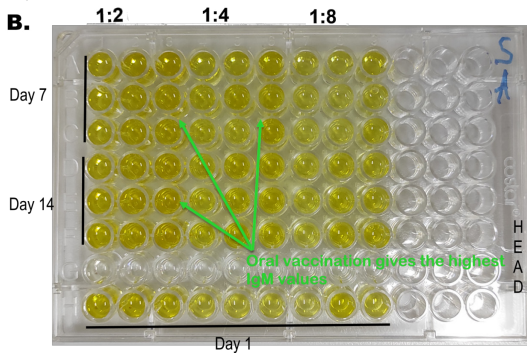
STREPTOCOCCUS AGALACTIAE (head)
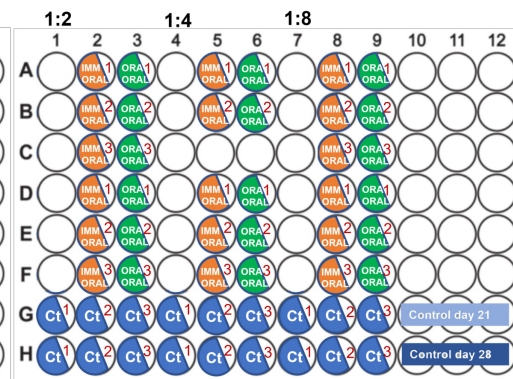
AEROMONAS VERONII (head)

D.

Figure 3.11

*Boxplots of the specific antibody IgM relative levels specific to antigens (**A**) S.agalactiae (**B**) A.veronii in whole head of 6 fry Nile tilapia per timepoint and per group immunization, assessed by indirect ELISA assay. Absorbance is on the y-axis and measured at a wavelength of 450nm. The x-axis represents the different timepoints: day 1, 7, 14, 21, 28, and 42 post-vaccination(s).*
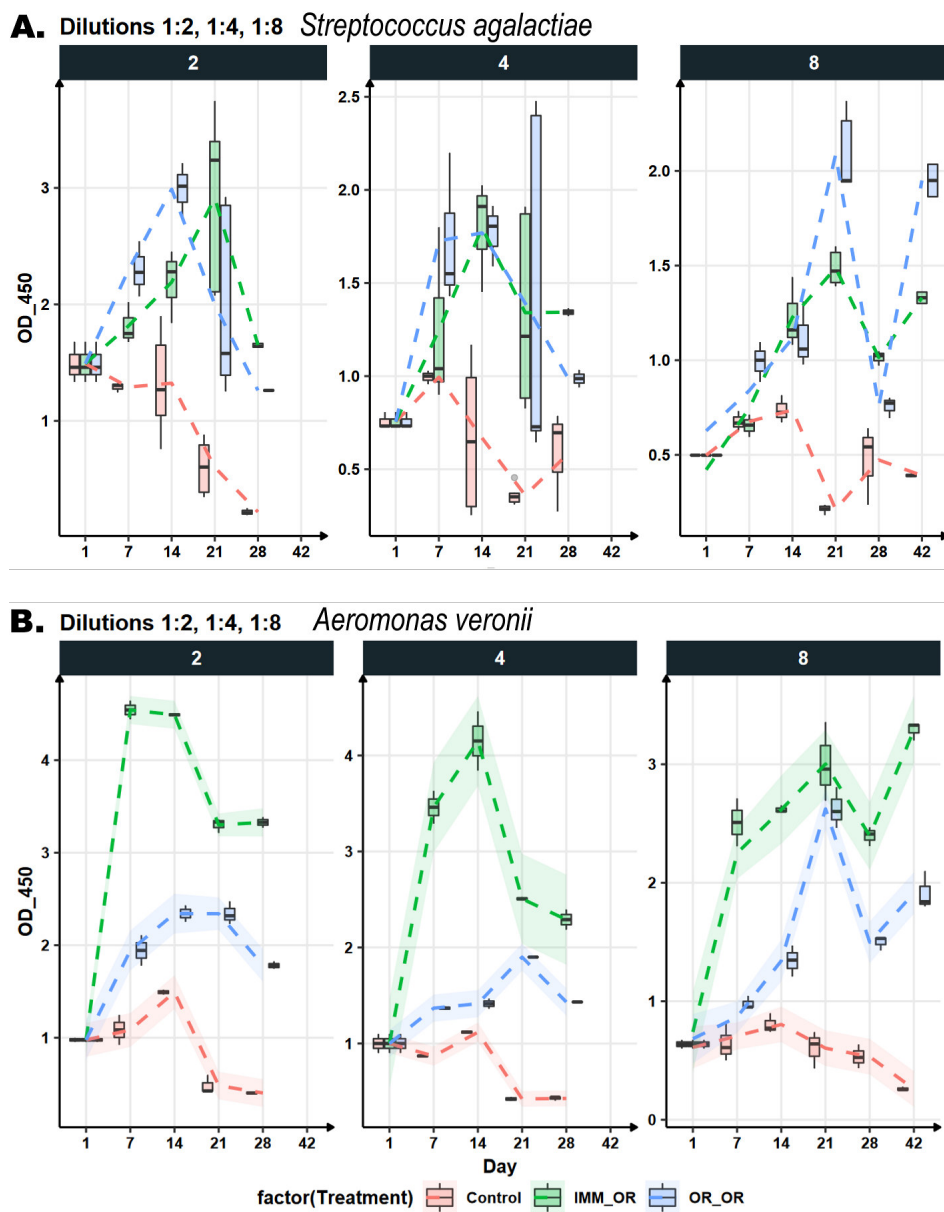
3.6.3 Indirect Elisa Assay for IgM Levels in the Gastrointestinal Tissues

Teleost fish mucosal surfaces are constantly exposed to a wide range of pathogens and are also habitat to large densities of microbes due to direct exposure with the aquatic environment. In teleost mucosa-associated lymphoid tissues (MALTs), B lymphocytes and immunoglobulins play critical roles in local mucosal adaptive immune responses. From their genomic sequence, three Ig isotypes (IgM, IgD, and IgT/Z) have been acknowledged (Yu et al., 2020). Following vaccination of the fish, gut-associated lymphoid tissue, or GALT, may have a high level of IgM specific for the antigens included in the vaccines. The purpose of indirect elisa using fry Nile tilapia gastrointestinal tissues is to see if antigen-specific IgM levels in the fish serum alter within groups following vaccination.

Figure 3.12

Specific antibody IgM levels against (A) S.agalactiae (B) A.veronii in whole gut of 6 fry Nile tilapia per timepoints and per group, assessed by indirect ELISA assay. The samples (primary antibody / serum) are diluted 8 times.
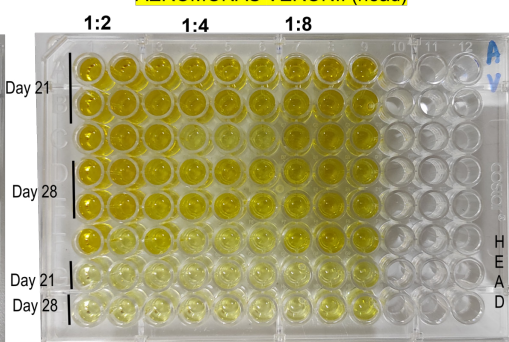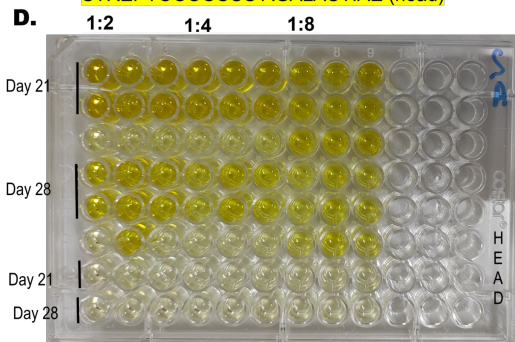

38
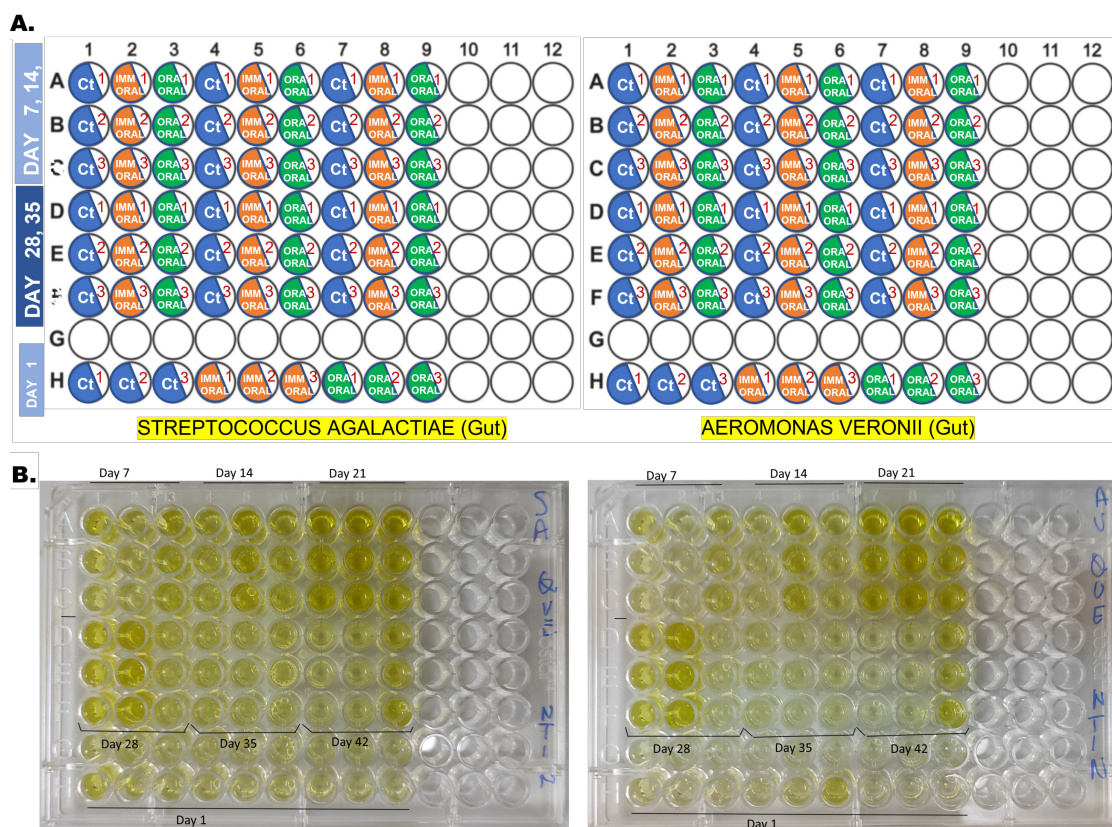
**Figure 3.13**

Boxplots of the specific antibody IgM relative levels specific to antigens (A) S.agalactiae (B) A.veronii in the GALT of 6 fry Nile tilapia per timepoint and per group immuniza-tion, assessed by indirect ELISA assay. Absorbance is on the y-axis and measured at a wavelength of 450nm. The x-axis represents the different timepoints: day 1, 7, 14, 21, 28, 35, and 42 post-vaccination(s).
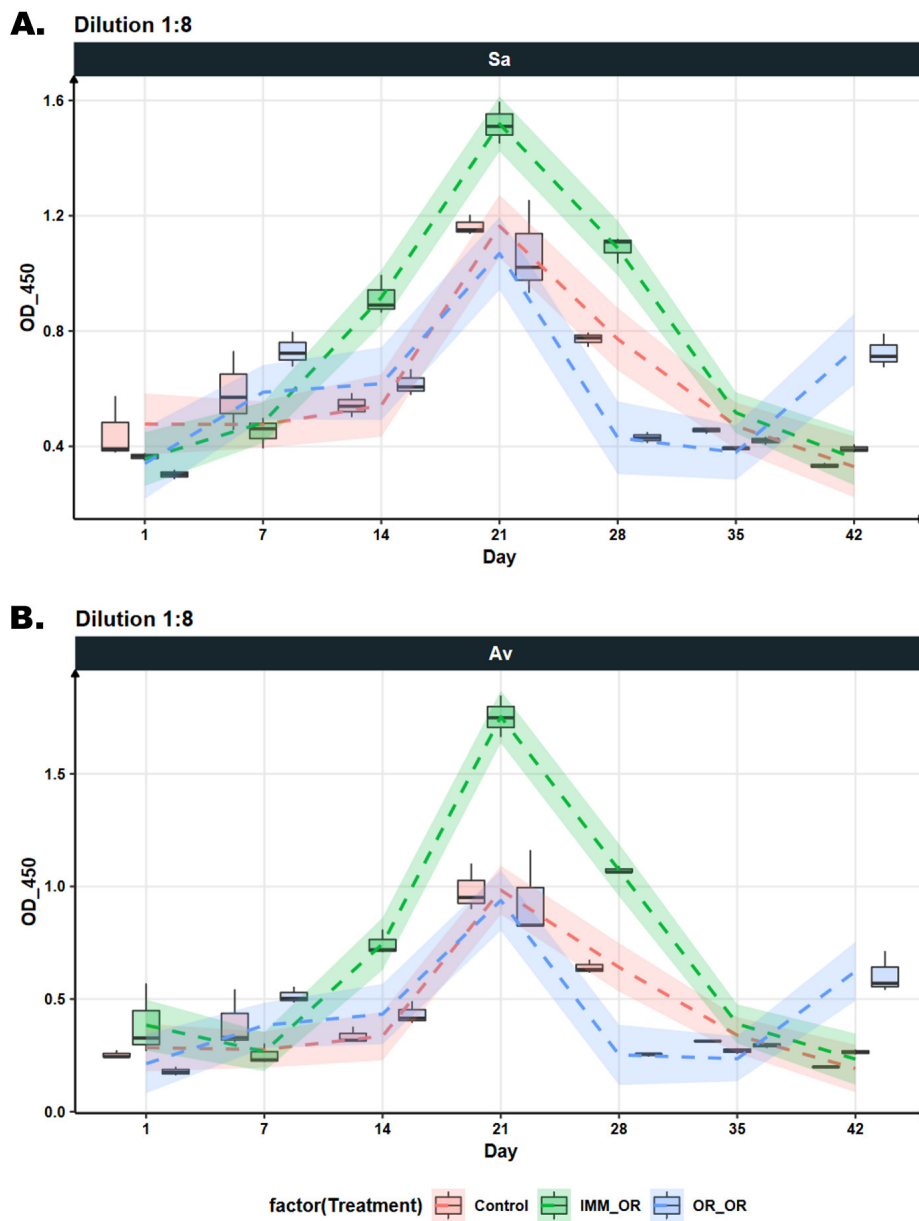
3.7 Bacterial Challenge Trials

A total of eight tanks are required for the challenge trials. There are two controls: a positive control (unvaccinated fish challenged with Sa or Av) and a negative control (only water without live bacteria). During the 10-day post-challenge phase, mortality is monitored on a daily basis. After four days of no mortality, the challenge trials were terminated, and the overall survival probabilities were computed.

Figure 3.14

Experimental design for the survival challenge trials.
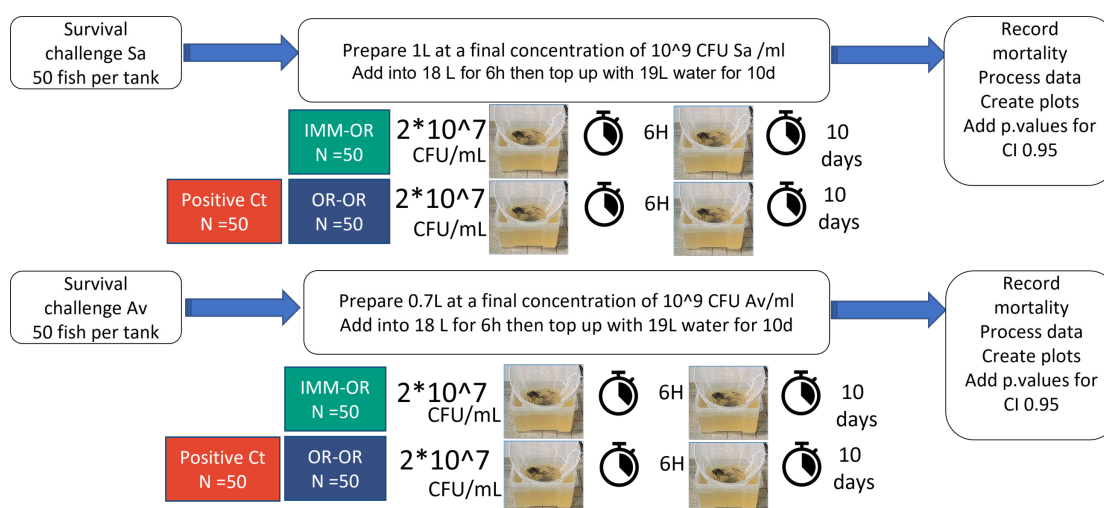
For *S.agalactiae*, in a first trial, groups of 50 fish are challenged from day 42 post vaccination for 10 days/240 hours. Fish received bacteria by bath immersion on day 42 at 6.15PM for 6 hours until 00:15AM, immersion consist in a lethal dose of pathogen in a volume of 1 Liter with 1 billion Streptococcus agalactiae /mL in TSB, diluted into the 18 liters of water contained in the trial tank (1+18L). Then 19 liters were added to the tank to top up to 38 liters, for the remaining 9 days. Oxygen is supplied with air stones and the fish are fed as usual with 3%BW a day.

For *A.veronii*, in a second trial, groups of 50 fish are challenged from day 42 post vaccination for 10 days/240 hours. Fish received bacteria by bath immersion on day 42 at 6.45PM for 6 hours until 01:00AM, immersion consist in a lethal dose of pathogen in a volume of 0.7 Liter with 1 billion *A.veronii* /mL in TSB, diluted into the 18 liters

of water contained in the trial tank (1+18L). Then 19 liters were added to the tank to top up to 38 liters, for the remaining 9 days. Oxygen is supplied with air stones and the fish are fed as usual with 3%BW a day.

During the challenge trials, the two tanks assigned to the duty of "control" encountered a material issue. After 6 hours, the bacteria concentration could not be kept at the same level as in the other tanks. It was decided to dump the bacteria-infested water from the two tanks and refill to a capacity of 38 liters. Despite this, the tanks were kept in observation because the fish had been exposed to the viruses for 6 hours. However, none of the fish perished during the next ten days, and there were no evidence of illness. It was determined that the fish require a longer period of exposure to become infected. Two weeks before the challenge trials, two groups of 50 unvaccinated fry were placed in a box holding 20 liters of water with the same bacteria content as trial 1 and 2, and the majority of the fish perished within 48 days of exposure. This result showed that bacteria with a concentration of around 20 million CFU/ml may infect and kill the majority of Nile tilapia fry. Despite this finding, it was determined that those two trials could not be used as a control group for trials 1 and 2 since they were conducted two weeks apart and the bacteria did not come from the same culture batch. Furthermore, because the mortality of uninfected fish following the challenge experiment could not be assessed, it was decided not to compute the relative percentage survival (RPS).

Figure 3.15

Tank setup for the challenge trial.

41
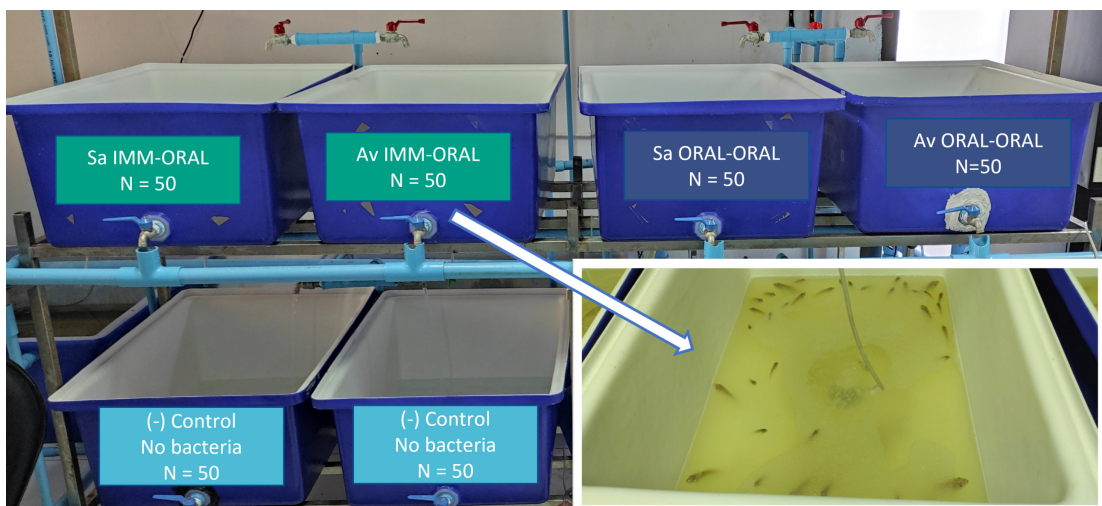
3.8 Methodology for Data Curation and Result Analysis

Because it is not logical to compare a population of fish of different ages (this is what a two way anova does if we consider the time as a continuous value), a one-way ANOVA or unpaired t-test between the different bootstrapped IgM or OD mean sub-populations values (corresponding to vaccination groups) for the same timepoint was decided to be the appropriate way to do the statistical analysis of ELISA results (Manfei et al., 2017). Post-hoc tests will be used to determine the significance of differences between groups.

For the statistical analysis of the challenge test results, in order to study the relevance of the results obtained we will use the statistical analyzes of Log-Rank. The Log-Rank, also called Peto-peto-Wilcoxon or Mentel-haenzel gives equal weights to all observations. It is optimal for highlighting the differences between survival curves whose risk functions are proportional. It is also possible to perform the statistical analysis directly using the appropriate R package survival (Therneau, 2021), and its corresponding function (Therneau, 2021; Jarp & Tverdal, 1997; RICH et al., 2010).

Relative percentage survival is calculated with the following formula (Amend, 1981):

$$RPS = (1 - (\% \text{ mortality of vaccinated batch})/(\% \text{ mortality of the control group})) * 100$$
(3.1)

3.8.1 Timeline

1. At the start, fish are acclimatized for 10 days, in a single tank. then the fish are moved to be raised the 6 experimental tanks A, B, C, D, E, F.
2. Bacterial culture and vaccine preparation : The 2 bacteria have been collected and cultured. The culture is inactivated to produce the heat killed vaccine solution.
3. Vaccination with the first dose at d0, either bath-immersion or through feed. Booster dose on day 21 through feed. Weekly sampling for gut and head from 6 fish per vaccine group.
4. Bacterial challenge on day 42 post vaccination for 10 days.
5. Wet-lab indirect elisa assays.

6. Results are interpreted, conclusions are drawn and recommendations are emitted.

Figure 3.16

Timeline of the research.
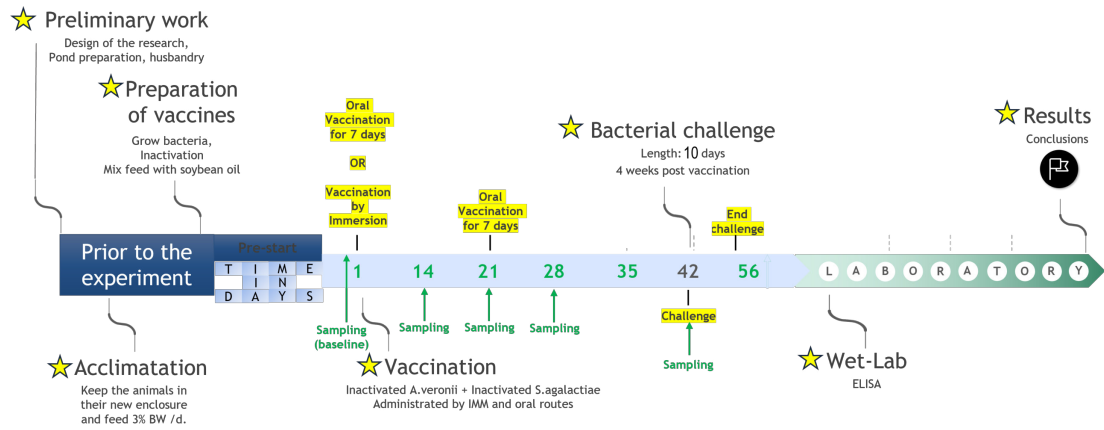
CHAPTER 4
EXPERIMENTAL RESULTS

4.1 Infection Challenge Trials for Survival

The following are the findings of two challenge trials in which the pathogens *Streptococcus agalactiae* and *Aeromonas veronii* were artificially inoculated into the fish. The mechanism employed was previously detailed in Chapter 3.7. The goal of this experiment is to determine the probability of survival in each fish population (vaccinated versus unvaccinated). The control group (in red) is from a two-week-old experiment and is included in the graph for informational purposes only. The findings of the two trials reveal that the two vaccinations had variable degrees of efficiency in protecting fish from illness. Indeed, it does not appear to be possible to calculate the relative percentage of survival (RPS) precisely, but we could estimate the RPS for the fish vaccinated with IMM+OR to be within a range of values, with the lowest value of the range being the RPS calculated as if the OR-OR group was the unvaccinated population to substract to the vaccinated population in the RPS formula using IMM+OR as the vaccinated group, and with the highest value of the upper range being the RPS calculated with the unvaccinated group having 0% survival.

4.1.1 Challenge Trial 1: Overall Survival Probability to Streptococcus agalactiae

In our first challenge trial, the real value of the RPS of IMM+OR bivalent vaccine against *S.agalactiae* is most likely to be somewhere between 67 and 87 percent. Similarly, if we apply the same logic to determine the RPS of the OR+OR bivalent vaccine. The RPS is most likely anywhere between 0% and 48%.

Figure 4.1

Overall survival probabilities following artificial infection with Streptococcus agalactiae: *Overall survival probabilities after a Streptococcus agalactiae infection: The survival rates are given as a chance of surviving through time. "Hours post injection" is how the time is displayed. With 95% confidence, the survival curves' confidence intervals presumably reflect the real mean of survival at a given timepoint. If their confidence intervals do not overlap for the same timepoint, the real means of two groups are likely to be statistically different.*

Legend:
- Negative control, without bacteria
- Positive control, unvaccinated fish with bacteria
- Immersion+Oral, with bacteria
- Oral+Oral, with bacteria

4.1.2 Challenge Trial 2: Overall Survival Probability to Aeromonas veronii

In our second challenge trial, the real value of the RPS of IMM+OR bivalent vaccine against *A.veronii* is most likely to be near 87.5 percent. Conversely, the RPS of OR+OR bivalent vaccine against *A.veronii* is 0% which means that this method does not protect the population in our trial.

Figure 4.2

Overall survival probabilities following artificial infection with Aeromonas veronii: Overall survival probabilities after a Streptococcus agalactiae infection: The survival rates are given as a chance of surviving through time. "Hours post injection" is how the time is displayed. With 95% confidence, the survival curves' confidence intervals presumably reflect the real mean of survival at a given timepoint. If their confidence intervals do not overlap for the same timepoint, the real means of two groups are likely to be statistically different.
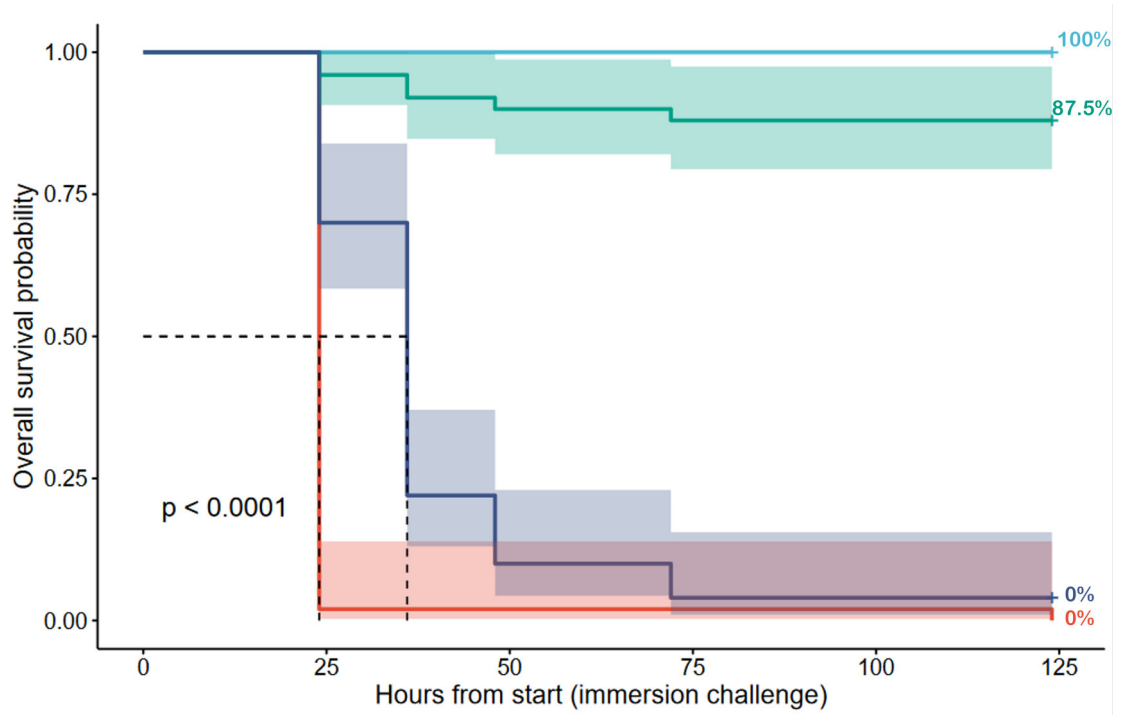
4.2 Immune Responses to Vaccination

The following are the results of an indirect ELISA against specific antigen antibodies of the immunoglobulin M class in the fish's gastrointestinal tissues and head. Only the findings from the indirect ELISA utilizing the 8-fold dilution of the samples have been included in this part, as indicated in the methodology (chapter 3.6).

4.2.1 *Streptococcus agalactiae Antigen-specific IgM Levels in the Head*

In the whole head, the generation of specific antibodies against *Streptococcus agalactiae* takes one to two weeks after vaccination, but the control has no discernible antibody production as relative levels of specific antibodies remain low throughout the 42-day period.

On day 21 after vaccination, the most significant difference between the vaccinated groups and the control group of fish can be seen, with relative levels of antibodies higher than the control for the IMM+OR group and extremely high for the OR+OR group. In both vaccinated groups, the relative amount of antibody to the control drops after day 21.

From day 21 to day 28, a booster dose of vaccination is given to the two groups that were immunized per os for one week (refer to the methodology, chapter 3.4.2). On day 42, the influence of the vaccination booster on the production of specific antibodies is confirmed by an evident peak of IgM in both vaccinated groups, but not in the control group.

Figure 4.3

Changes in antigen-specific IgM levels in whole head extract after immersion-oral and oral-oral bivalent vaccines caracterized by ELISA. Optical Density (OD) at $450nm$ reveal IgM relative specific antibody levels for S.agalactiae (diluted 1:8). Values are for 6 randomly selected fish and are the mean of 3 technical replicates.

Dilution 1:8
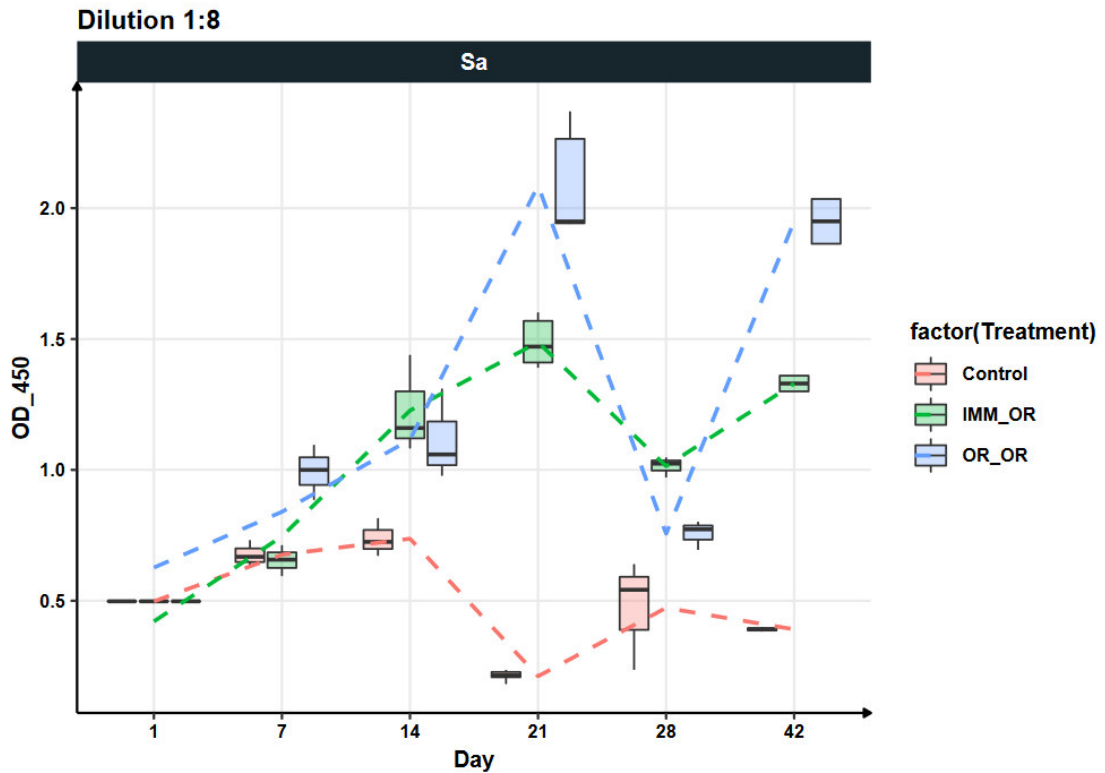
4.2.2 *Aeromonas veronii Antigen-specific IgM Levels in the Head*

In the whole head, the generation of specific antibodies against *Aeromonas veronii* takes one to two weeks after vaccination, immersion is very efficient for immunizing the fish with a very high level of IgM detectable only a week after vaccine exposure, the control has no discernible antibody production as relative levels of specific antibodies remain low throughout the 42-day period in contrast to vaccinated groups.

On day 21 after vaccination, the most significant difference between the vaccinated groups and the control group of fish can be seen, with relative levels of antibodies higher than the control for the OR+OR group and extremely high for the IMM+OR group. In both vaccinated groups, the relative amount of antibody to the control drops by a little after day 21. IMM+OR is able to elevate IgM levels with the best results.

From day 21 to day 28, a booster dose of vaccination is given to the two groups that were immunized per os for one week (refer to the methodology, chapter 3.4.2). On day 42, the influence of the vaccination booster on the production of specific antibodies is confirmed by an evident peak of IgM in both vaccinated groups, but not in the control group.

48

Figure 4.4

Changes in antigen-specific IgM levels in whole head extract after immersion-oral (green) and oral-oral (blue) bivalent vaccines caracterized by ELISA, unvaccinated fish are the control group (red). Optical Density (OD) at $450nm$ reveal IgM specific antibody relative levels for A.veronii(diluted 1:8). Values are for 6 randomly selected fish and are the mean of 3 technical replicates.
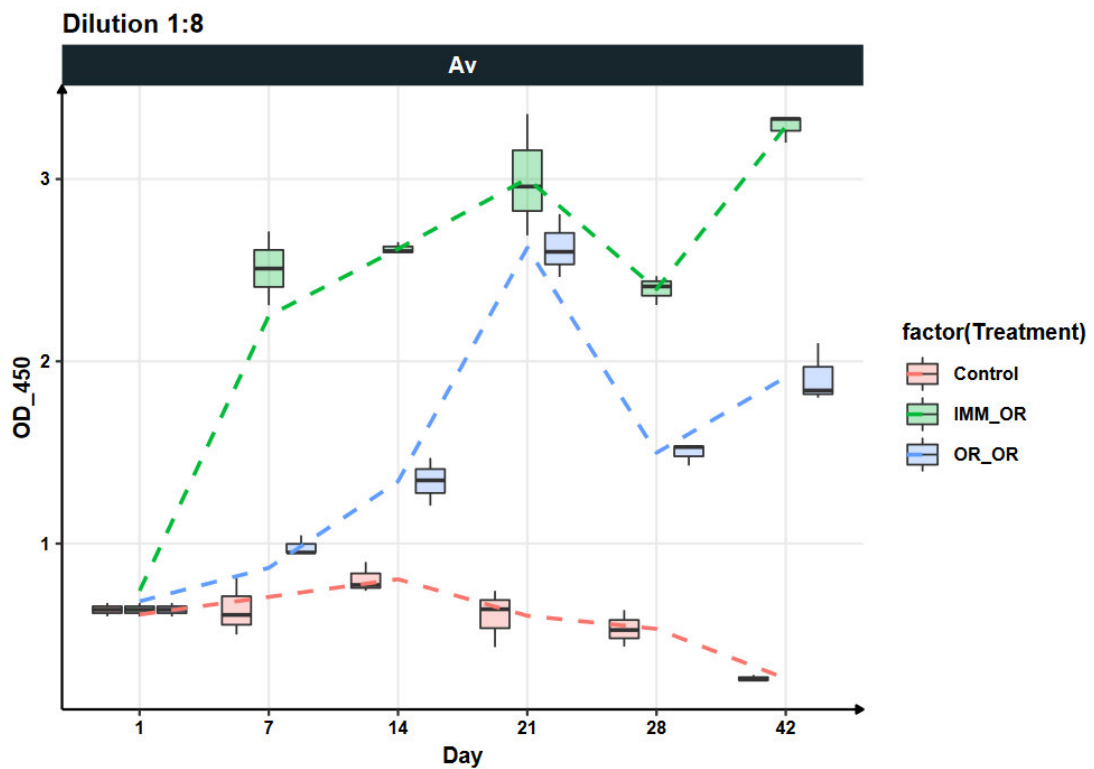
4.2.3 Aeromonas veronii or Streptococcus agalactiae Antigen-specific IgM Levels in the Head (alt')

The following figure is simply the two previous figures, side by side, with *Aeromonas veronii* on the left hand side and *Streptococcus agalactiae* on the right hand side.

Figure 4.5

Changes in antigen-specific IgM levels in whole head extract after immersion-oral (green) and oral-oral (blue) bivalent vaccines caracterized by ELISA, unvaccinated fish are the control group (red). Optical Density (OD) at $450nm$ reveal IgM specific antibody relative levels for A.veronii(diluted 1:8). Values are for 6 randomly selected fish and are the mean of 3 technical replicates.

4.2.4 Streptococcus Agalactiae Antigen-specific IgM Levels in the Gut

In the entire gut of the fish, the generation of specific antibodies against *Streptococcus agalactiae* is observable but to a lesser extent than in the head. Indirect elisa gave high background and also showed little difference between treatment groups, this effect is probably due to the sample preparation because the samples were of different sizes and it is not possible to normalize them by their mass. However we observe that all of 3 groups have approximately the same baseline for their relative IgM levels at day 0. Then two weeks after vaccination the relative levels of IMM+OR group are higher than the control and OR+OR.

On day 21 after vaccination, the most significant difference between the IMM+OR groups and the control group with OR+OR can be seen, with relative levels of antibodies higher than the both groups for the IMM+OR group. Our results doesn't show that the OR+Or group generates more specific antibodies during the first 35 days.

From day 21 to day 28, a booster dose of vaccination is given to the two groups that were immunized per os for one week (refer to the methodology, chapter 3.4.2). On day 42, the influence of the vaccination booster on the production of specific antibodies is confirmed by an evident peak of IgM in OR+OR vaccinated groups, but not in IMM+OR and in the control group.

Figure 4.6

Changes in antigen-specific IgM levelsin whole gut extract after immersion-oral and oral-oral bivalent vaccines caracterized by ELISA. Optical Density (OD) at $450nm$ reveal IgM relative specific antibody levels for S.agalactiae (diluted 1:8). Values are for 6 randomly selected fish and are the mean of 3 technical replicates.
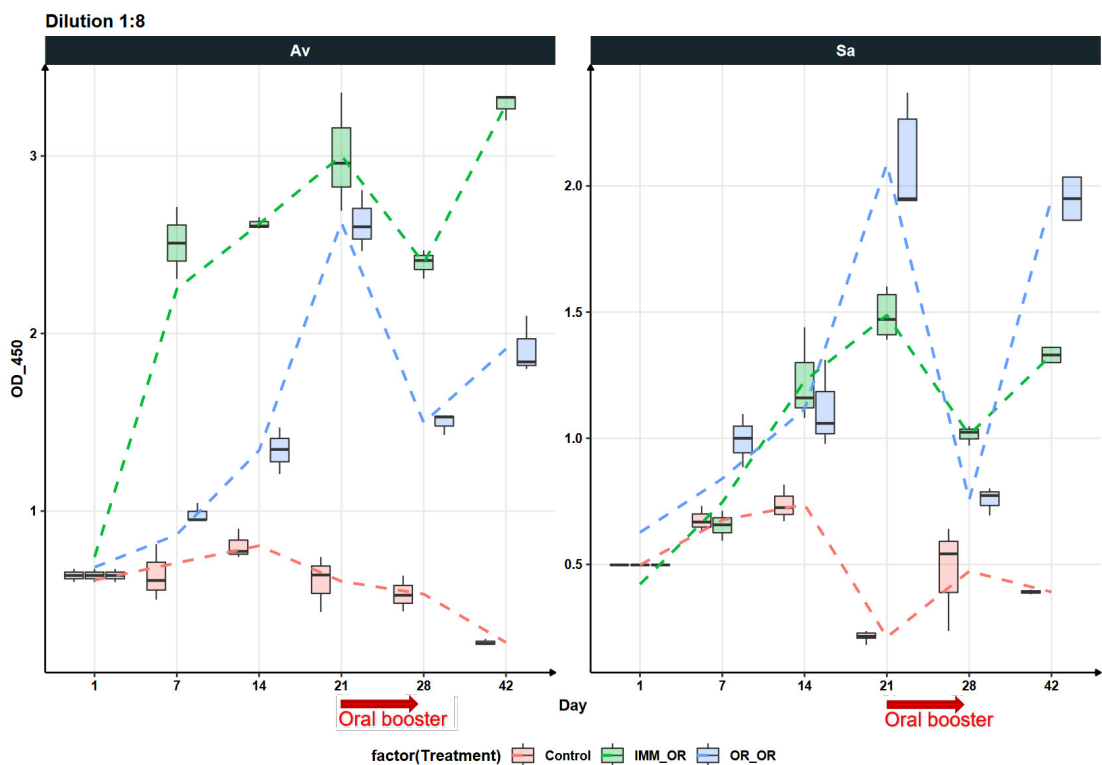
Dilution 1:8

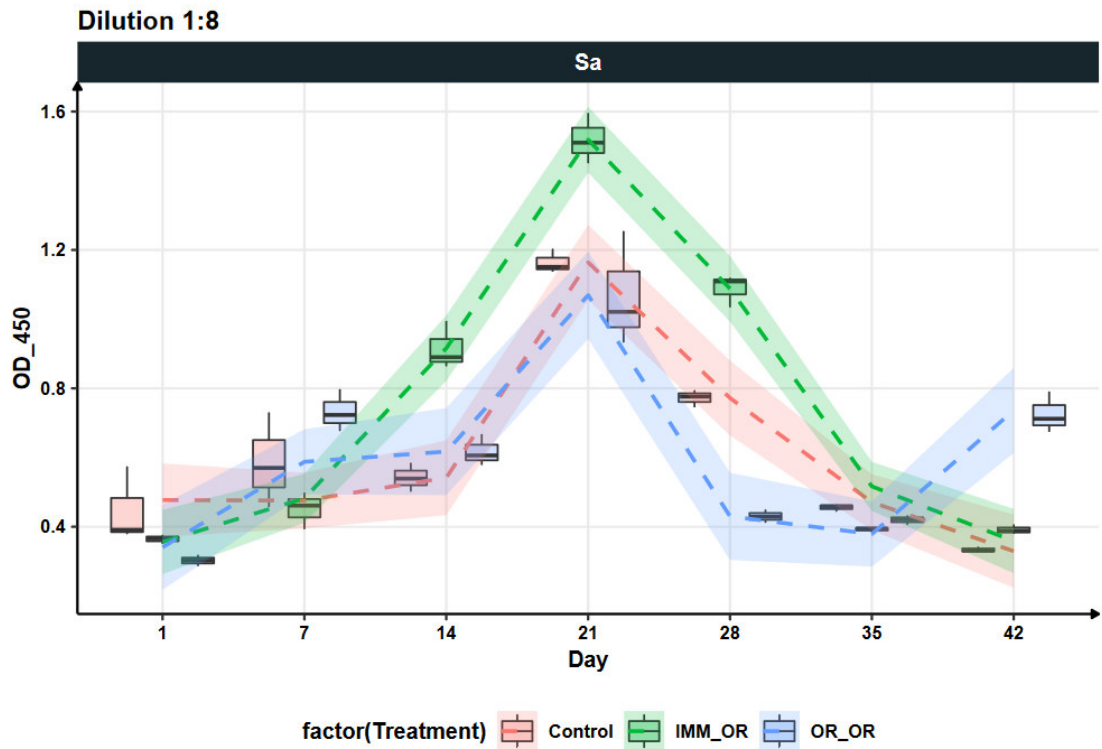### 4.2.5 *Aeromonas veronii Antigen-specific IgM Levels in the Gut*

In the entire gut of the fish, the generation of specific antibodies against *Aeromonas veronii* is observable but to a lesser extent than in the head. Indirect elisa gave high background and also showed little difference between treatment groups, this effect is probably due to the sample preparation because the samples were of different sizes and it is not possible to normalize them by their mass. However we observe that all of 3 groups have approximately the same baseline for their relative IgM levels at day 0. Then two weeks after vaccination the relative levels of IMM+OR group are higher than the control and OR+OR.

On day 21 after vaccination, the most significant difference between the IMM+OR groups and the control group with OR+OR can be seen, with relative levels of antibodies higher than the both groups for the IMM+OR group. Our results doesn't show that the OR+Or group generates more specific antibodies during the first 35 days.

From day 21 to day 28, a booster dose of vaccination is given to the two groups that were immunized per os for one week (refer to the methodology, chapter 3.4.2). On day 42, the influence of the vaccination booster on the production of specific antibod-

ies is confirmed by an evident peak of IgM in OR+OR vaccinated groups, but not in IMM+OR and in the control group.

Figure 4.7

Changes in antigen-specific IgM levels in whole gut extract after immersion-oral (green) and oral-oral (blue) bivalent vaccines caracterized by ELISA, unvaccinated fish are the control group (red). Optical Density (OD) at $450nm$ reveal IgM specific antibody relative levels for A.veronii(diluted 1:8). Values are for 6 randomly selected fish and are the mean of 3 technical replicates.
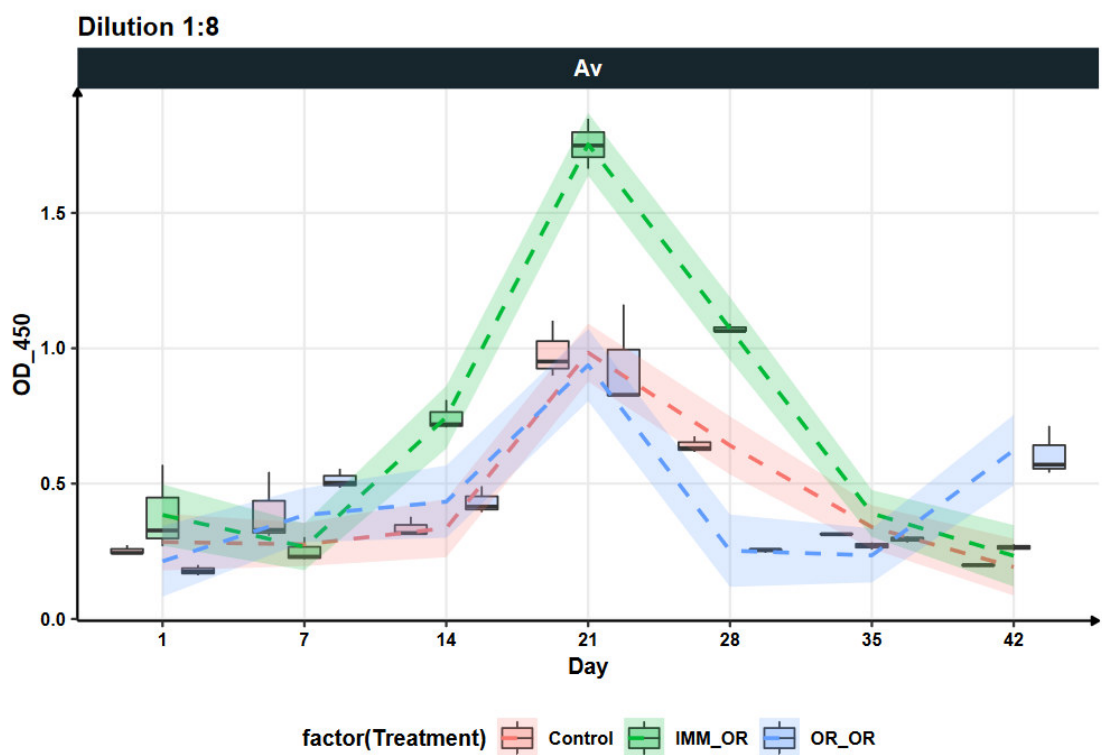
4.2.6 *Aeromonas veronii or Streptococcus agalactiae Antigen-specific IgM Levels in the Gut (alt')*

The following figure is simply the two previous figures, side by side, with *Aeromonas veronii* on the left hand side and *Streptococcus agalactiae* on the right hand side.

Figure 4.8

Changes in antigen-specific IgM levels in whole gut extract of Nile tilapia after immunizations by immersion-oral (green) or oral-oral (blue) bivalent vaccines caracterized by ELISA, unvaccinated fish form a control group (red). Optical Density (OD) at $450nm$ reflect IgM specific antibody relative levels (diluted 1:8). Values are for 6 randomly selected fish and are the mean of 3 technical replicates.
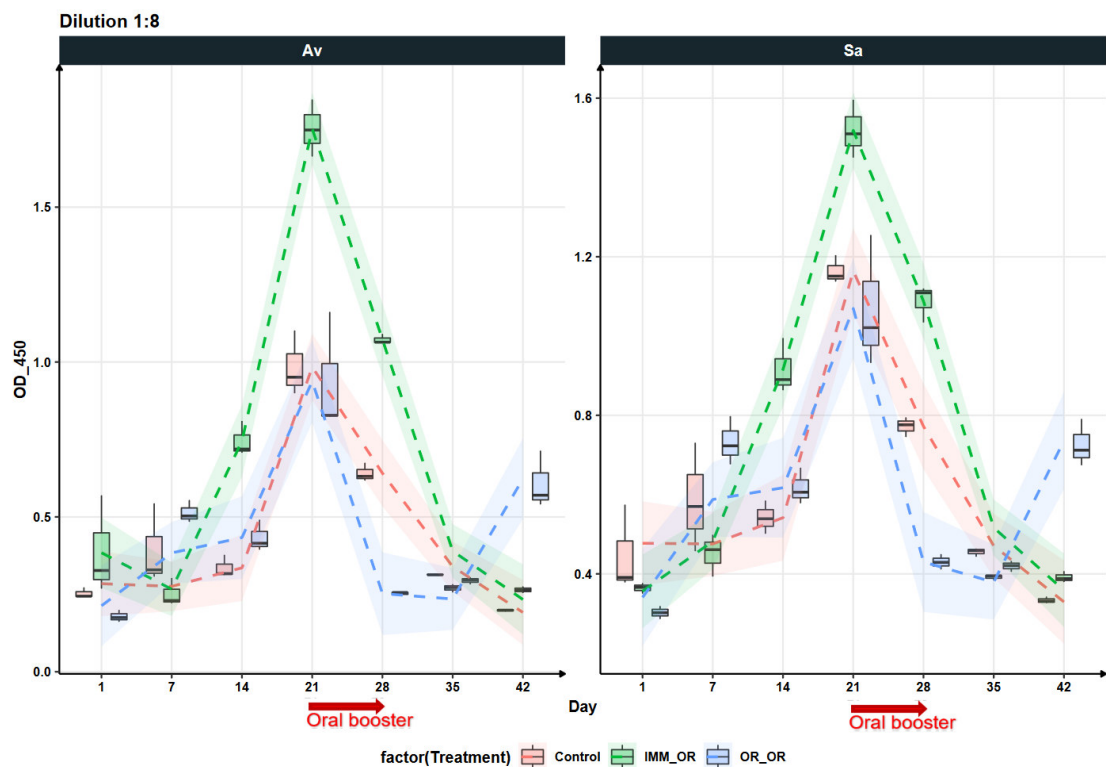
CHAPTER 5

DISCUSSION

The most effective way to prevent the occurrence of streptococcal and aeromonas diseases in aquaculture ponds is by vaccination prophylaxis of Nile tilapia. Vaccinating fish in a bath or via oral routes require a lesser labor than vaccinating by hand and can be done to young fish on their few weeks of life, when at this age the fish are more susceptible to infectious diseases and vaccinating by injection is impossible to administrate (Adams, 2019; Embregts & Forlenza, 2016; Plant & Lapatra, 2011; MuAtienza et al., 2021). Furthermore, our findings show that the two vaccine techniques, IMM+OR OR+OR, do not provide comparable protection. Our findings are in line with those of several previous research that suggest that bath vaccination is more efficient than oral immunization in mass vaccination of juvenile tilapia fry. It is commonly known that vaccinations administered via injection have a higher efficiency than immunizations administered through bath immersion, being about twice as effective. (J. J. Evans et al., 2004). Furthermore, recent *S.agalactiae* vaccination strategies tried in Nile tilapia that attempted to induce long-lasting immunity by oral vaccination but required administering a booster dose by feeding every 6 weeks and therefore changing the regular fish feed regime for a "feed-based vaccination regime" (Ismail et al., 2016; MuAtienza et al., 2021). Oral vaccination in Atlantic salmon (*S.salar*) has also been employed to generate long-lasting immunity with detectable specific antibodies in the gut mucosa almost a year after a prime injection of vaccine (J. A. Tobar et al., 2011; MuAtienza et al., 2021).

According to our findings, IMM+OR and OR+OR are able to stimulate the fish's specific immune system, however only IMM+OR but not OR+OR could protect against all of the pathogens when the fish were artificially infected and put in the same condition as the oral vaccination. As explained before, oral vaccines are less effective than immersion vaccines and injection counterparts (Vinitnantharat et al., 1999). Rather than being a technique of immunization in and of itself, oral vaccination has been advocated as a booster to provide longer protection against i.p injected antigens. In this regard, in another study Atlantic salmon, rainbow trout, and coho salmon (Oncorhynchus kisutch) were boosted orally after being vaccinated with an injectable monovalent or polyvalent

vaccine against salmonid rickettsial septicemia (SRS), the production of specific IgM antibodies was significantly increased (I. Tobar et al., 2015; MuAtienza et al., 2021). Oral boosters not only boosted the size of the response, but also extended the duration of the antibody response, up to 2800-3200 degree-days in their experiment. (I. Tobar et al., 2015; MuAtienza et al., 2021).

Our survival challenge trials let to the observation that IMM+OR achieved to protect more than 80% of the animals against both pathogens when OR+OR failed to protect against *Aeromonas veronii* (0% survival after challenge). However, the IMM+OR vaccination approach has yet to improve and does not appear to be suitable for use in fry (3 percent morality after bath vaccination). Fry are sensitive, and if they are left in the diluted TSB vaccine solution for longer than 2 hours, they will begin to die (in our design the bath immersion took 4.5 hours). The study of antigen-specific antibody levels in pooled serum revealed that both vaccination strategies did activate the fish immune response. Immersion and Oral vaccination both outperformed the unvaccinated group in the head. It is also possible to observe a peak in IgM levels on day 42, two weeks after the oral booster in both methods, which means that soybean-oil coated feed pellets containing inactivated bacteria is able to stimulate the immune response after a week of feeding. Anti *S.agalactiae*-specific IgM levels were found to be the highest in the gut but not in the head samples of fry following IMM+OR immunization. Anti *A.veronii*-specific IgM levels were found to high in the gut and in the head of the fry following IMM+OR immunization.

It is possible that the indirect Elisa assay was not accurate because IgM levels in the head samples were heightened in fry following OR+OR immunization as compared to the IgM levels of the unvaccinated group, and despite the low to negligible protection confered by OR+OR immunization (Respectively, 0% of overall survival probability and 51 % against *A.veronii* and *S.agalactiae*). The study of antigen-specific immune response in gut by indirect elisa also needs improvement as the values of the control are not usual and linear in contrast to in the whole head, probably due to manipulation mistakes or inadequate protocol for this type of sample. Another possibility would be that the supernatant from the gut contains gastric proteolyic enzymes such as trypsin and pepsin which would lead to antibody fragmentation in our samples. such as However specific immune response following IMM+OR seems to correlate more accurately with

the survival of the fish upon challenge trials. Regarding the survival challenge trial, it is possible that our results were influenced because our control fish did not receive the same treatment as the vaccinated fish, making it impossible to know precisely what the mortality of the unvaccinated fish would be after being exposed for 6 hours to a high concentration of bacteria and then for 12 days to a lower concentration. Nonetheless, the bacteria are clearly exceedingly pathogenic, with *A.veronii* capable of decimating the whole population vaccinated with OR+OR. Although we were able to take inspiration from previously developed immersion challenge model for *S.agalactiae* in Nile tilapia (R. Z. He et al., 2021), it would be interesting to be able to study the effectiveness of OR+OR and IMM+OR challenge by intraperitoneal injection of live bacteria because it is difficult to standardize and undertake immersion challenge trials.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Two simple and low-cost vaccination techniques for preventing streptococcus infection and motile aeromonas septicemia were tested in a controlled experiment to see how they affected disease-specific antibody levels and survival rates in fry tilapia (*Oreochromis niloticus*)

In our study, the protection provided by OR+OR was found to be minimal in terms of disease protection. Conversely, we demonstrated that immunizing the fish with a 4.5-hour bath immersion followed by a week of feeding oral vaccine 21 days later (IMM+OR), was efficacious in preventing mortality from *Streptococcus agalactiae* and *Aeromonas veronii*. To further understand how to optimize the bath immersion process, more study and effort is required. Despite the fact that the oral booster administrated after a bath immersion but also after oral vaccine efficiently activates the fish immune system as measured by indirect ELISA, we were unable to identify to what degree it enhances vaccination efficiency.

Vaccination, like it is in the salmon and cattle sectors, will become an essential component of commercially successful and sustainable aquaculture in Asia. However, it is crucial to note that only a combination of vaccination and other factors, including as high-quality seed, suitable feeding, competent management, and good husbandry procedures, can produce the best potential survival rate and profit margins.

The best takeaways stressed by the results of the research is that vaccination of fry tilapia is simple and low cost to perform,

We advocate bath immersion vaccination of fish fry or fingerling (heavier than 1 g if possible) using our approach to immunize nursery-sized Nile tilapia. This finding offers up new possibilities for hatcheries, nurseries, and small-scale farmers who wish to use the same processes to vaccinate against our two bacteria, or any other pathogen, by making their own autogenous vaccines from a strain of bacteria found on their own diseased fish. Our inexpensive one-of-a-kind approach of immunizing and safeguarding Nile tilapia fry is both practical and effective.

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APPENDIX A : OVERVIEW OF LICENSED FISH VACCINES

Overview of licensed fish vaccines to date against viruses. Credits: Ma et al. (2019)

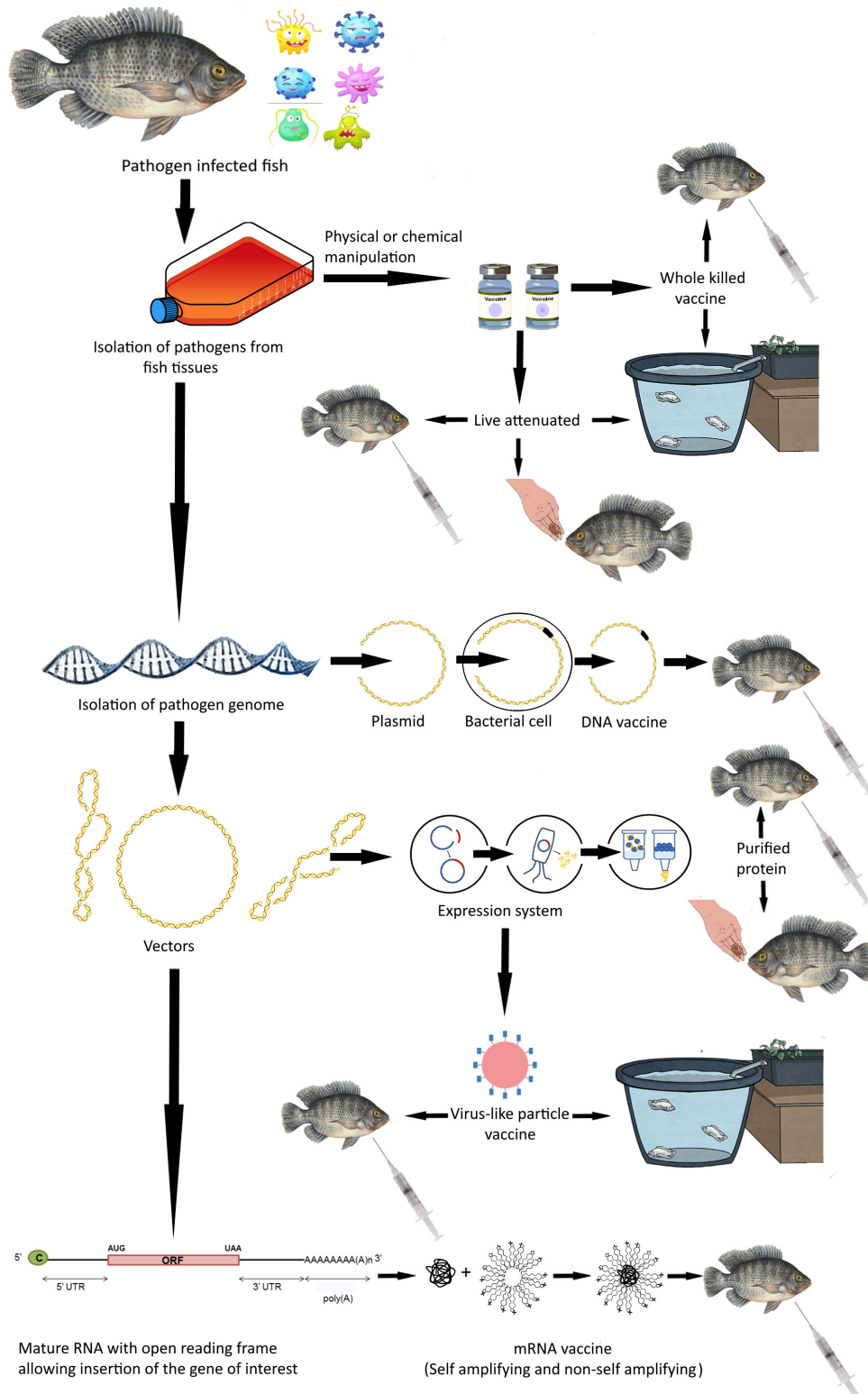| Disease	Pathogen	Major Fish Host	Vaccine Type	Antigens/Targets	Delivery Methods	Country	Links
Infectious hematopoietic necrosis	IHNV Rhabdovirus	Salmonids	DNA	G Glycoprotein	IM	Canada	
Infectious pancreatic necrosis	IPNV Birnavirus	Salmonids, sea bass, sea bream, turbot, cod	Inactivated	Inactivated IPNV	IP	Norway, Chile, UK	link
			Subunit	VP2 and VP3 Capsid Proteins	Oral	Canada, USA	link
			Subunit	VP2 Proteins	IP	Canada, Chile, Norway	link
Infectious salmon anemia	ISAV Orthomyxovirus	Atlantic salmon	Inactivated	Inactivated ISAV	IP	Norway, Chile, Ireland, Finland, Canada	link
Pancreatic disease virus	SAV alphaviruses	Salmonids	Inactivated	Inactivated SAV	IP	Norway, Chile, UK	link
Spring viremia of carp virus	SVCV Rhabdovirus	Carp	Subunit	G Glycoprotein	IP	Belgium	
			Inactivated	Inactivated SVCV	IP	Czech Republic	
Koi herpesvirus disease	KHV Herpesvirus	Carp	Attenuated	Attenuated KHV	IMM or IP	Israel	
Infectious spleen and kidney necrosis	ISKNV Iridovirus	Asian seabass, grouper, Japanese yellowtail	Inactivated	Inactivated ISKNV	IP	Singapore	link

APPENDIX A : OVERVIEW OF LICENSED FISH VACCINES

Overview of licensed fish vaccines to date against bacteria and parasites. Credits: Ma et al. (2019)

Disease	Pathogen	Major Fish Host	Vaccine Type	Antigens/Targets	Delivery Methods	Country	Links
Enteric redmouth disease (ERM)	Yersinia ruckeri	Salmonids	Inactivated	Inactivated Y. ruckeri	IMM or oral	USA, Canada, Europe	link link 2
Vibriosis	Vibrio anguillarum; Vibrio ordalii; Vibrio salmonicida	Salmonids, ayu, grouper, sea bass, sea bream, yellowtail, cod, halibut	Inactivated	Inactivated Vibriosis spp.	IP or IMM	USA, Canada, Japan, Europe, Australia	link
Furunculosis	*Aeromonas salmonicida subsp. salmonicida*	Salmonids	Inactivated	Inactivated A. salmonicida spp.	IP or IMM	USA, Canada, Chile, EU Australia	link
Bacterial kidney disease (BKD)	Renibacterium salmoninarum	Salmonids	Avirulent live culture	Arthrobacter davidanieli	IP	Canada, Chile, USA	
Enteric septicemia of catfish (ESC)	Edwarsiella ictaluri	Catfish	Inactivated	Inactivated E. ictaluri	IP	Vietnam	link
Columnaris disease	Flavobacterium columnaris	All freshwater finfish species, bream, bass, turbot, salmon	Attenuated	Attenuated F. columnare	IMM	USA	
Pasteurellosis	Pasteurela piscicida	Sea bass, sea bream, sole	Inactivated	Inactivated P. pscicida	IMM	USA, Europe, Taiwan, Japan	link
Lactococciosis	Lactococcus garviae	Rainbow trout, amberjack, yellowtail	Inactivated	Inactivated L. garviae	IP	Spain	link
Streptococcus infections	*Streptococcus spp.*	Tilapia, yellow tail, rainbow trout, ayu, sea bass, sea bream	Inactivated	Inactivated *S.agalactiae* (biotype 1)	IP	Taiwan, Japan, Brazil, Indonesia	link
				Inactivated *S.agalactiae* (biotype 2)	IP		link
				Inactivated S. iniae	IP or IMM		link
Salmonid rickettsial septicemia	Piscirickettsia salmonis	Salmonids	Inactivated	Inactivated P. salmonis	IP	Chile	link
Motile Aeromonas septicemia (MAS)	*Aeromonas spp.*	Striped catfish	Inactivated	*A. hydrophila (serotype A and B)*	IP	Vietnam	link
Wound Disease	Moritella viscosa	Salmonids	Inactivated	Inactivated M. viscosa	IP	Norway, UK, IR, Iceland	link
Tenacibaculosis	Tenacibaculum maritimum	Turbot	Inactivated	Inactivated T. maritimum	IP	Spain	link

APPENDIX B : TYPES OF VACCINES FOR AQUACULTURE

Different types of fish vaccines can be made from pathogens. Adapted from: Ma et al. (2019)

Pathogen infected fish

Physical or chemical manipulation

Isolation of pathogens from fish tissues

Whole killed vaccine

Live attenuated

Isolation of pathogen genome

Plasmid   Bacterial cell   DNA vaccine

Vectors

Expression system

Purified protein

Virus-like particle vaccine

Mature RNA with open reading frame allowing insertion of the gene of interest

mRNA vaccine (Self amplifying and non-self amplifying)

APPENDIX C : AGENDA

APPENDIX D : MATERIALS EQUIPMENTS AND ASSOCIATED COSTS

Materials and equipments, associated costs and amount required for the research project.

Role	Name and models	Vendor / supplier	Total (THB ฿)	Amount (pc)
Pond maintenance	Air compressor HAILEA ACO-388D	FISH STORIES	1250.00	1
Pond maintenance	PE aquarium filter mesh square/board	FISH STORIES	450.00	3
Pond maintenance	Air Bubble Stone Fish Tank Oxygen Aerator	FISH STORIES	250.00	30
Pond maintenance	Green air valve (1 pack)	FISH STORIES	280.00	100
Pond maintenance	5M x 5mm Clear Soft Plastic Aquarium Air Line	FISH STORIES	100.00	1
Pond maintenance	4M of gas hose (size 8*13.5)	Somthrong talaadthai	140.00	4
Pond maintenance	Blue pipes (plastic elbows) valve stocket	Somthrong talaadthai	190.00	38
Pond maintenance	Blue pipes (plastic elbows) faucet stocket	Somthrong talaadthai	380.00	38
Feed for fish	CP 9920 Nursery catfish feed (20 kg)	Buachan pet shop	475.00	1
Aquarium	Aquarium 100L	Asian institute of technology		14
Aquarium	Buckets-50L HDPE boxes	Asian institute of technology		4
Pond maintenance	Blue plastic pipe HDPE	Asian institute of technology		
Pond maintenance	Polystyrene board 1M^2	Asian institute of technology		11
Laboratory	Plastic box	Asian institute of technology		20
Laboratory	Agitator/incubator	Asian institute of technology		1
Laboratory	Micropipettes	Asian institute of technology		3
Laboratory	Plate reader	Asian institute of technology		1
Laboratory	Spectrophotometer	Asian institute of technology		1
Bacteria and vaccine	Semi-log paper			
Pond maintenance	Aqua test kit	Asian institute of technology		1
Sampling	Eppendorf 2mL	Asian institute of technology		
Laboratory	Micropipette cones	Asian institute of technology		
Bacteria and vaccine	Microscope x100			1
Sampling	Needles (insulin 1mL)			
Pond maintenance	pH meter	Asian institute of technology		1
Bacteria and vaccine	Petri dish	Asian institute of technology		400
Sampling	plastic bags			400
Sampling	Sterile tubes	Asian institute of technology		400
Laboratory	Ice	Asian institute of technology		
Laboratory	Flat-bottom microplate wells	Asian institute of technology		4
Sampling	Dissection tools	Asian institute of technology		1
Laboratory	Centrifuge	Asian institute of technology		1
Laboratory	Vortex machine	Asian institute of technology		1
Laboratory	Erlenmeyer 500mL	Asian institute of technology		4
Laboratory	Erlenmeyer 200mL	Asian institute of technology		4
Laboratory	Hemocytometer	Asian institute of technology		1
Laboratory	Hot-bath bain marie	Asian institute of technology		1
Laboratory	Erlenmeyer 1000mL	Asian institute of technology		2
Laboratory	Distilled water dispenser	Asian institute of technology		1
Laboratory	Sterilization hot steam bath	Asian institute of technology		1
Laboratory	Fridge	Asian institute of technology		1
Totals (THB ฿)			**3515.00**	

APPENDIX E : LIST OF MATERIALS: LABORATORY CONSUMABLES AND REACTANTS

List of consumables and reactants and their associated cost estimate for the master thesis research project.

Role	Name and models	Vendor	Reagent costs ($)	Cost for 1 ($)
Bacteria and vaccine	Glycerol			
Bacteria and vaccine	tryptone soya agar (TSA) - Culture medium (solid)			
Bacteria and vaccine	TSB - Culture medium (liquid)			
Bacteria and vaccine	Soybean oil - Adjuvant			
Bacteria and vaccine	phosphate buffer saline (PBS) - saline buffer			
Sampling	Clove oil. - Anaesthesia			
Sampling	Sodium azide			
ELISA	Aeromonas.veronii whole-cell antigen			
ELISA	S. agalactiae whole-cell antigen			
ELISA	Carbonate coating buffer			
Others	NaOH			
Others	H3O+			
Others	Tween-20 -Detergent			
ELISA	PBST			
ELISA	0.2% Skimmed milk (PBSTM)			
ELISA	Anti-Tilapia IgM secondary Ab			
ELISA	Goat anti-mouse Ab HRP conjugate			
ELISA	3, 3 , 5, 5 -tetramethylbenzidine (TMB) chromatic agent			
ELISA	H2SO4			
Others	Ethanol - Prepared from stock EtOH95%			
ELISA	0.01% poly-L-lysine - Coating agent for ELISA			
ELISA	1% Bovine serum albumin			
Totals (THB ฿)			**TBD**	