EVALUATION OF MONTANIDE GR01 FOR ORAL VACCINATION IN NILE TILAPIA (*Oreochromis niloticus*): LABORATORY AND ON-FARM TRIALS

Sirinya Pholchamat

A Thesis Submitted in Partial Fulfillment of Requirements for
degree of Master of Science in Biotechnology and Biobusiness

May 2023

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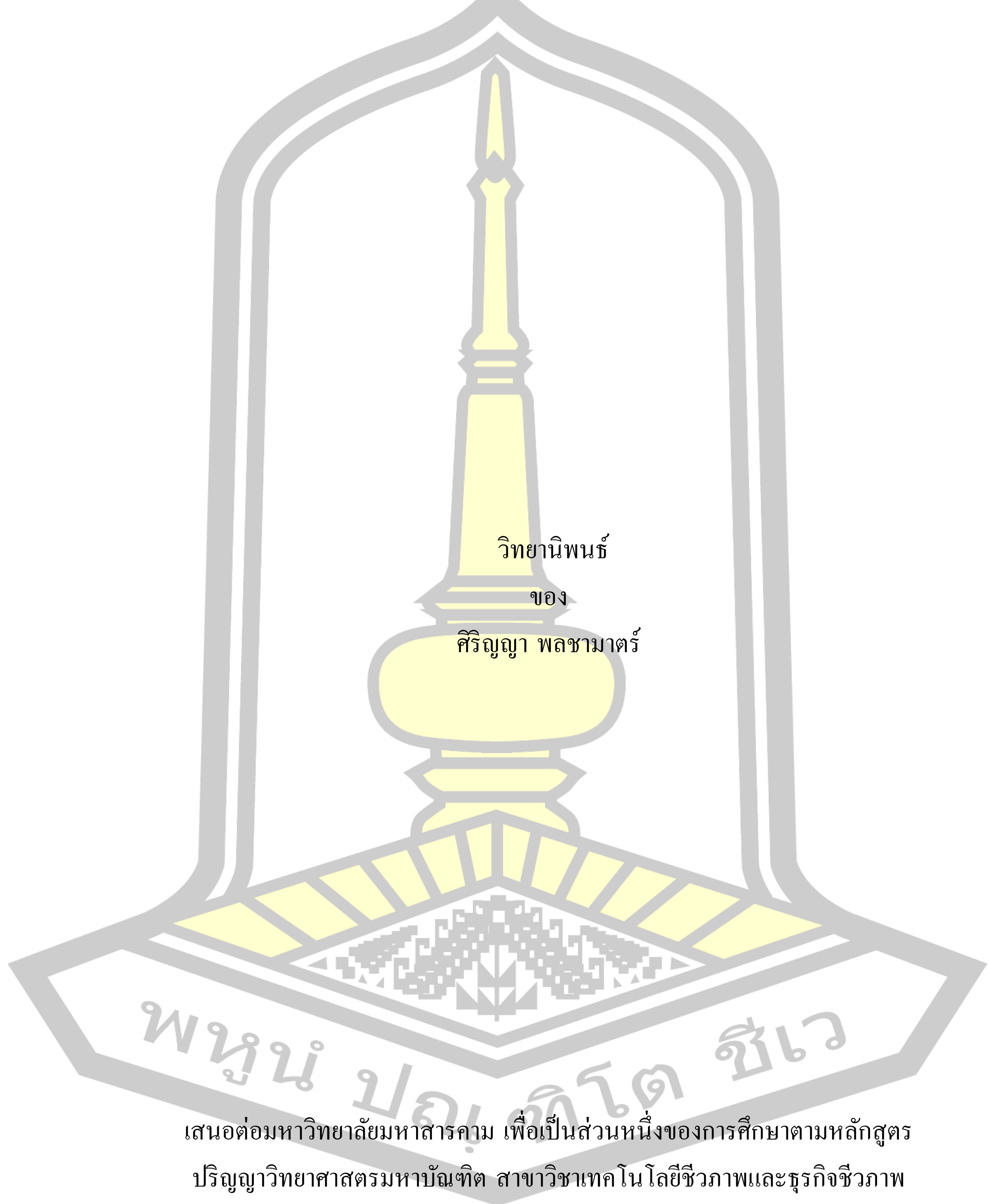
การศึกษาประสิทธิภาพของสารเสริมฤทธิ์ Montanide GR01 ในการให้วัคซีนด้วยวิธีผสมอาหารในปลานิล (*Oreochromis niloticus*): การศึกษาระดับห้องปฏิบัติการและระดับฟาร์มเลี้ยง



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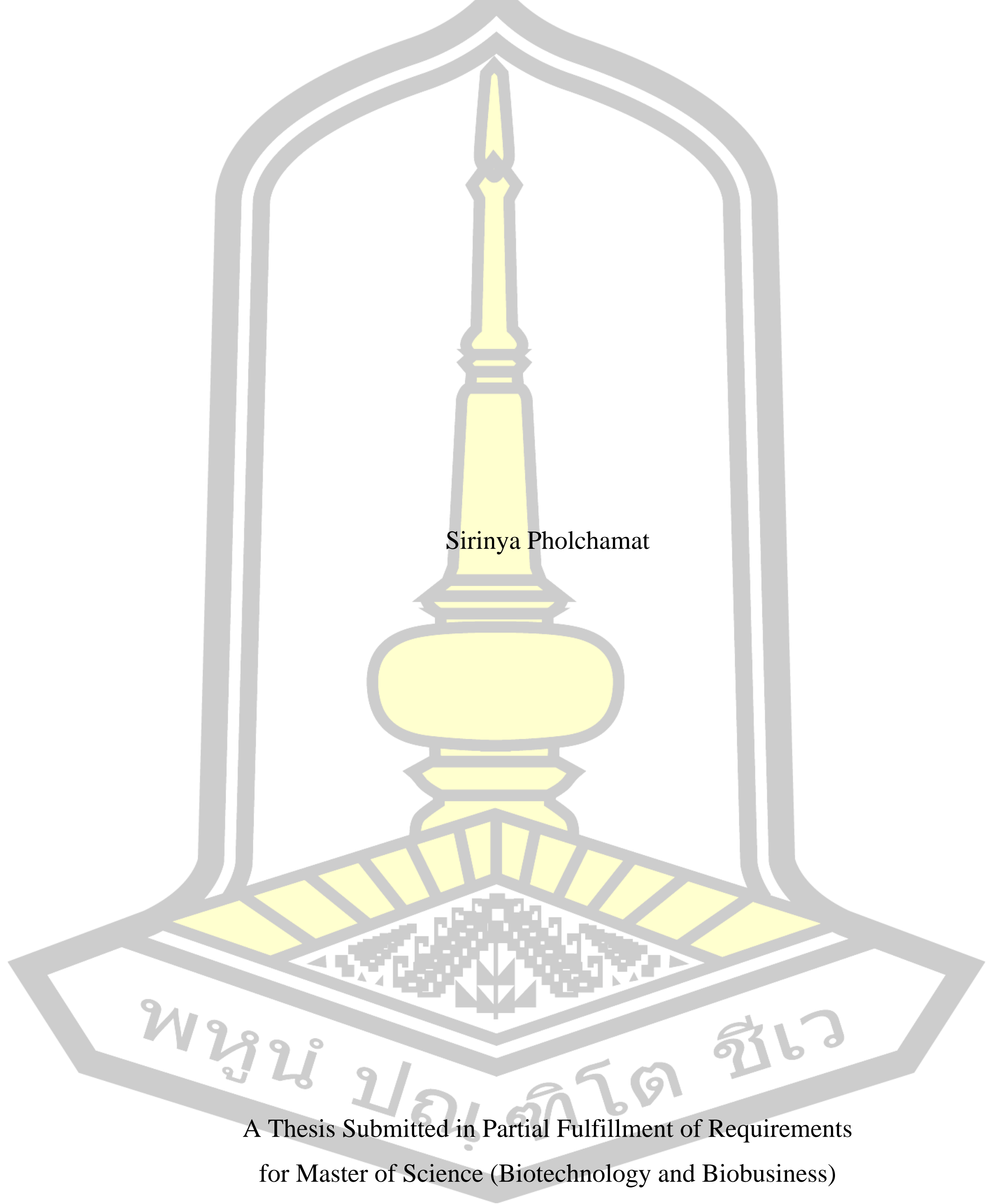

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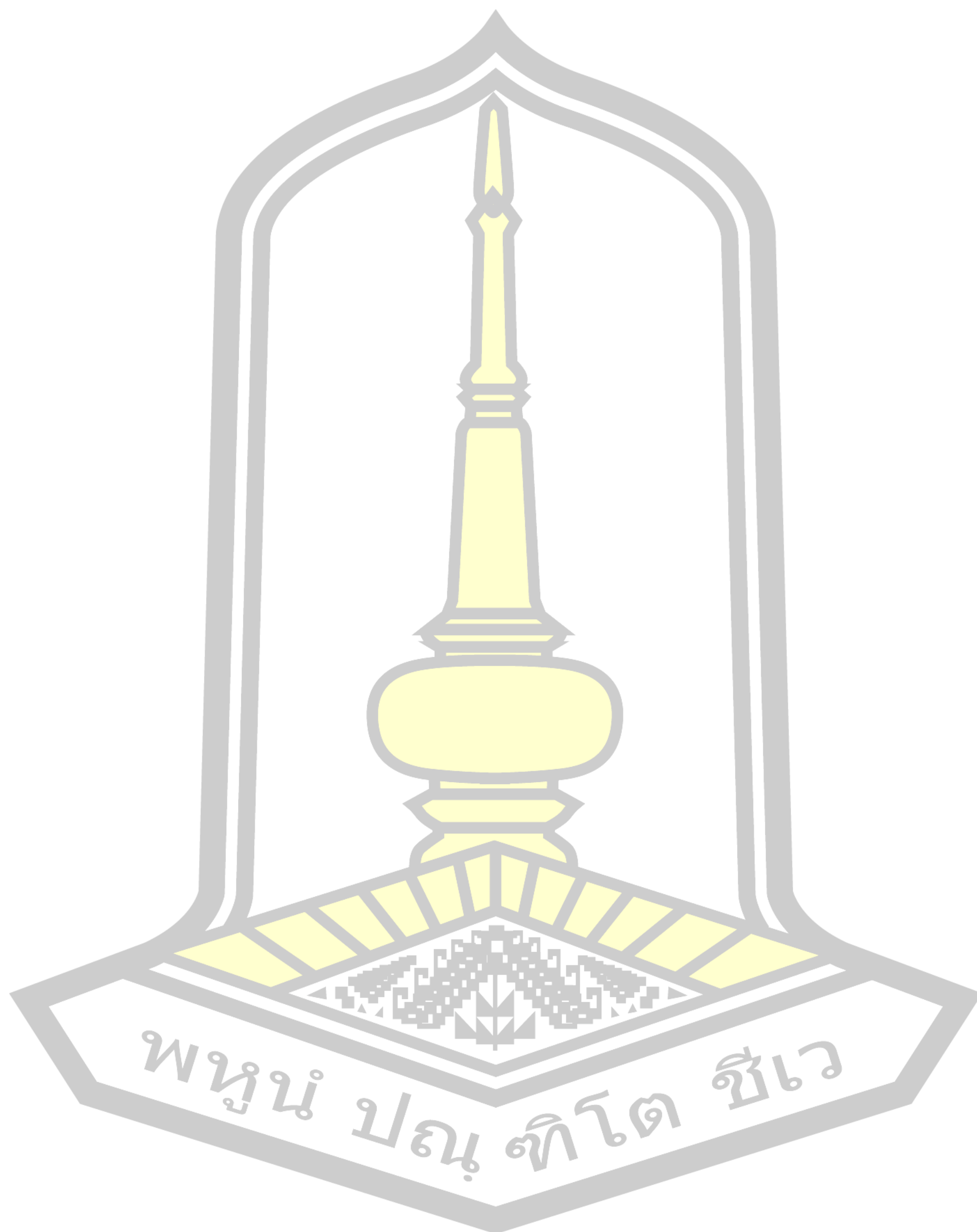
ABSTRACT

An emerging infectious disease called Streptococcosis caused by *Streptococcus agalactiae* has adversely affecting Nile tilapia (*Oreochromis niloticus*) aquaculture. The vaccines against this disease have been developing to prevent disease and increase the specific immune response in fish to a particular pathogen. The application of adjuvants registered under the trademark Montanide have been optimized to improve vaccine formulation efficacy and stability while reducing side effects. In this study, this study plans to optimize the use of a novel adjuvant Montanide GR01 for use in fish, Nile tilapia. Initially, stable formulations containing a killed *S. agalactiae* was prepared. Three formulations of feed-based vaccine were compared the responses elicited in the fish with those elicited by oral vaccine to this disease. The immune response and protective efficacy of formalin-killed cell (FKC) vaccine only and mixed FKC vaccine with a new adjuvant Montanide™ GR01 in Nile tilapia for oral vaccination were investigated. The present study found that vaccination of FKC emulsified with Montanide GR01 could significantly improve innate and adaptive immune parameters tested in both the laboratory and field-trial experiments. Moreover, it was clearly demonstrated that Montanide GR01, which was formulated in fish feed as adjuvant, effectively improve Nile tilapia immunity and clearly elevate disease resistance against streptococcosis. The levels of immune responses in fish fed dietary vaccine formulated with Montanide GR01 for four months were higher compared to those fed with vaccine alone or commercial diet (control group); although it was not effective to enhance growth performance of tilapia in this study. In overall, Montanide GR01 is a very useful adjuvant for oral vaccination and could be applied as potential adjuvants for vaccine development to control and prevent of streptococcosis in Nile tilapia aquaculture.

Keyword : Streptococcosis, Montanide GR 01, Immune Response, Formalin-killed Cell Vaccine (FKC), Nile tilapia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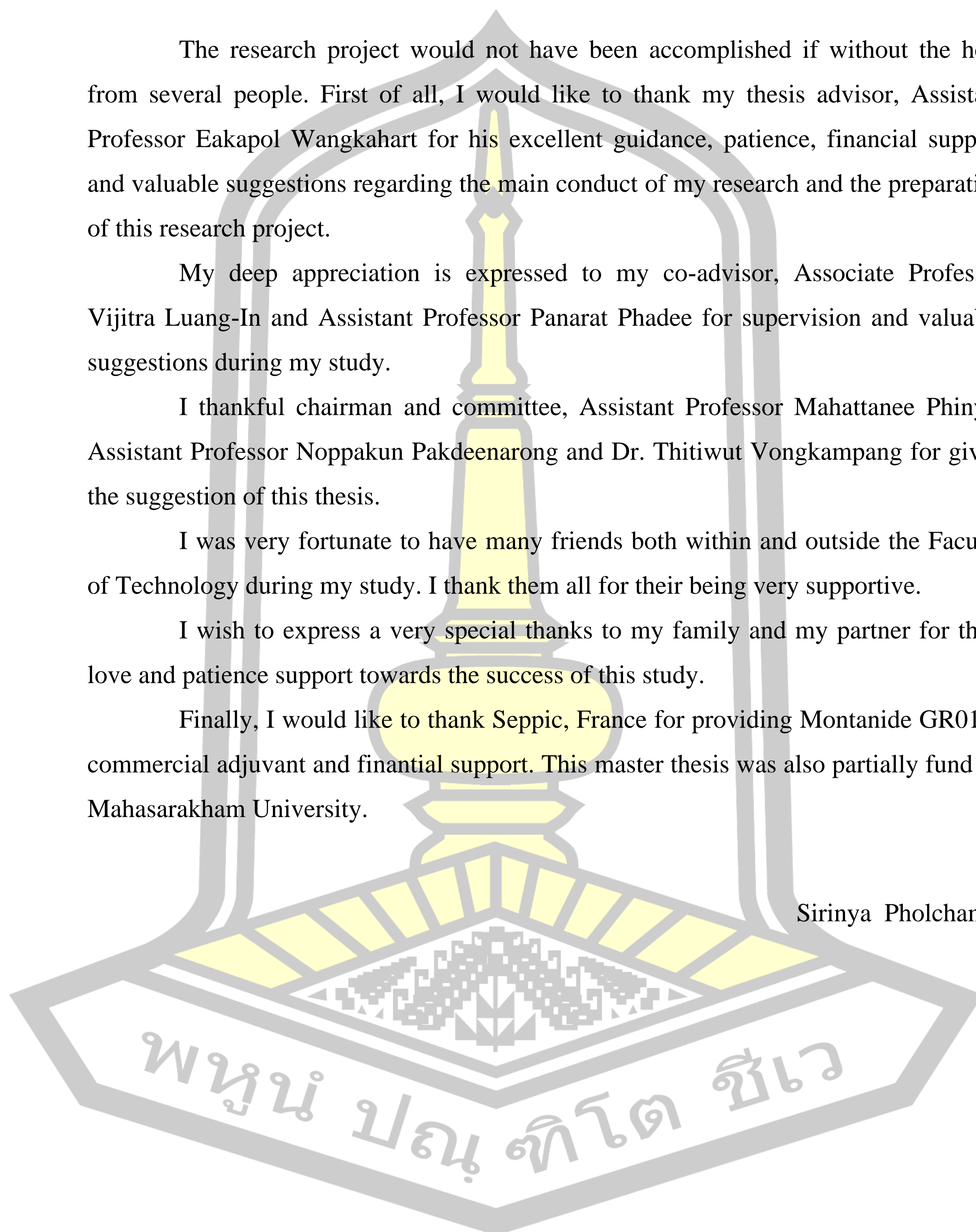

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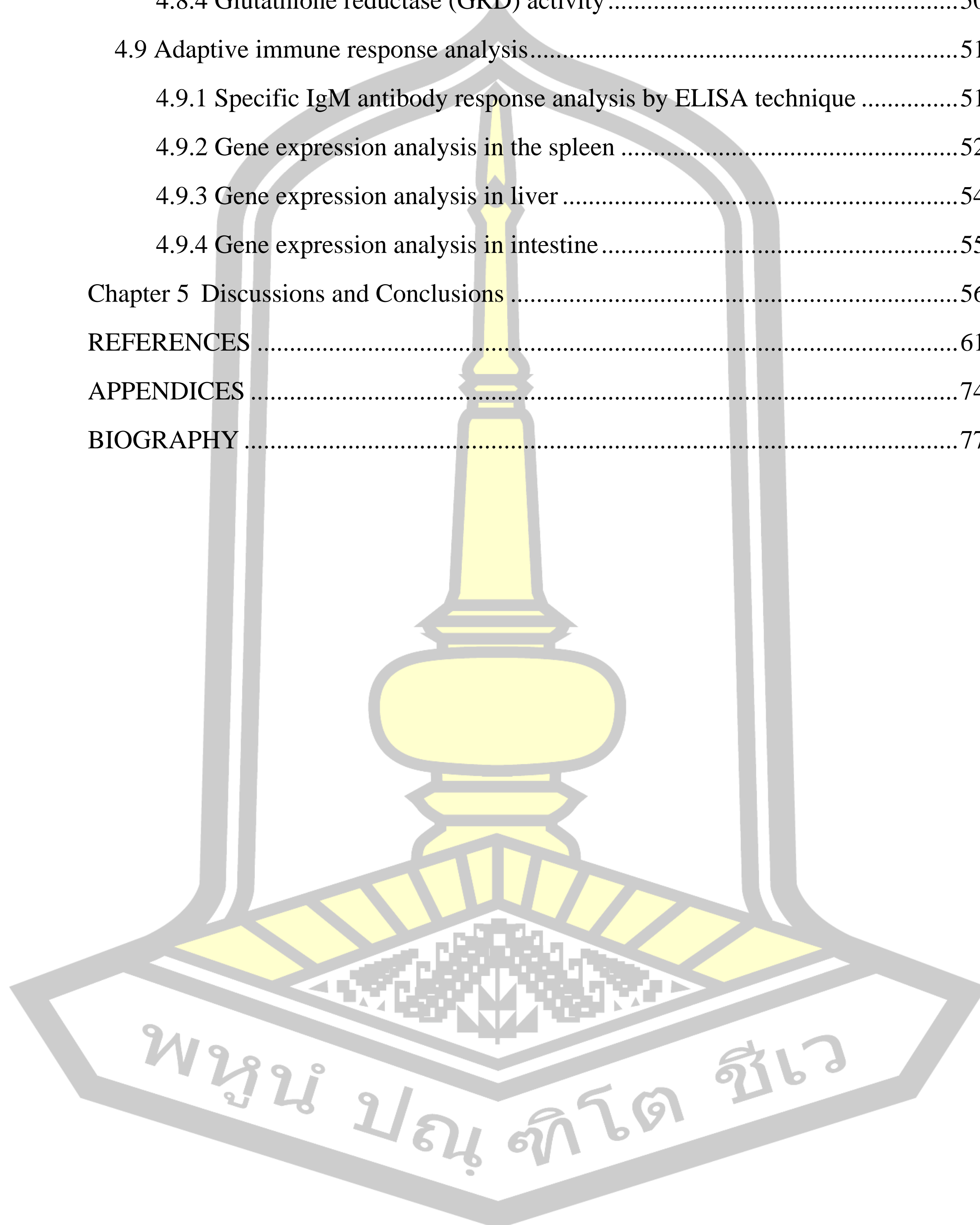

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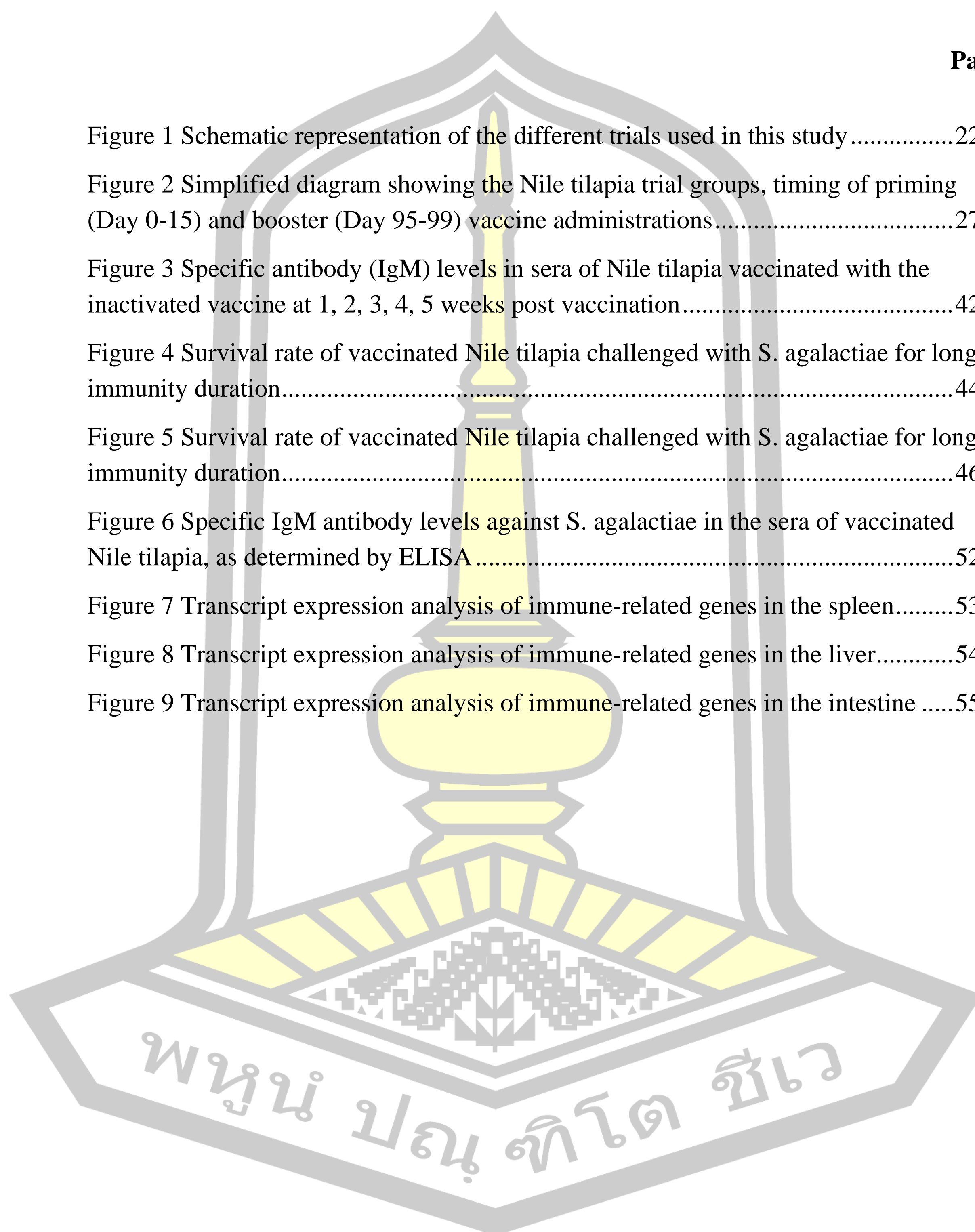

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Chapter 1
Introduction

1.1 Introduction

The major objective of vaccination is to prevent disease and increase the specific immune response in fish to a particular pathogen. The key advantage of vaccine application in aquaculture is that it is a prophylactic treatment applied before a disease outbreak, rather than trying to solve a disease problem after infection. At present, vaccine administration in fish is a critical component of fish health management, and it is generally accepted that vaccination is the most practical tool to control infectious diseases that threaten the aquaculture industry worldwide (Evensen and Leong, 2013; Huang *et al.*, 2014). Vaccines can also have a significant positive impact on reduced usage of antibiotics in fish farming, resulting in a rapid decline of antibiotic consumption (LaFrentz *et al.*, 2008; Pridgeon and Klesius, 2013). Nevertheless, fish vaccines must be safe, cheap to produce and induce long lasting immunity. Moreover, the vaccines should be stable and easily administered for mass production (Austin, 2012).

In general, administration of vaccines is via a number of different routes in fish, including immersion, injection, or orally (Plant and LaPatra, 2011; Chettri *et al.*, 2013). Injection vaccination is the most commonly used method for administration of commercial fish vaccines. It provides a long duration of protection and allows the incorporation of adjuvants (Mutoloki *et al.*, 2008). However, the disadvantage of injection vaccin are time consuming and personal skilled requirement or from side effects of the vaccine itself (e.g. adhesions). Developing alternative vaccine delivery systems would address many of the problems associated with IP (Intraperitoneal) injection with the potential to 1) decrease the costs of fish vaccination, 2) improve vaccinator safety, 3) reduce losses associated with side effects and/or opportunistic infections, and as a result 4) improve animal welfare. Some oral vaccines have been developed and are designed to be administered with the feed. This gives a simple method of application, improved safety, and substantial reduction on the stress imposed to the fish. The need for effective adjuvants has largely driven injection

vaccination, and this needs to be revisited with the appearance of several highly efficacious mucosal adjuvants being developed for use in mammals or humans.

This research is focusing on the optimize of a novel adjuvant Montanide GR01 Nile tilapia. Initially, stable formulations containing a killed bacterial pathogen (*Streptococcus agalactiae*) was prepared. Three formulations of feed-based vaccine were compared the responses elicited in the fish by oral vaccine. The ability to enhance the expression of a number of important immune genes, thought to be involved in protective responses, as well as blood antibody levels was also evaluated. The best formulation in terms of the responses elicited was selected, to be used in a further experiment where the fish was vaccinated and then exposed to the live pathogen (challenge test), to see if the responses give good protection or immunity. If successful, the results would have the potential to revolutionize oral vaccination of fish. Enhanced immune responses or protection obtained with effective adjuvant may avoid the need for booster vaccination following oral vaccination, or allow protective responses to be induced to diseases that currently require adjuvanted IP injection vaccination. Most importantly, for practical application, performance of adjuvant Montanide GR01 in the feed-based vaccine of the Nile tilapia was further investigated in farm-scale trials. The cage culture system, which is normally susceptible to pathogenic bacterial infections in particularly hot seasons, will be chosen. This research intends to evaluate the capacity of oral vaccination method in fish.

1.2 Objectives

1.2.1 To develop an inactivated vaccine using formalin-killed cell (FKC) of *Streptococcus agalactiae* in prevention of Streptococcosis in Nile tilapia

1.2.2 To evaluate the efficacy of a commercial adjuvant Montanide GR01 on FKC vaccine by oral vaccination in Nile tilapia

1.2.3 To generate adjuvanted vaccine for prevention of streptococcosis that provide several advantages with applicability to Nile tilapia cultures in order to increase the fish immune response, reduce the utilization of antimicrobial agents by fish farmers and decrease unprofitable negative impacts, especially culture costs to the farmers

1.3 Scope of thesis

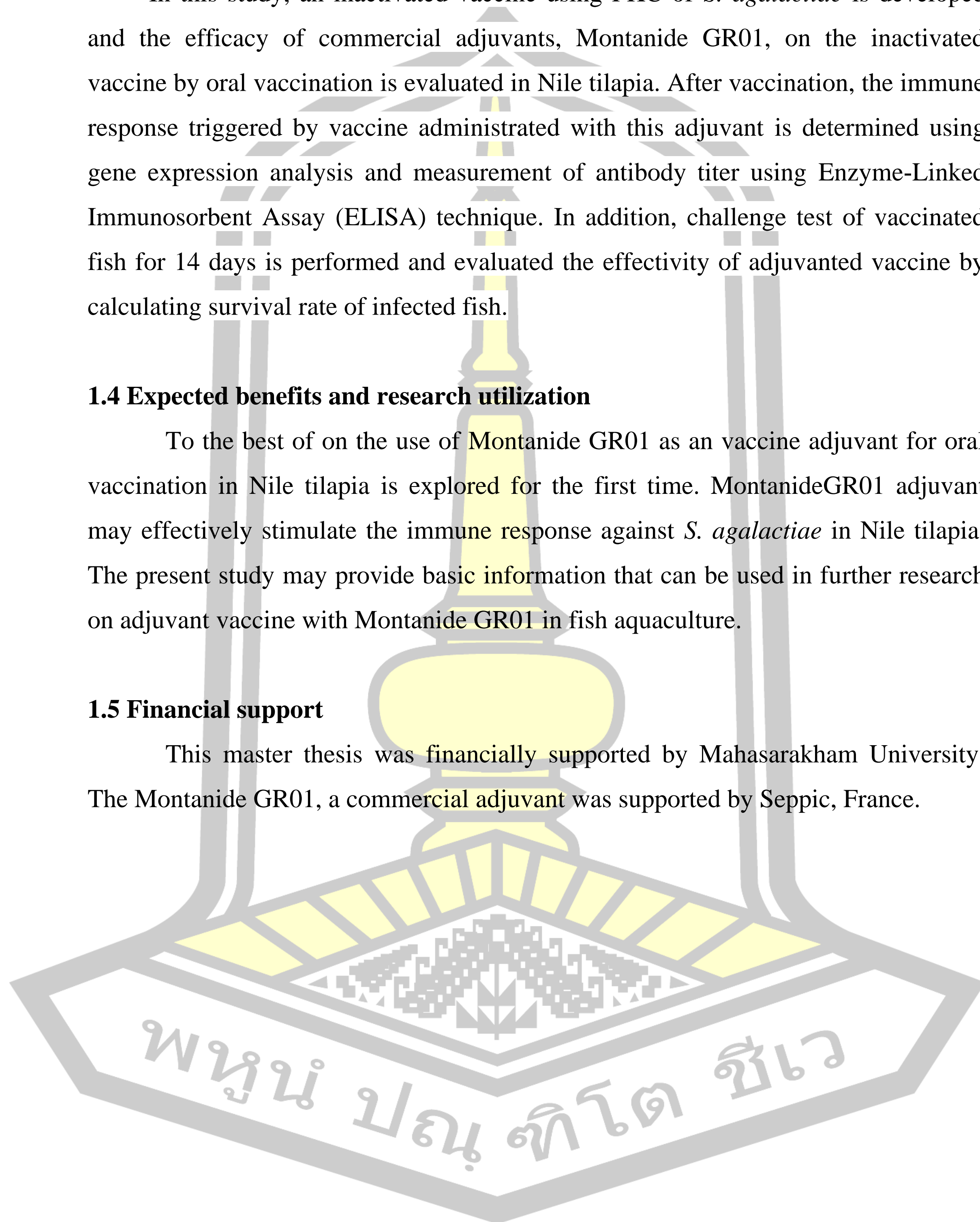
In this study, an inactivated vaccine using FKC of *S. agalactiae* is developed and the efficacy of commercial adjuvants, Montanide GR01, on the inactivated vaccine by oral vaccination is evaluated in Nile tilapia. After vaccination, the immune response triggered by vaccine administrated with this adjuvant is determined using gene expression analysis and measurement of antibody titer using Enzyme-Linked Immunosorbent Assay (ELISA) technique. In addition, challenge test of vaccinated fish for 14 days is performed and evaluated the effectivity of adjuvanted vaccine by calculating survival rate of infected fish.

1.4 Expected benefits and research utilization

To the best of on the use of Montanide GR01 as an vaccine adjuvant for oral vaccination in Nile tilapia is explored for the first time. MontanideGR01 adjuvant may effectively stimulate the immune response against *S. agalactiae* in Nile tilapia. The present study may provide basic information that can be used in further research on adjuvant vaccine with Montanide GR01 in fish aquaculture.

1.5 Financial support

This master thesis was financially supported by Mahasarakham University. The Montanide GR01, a commercial adjuvant was supported by Seppic, France.



Chapter 2
Literature reviews

2.1 Diseases of Nile tilapia

Infectious causes of Nile tilapia diseases include viral diseases, bacterial diseases, fungal diseases, and parasitic diseases (Ibrahim, 2020). Bacterial diseases are the important disease for both cultivated and wild fish, causing massive economic losses in fish production for example, Septicaemic bacterial diseases (Motile aeromonas septicemia, Pseudomonas septicemia, Vibriosis, Edwardsiellosis, Enteric Redmouth Disease (ERM), Streptococcosis, and Staphylococcosis), Columnaris disease, and Mycobacteriosis (Ibrahim, 2020; Mishra *et al*., 2018).

2.2 Streptococcosis

2.2.1 Bacterial characteristics

Streptococcosis is an infectious disease caused by *Streptococcus* spp. and is a common pathogenic species found in Nile tilapia (Mzula *et al*., 2021). The disease may be subacute but is often chronic. Although multiple species of *Streptococcus*, such as *S. agalactiae*, *S. iniae*, *S. dysqalactiae* subsp. *dysgalactiae*, *S. dysgalactiae* subsp. *equisimilis*, *S. pyogenes*, *S. parauberis*, *S. equi* subsp. *equi*, and *S. equi* subsp. *zooepidemicus*. (Plumb and Hanson, 2011). Both *S. iniae* and *S. agalactiae* are the most common pathogens that cause serious disease in Nile tilapia and affect a variety of fish species (Plumb and Hanson, 2011, Wongsathein *et al*., 2019).

Streptococcus spp. are Gram-positive bacteria, cocci, and arranged in chains. *S. agalactiae* is another Nile tilapia pathogen linked to intensive brood stock culture (Mzula *et al*., 2021). The *Streptococcus* spp. are β-hemolytic, nonhemolytic, and are biochemically less reactive (Plumb and Hanson, 2010). Streptococcosis can cause 50-70% mortality in Nile tilapia aquaculture, resulting in massive economic losses from outbreaks. As a result, extensive surveillance should be attempted (Mzula *et al*., 2021). *S. agalactiae* specifically serotype III has become a particularly major Nile tilapia pathogen in the past decade (Wongsathein *et al*., 2019, Sapugahawatte *et*

al., 2022). Streptococcosis is currently endemic in several Southeast Asian tilapia-producing countries, including Malaysia, Indonesia, Vietnam, and Thailand, hampering the development of this business sector (Sapugahawatte *et al*., 2022).

2.2.2 Clinical signs of streptococcosis

In general, fish infected by *Streptococcus* sp. exhibit one or more with the following clinical signs, depending on the species. The most common symptoms in Nile tilapia and pathological signs include erratic swimming (such as spiraling or spinning), loss of orientation; lethargy, darkening; unilateral or bilateral exophthalmia (also named “pop-eye”, in one or both eyes), corneal opacity (whitish eyes), anorexia, abdominal distention, darkening of the skin, and hemorrhaging skin around the anus or at the base of the fins. Moreover, some syndromes abdominal dropsy, pale, e.g. haemorrhagic liver as well as some haemorrhage around the eyes, operculum, fin and/or body, inflammations around the heart and kidney, and meningitis were also found (Yanong and Francis-Floyd, 2002, Zhang, 2021, Wongsathein *et al*., 2019).

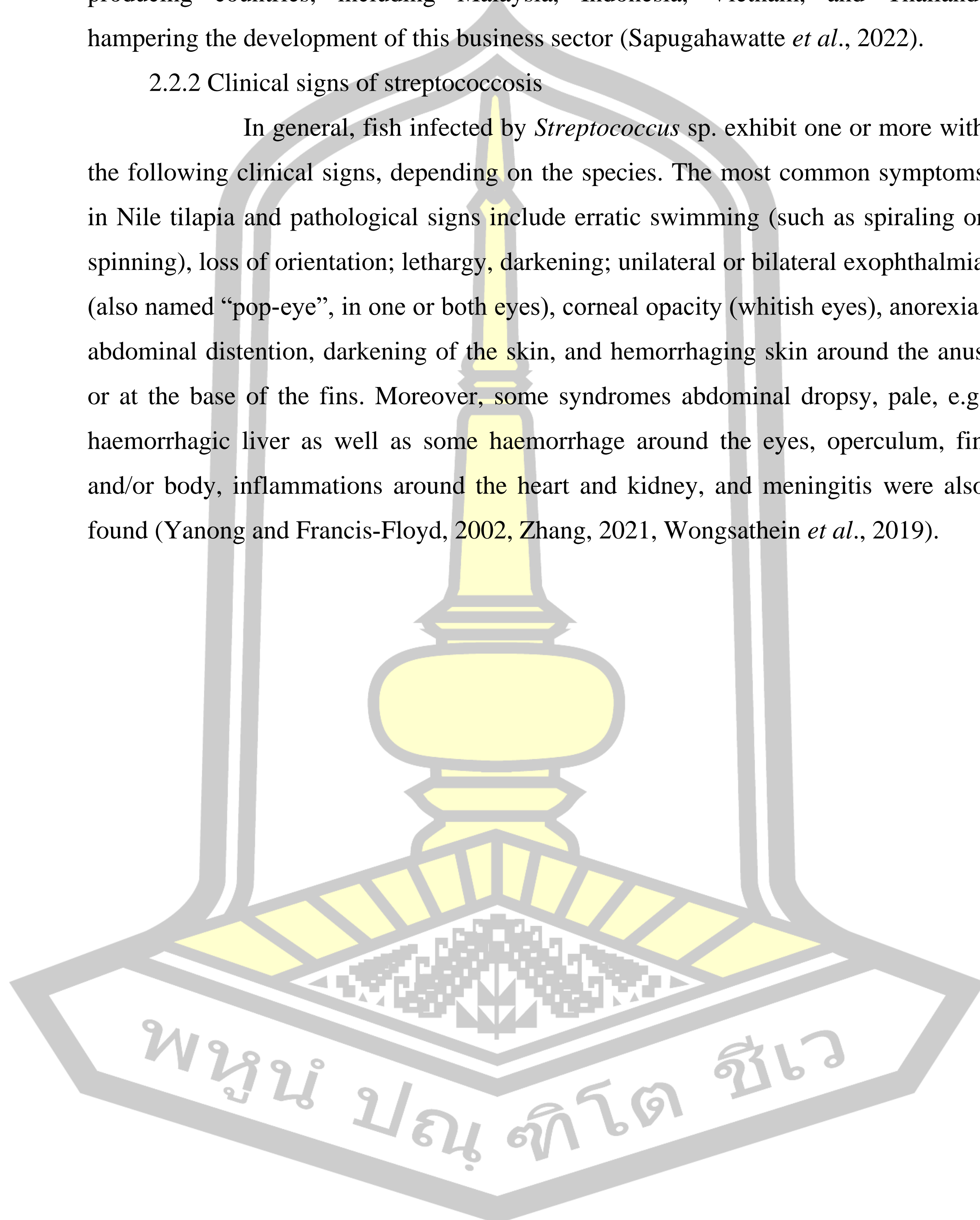


Table 1 The bacterium in the genus *Streptococcus* spp. that affected in fish and clinical signs

Species	Fish species	Clinical signs
S. iniae	Nile tilapia, Hybrid tilapia, Rainbow trout, Red drum, Rabbitfish, Sea bass, Olive Flounder, Wild fish	Hemorrhage, exophthalmia, abdominal distension, ascites, lesions (liver, kidney, spleen and intestine)
S. agalactiae	Nile tilapia, Rainbow trout, Cultured turbot, Hybrid striped bass	Erratic swimming, appetite, lethargy, uncoordinated movements, exophthalmia (uni- or bi-lateral), intraocular hemorrhage, opaqueness of cornea, ascites
S. parauberis	Olive flounder, Rainbow trout, Cultured turbot, Hybrid striped bass	Chronic wasting syndrome, hemorrhagic septicemia, exophthalmia, meningitis with abnormal swimming
S. dygalactiae	White spotted snapper, Kingfish, Grey mullet, Cobia, Hybrid red tilapia, Pompano, Basket mullet, Golden pomfret, Amur sturgeon, Nile tilapia, Yellow tail, Rainbow trout, Atlantic salmon	Loss of equilibrium, exophthalmia, melanosis, bleeding (jaw, eye, mouth, abdomen, fins and anus), necropsy, transparent fluid accumulation, fibrinous deposits (heart, liver, spleen)

Modified from: Mishra *et al*. (2018)

2.3 The fish immune systems

Basically, the fish immune system can be divided in to two categories, innate and adaptive immunity. Innate immunity is the first line of defense in several organism including invertebrate and vertebrates. In teleost fish, the epithelial barriers including scales, skin, gill and gut act as the first barrier against infection (Shephard, 1994; Ellis, 2001). Some molecules such as mucus, glycoproteins, lytic enzyme can be released from fish skin to protect the infection (Van der Marel *et al*., 2010). The most important function of these mucus is preventing attachment of bacteria, fungi, parasites and viruses at the epithelial surfaces (Van Muiswinkel and Nakao, 2014).

Apart from efficient trapping and removing of pathogens, fish mucus also contains immune parameters such as lectins, pentraxins, lysozyme, complement proteins, and antimicrobial peptides (Alexander, 1992; Rombout *et al*., 1993; Aranishi and Nakane, 1997). Additionally, monocytes and neutrophils, are the most common type of white blood cells worked as the first immune cells to defense against infection. Macrophages are the important effectors of inflammation and host defense against microbial infection by destroying pathogens through phagocytosis. Reactive oxygen species (ROS) and nitric oxide (NO) are fundamental macrophages to eliminate invasive microorganisms. These macrophages can stimulate the host immune response by releasing of pro-inflammatory cytokines and chemokines (Smith *et al*., 2019).

2.3.1 The immune relevant organs in fish

2.3.1.1 Head kidney

Head kidney is the bone marrow equivalent of teleost fish acting as a hematopoietic site. The immune cells are present over entire kidney whereas anterior or head kidney has the highest concentration of developing B-lymphoid cells. It is the principal organ for phagocytosis, antigen processing, and formation of IgM and immune memory through melanomacrophage centres (Bjørgen and Koppang, 2020; Sahoo *et al*., 2021).

The head kidney is also the haematopoietic-lymphoid organ in teleostean or "true bony" fish, homologs to adrenal gland in mammals and release corticosteroids and other hormones (Bjørgen and Koppang, 2020). Furthermore, anterior kidney is the major site for antibody production (Sahoo *et al*., 2021; Geven and Klaren, 2017). Sinusoidal endothelial cells, macrophages and fibroblastic reticular cells can form the actively phagocytic elements. The head kidney contains mostly proliferating B cell precursors and plasma cells, whereas the trunk kidney contains abundant B cells, some of which are activated, and in addition plasmablasts (Bjørgen and Koppang, 2020).

2.3.1.2 Thymus

Thymus is the most important lymphoid organ and found in all vertebrates including teleost fish. The lymphoid cells are actually major immune blood cells initially and are not differentiated in the head kidney. Unlike mammals,

thymus appears to carry and develop precursor cells migrating from bone marrow for T cells formation (Sahoo *et al*., 2021).

2.3.1.3 Spleen

The spleen is the major lymphoid organ, and plays the major role in the clearance of antigens in the adaptive immune response in teleost fish (Sahoo *et al*., 2021). The spleen has the functions of degrading and processing antigens and producing antibodies. Additionally, the spleen is also a major hematopoietic organ that can produce blood cells, endothelial cells, reticulocytes, macrophages and melanin macrophages (Long *et al*., 2021).

2.3.2 Innate immunity

The innate immune system in fish is of prime importance in the immune response. It is commonly divided into three compartments, the epithelial/mucosal barrier, the humoral parameters, and the cellular components. The epithelial and mucosal barrier of the skin, gills, and alimentary tract are the first line of defense in fish, being constantly immersed in media containing potentially harmful agents. These barriers contain several immune defense parameters such as antimicrobial peptides, complement components, and immunoglobulins (Magnadottir, 2010). The skin surface of fish differs from that of higher vertebrates whereas the epidermis composed of several non-keratinised living cells (Roberts, 2012). Fish gill as an important route of entry of microorganisms. This organ can protect fish by mucus produced and is a highly responsive epithelium resulting in hyperplasia, frequently seen in many gill infections, for example costiasis and myxobacterial gill disease (Roberts, 2012).

The gastrointestinal tract or mucous membrane layer is the lines of digestive tract containing epithelium similar to the skin. However, the digestive function of the gut provides an extremely hostile environment to potential pathogens by virtue of the low pH (in species with a stomach) and often encountering strong and mild acidic environments (Roberts, 2012). Lysozyme (LZM) is one of the important bactericidal enzymes of innate immunity and usually found in fish. It is involved in the overall alarm responses during the infection as well as stress conditions and acts as an acute phase protein (Swain and Nayak, 2009). Moreover, LZM has been widely studied as biomarker of innate immunity in many organisms. Functionally, LZM

presents in mucus and lymphoid tissue and can lysis of peptidoglycan layer of bacterial cell walls. Recently, LZM has been found in fish and plays an important role in the defense mechanisms, such as bacteriolysis, opsonization, and antiviral. Lectins are proteins which agglutinate cells and precipitate glycoconjugates. It could act as opsonin for phagocytosis of bacteria and also involved in activation of the complement system (Ruata *et al*., 2012).

The macrophages and neutrophilic granulocytes are the principal phagocytic cells in fish. When stimulation, these cells participate phagocytose antigenic material and/or exert cytotoxic activity. The killing of intracellular or extracellular pathogens is based upon the release of a number of ROS and NO (Van Muiswinkel and Nakao, 2014). Antibacterial peptides (AMPs) as an important feature regarding antibacterial activity in several species including fish (Smith *et al*., 2019). These are low molecular molecules with the ability to disrupt antigens like bacterial membranes. AMPs are secreted by fish skin and can be activated against microorganisms (Jia *et al*., 2000; Cuesta *et al*., 2008; Chia *et al*., 2010).

2.3.3 Adaptive immunity

Adaptive immunity plays an important role in prevention of pathogens in vertebrates. When pathogen invade into the body, the adaptive immunity will be activated. The adaptive immunity includes humoral and cellular immune response. Lymphocytes (B cells and T cells) are key regulators for both humoral adaptive immune response and in all vertebrates. The major role of B cells is to produce antibody against foreign antigens, while T cells are key regulators of cellular immune response (Flajnik and Kasahara, 2010; Smith *et al*., 2019). Humoral immunity with assistance from helper T cells, B cells will differentiate into plasma B cells that can produce antibodies against a specific antigen. Antibodies produced by the B cells will bind to antigens, neutralizing, causing lysis or phagocytosis (Smith *et al*., 2019).

In teleost fish, adaptive immunity mainly relies on the functional potential of T and B cells and their sub-populations. Nevertheless, the key role in protecting fish against infection is limited (Mutoloki *et al*., 2014). T cells are the subtypes that orchestrate cell-mediated immune responses. T lymphocytes constitute a minor population in circulation, although they are believed to be present in greater numbers in mucosal areas (Mutoloki *et al*., 2014). Moreover, B cells in teleost fish are

produced and maturate in the central lymphoid organ, i.e the pronephros of the kidney. In this organ, all developmental forms of B cells are present, including proliferating precursor cells B, plasmablasts and plasma cells, which synthesize and secrete antibodies (Stosik *et al*., 2021).

2.3.3.1 Humoral immune response

Immunoglobulins (Igs) are mainly produced by plasmablasts and plasma cells, and secreted into body fluids (including serum and mucosal secretions) as antibodies (soluble form), or present on the surface of B cells as B cell receptors (BCR) (membrane-bound form) (Salinas *et al*., 2011). The principal components of the humoral immune response in adaptive immunity are the Igs. Due to the particular characteristics of mucosal surfaces, vertebrates have specialized Igs in their mucosal surfaces. In mammals, five classes of Igs (IgM, IgD, IgE, IgG, and IgA) have been found. However, three Ig isotypes have been reported in teleosts so far, e.g. IgM, IgD, and IgT/IgZ (Gomez *et al*., 2013).

IgM is a universal isotype in all vertebrate species and found in the sera (Ye *et al*., 2013). Fish antibodies are found in the skin, intestine, gill, mucus, bile and systemically in the plasma (Uribe *et al*., 2011). IgM antibody has also been demonstrated to mediate antigen-specific opsonization and phagocytosis of bacterial pathogens (Ye *et al*., 2013). While IgD is found in several vertebrates including teleost fish (Smith *et al*., 2019). The IgT/IgZ is reported only in bony fish and first identified in rainbow trout (IgT) and zebrafish (IgZ). IgT/IgZ is specialized for mucosal immunity and functions analogously to mammalian IgA. In addition, IgT/IgZ and B cells are also found in teleost skin associated lymphoid tissue (SALT) where they secrete IgT into skin mucus (Smith *et al*., 2019).

Cytokines are small molecules that play a crucial roles in the adaptive immunity in mammals and teleost fish (Zhu *et al*., 2013). Cytokines are mainly secreted by cells of both the innate and adaptive immune systems. These molecules effect small physiological changes via specific receptors present on target cell surfaces and are responsible for signal transmission between cells (Sakai *et al*., 2021). Many cytokines that play critical roles in mammalian adaptive immunity have been identified in teleost fish including the CC cytokine family, a number of ILs, and transforming growth factor-β that contributes significantly to adaptive immune

responses. One of the major type I cytokines is the CC family. These contain four a-helical bundles and share the same CC receptor chains. Various ILs, including IL-2, IL-4/13, IL-7, IL-15, and IL-21 belong to this family (Zhu *et al*., 2013; Venkatachalam, 2019).

2.3.2.2 Cellular immune response

The first evidence of the existence of T-cell populations has been revealed in bony fish since the 1970s (Randelli *et al*., 2008). T cells play an important role in cell-mediated immune responses by either regulating the functions of other leukocytes or directly killing infected host cells (Kordon *et al*., 2021). T cells secrete factors that help other immune cells coordinate their responses or cytotoxic factors that directly kill infected or abnormal cells. Many genes associated with T cell functioning and signaling have been identified in fish. However, understanding of the functional correlation between presumed T cell associated genes and T cell activity in adaptive immunity in fish is under investigated (Rauta *et al*., 2012).

2.4 Fish vaccination

From many decades, vaccination has been practiced against infectious diseases (Mondal and Thomas, 2022). Vaccine is a biological tool that is used for training of fish immunity against the diseases. Fish vaccines are typically prepared from weakened or killed forms of the microbes (Kumar *et al*., 2018). There are various different vaccines, which are generally divided into four categories, e.g. inactivated whole cell vaccines, live attenuated vaccines, DNA vaccines, and subunit vaccines.

2.4.1 Live attenuated vaccines

Live vaccines are prepared from microorganisms showing attenuated virulence or natural low virulence. Pathogens can be attenuated using physical or chemical processes, serial passage in cell culture, culture under abnormal conditions, or genetic manipulation (Desmettre and Martinod, 1997; Ma *et al*., 2019. Live vaccines are similar to natural infection, and can enhance both innate and adaptive immunity (Levine and Sztein, 2004; Ma *et al*., 2019. Basically, live-attenuated vaccines can also induce cell-mediated and humoral immune responses (Mohd-Aris *et al*., 2019).

2.4.2 Inactivated vaccines

Inactivated or killed vaccines are typically produced by a virulent microbe losing the ability to infect the host. This type of vaccine can be produced by physical, chemical, or radiation processes without compromising the antigenicity of the microbial agents (Tlaxca *et al*., 2015) . Formalin-inactivated and heat-killed whole-cell vaccines have been commonly used in most studies (Zhang, 2021). Inactivated vaccine is administered via inoculation for conferring protective immunity (Mondal and Thomas, 2022), and found to be safe when compared live activated vaccine (Baxter, 2007). This vaccine can necessitate the use of adjuvants or multiple booster vaccination to induce protective immunity. Once administered, phagocytic antigen presenting cells (APCs) begin the process of removing activated immune cells and eliciting a humoral immune response with memory B cells (Ma *et al*., 2019). The killed whole-cell vaccine, also known as bacterin, is a common type of bacterial vaccine. Recently, bacterin and inactivated vaccines are commercially available and authorized to be used in the aquaculture industry. Adjuvants are often added to these vaccines, as immune potentiators or vaccine carriers to increase efficiency of inducing a potent immune response (Mohd-Aris *et al*., 2019).

2.4.3 Subunit vaccines

Subunit vaccines take advantage of using only antigenic components for vaccination (Hansson *et al*., 2000). It can be produced in a highly characterized state, and they target immune responses toward specific microbial determinants. In addition, subunit vaccines have many desirable qualities; however, their ability to stimulate a potent immune response is weaker than killed or live whole cell preparations (Ma *et al*., 2019) . Subunit antigens are mainly produced from heterologous protein expression systems and offer the safest and most attractive means of antigen production. In fish vaccination, the most widely used protein expression systems for the production of subunit antigens are *E. coli* and yeast (Mutoloki *et al*., 2015). Instead of the entire microbe, subunit vaccines include only the antigens that show the good stimulate of the immune system. Sometimes, these vaccines use the very specific parts of the antigen as epitopes that recognize the antibodies or T and bind to them (Kumar *et al*., 2018).

2.4.4 DNA vaccine

The genes of microbe are introduced into cells will take up that DNA, instructs those cells to make antigen molecules. Bacterial plasmid DNA containing a construct for a given protective antigen, is to establish specific and long protective immunity against diseases (Tonheim *et al*., 2008; Kumar *et al*., 2018). DNA vaccines are defined as the intentional transfer of genetic material to somatic cells for the purposes of influencing the immune system (Tonheim *et al*., 2008). Moreover, DNA vaccines are made up of an expression plasmid that contains a specific gene that codes for a specific antigenic protein, which is expected to elicit a strong immune response when expressed in the host (Ma *et al*., 2019). Thus, after delivery to the fish, the DNA might; i) induce fish immune responses and protection, and ii) be released to the environment or to consumers either from the vaccinated fish and/or from accidental spilling (Gomez-Casado *et al*., 2011). DNA vaccines are able to strongly activate cellular and humoral immunity against various fish pathogens (Ma *et al*., 2019, Mondal and Thomas, 2022). DNA vaccines are often administered by intramuscular injection such as DNA vaccine against infectious hemorrhagic necrosis virus, IHNV (Brudeseth *et al*., 2013). For DNA vaccination, a short-term expression is sufficient for evoking an immune response (Tonheim *et al*., 2008). Lastly, DNA vaccines are safer to the fish health than attenuated live virus or even inactivated virus (Gomez-Casado *et al*., 2011).

2.5 Methods of administration vaccines to fish

In general, there are three vaccines delivery methods have been used for fish vaccination. Administration of vaccines is either performed by oral vaccination through feed, by immersion vaccination in diluted vaccine suspensions or by injection vaccination via intraperitoneal or intramuscular rout (Brudeseth *et al*., 2013).

2.5.1 Injection vaccination

This method is slow, and may require prior anaesthesia of the animals. Injection is feasible only for large and/or valuable fish (Austin, 2012). In this method, a small volume of vaccine is directly delivered into the fish via intramuscular (IM) or intraperitoneal injection (IP), allowing for direct stimulation of immune defense. In this approach, the time period of protection is more prolonged when compared to

immersion method (Vinitnantharat *et al*., 1999, Mondal and Thomas, 2022). Injection vaccination is effective for many pathogens that cause systemic disease and protect the animal more than 6 months. This method only used for larger size fishes. However, a number of limitations to injection vaccination is limited. Not only is the process of handling, anesthetizing and injecting stressful for the fish but injection vaccination also requires more time, labor and skilled personnel (Horne, 1997; Plant and LaPatra, 2011; Kumar *et al*., 2018). Other disadvantages of injection vaccination such as adhesion formation, temporary reduced feeding, inadvertent intestine puncture, and the formation of a wound that could serve as a portal of entry for infectious agents (Vinitnantharat *et al*., 1999). IP is the most productive route and efficient way of immunizing fish. Adjuvants are employed in IP injection because they provide better protection than the immersion approach, particularly oil adjuvants (Mondal and Thomas, 2022).

2.5.2 Immersion vaccination

Immersion vaccination (IMM) is probably the simplest method of vaccination, but it is not suitable for all farming situations. This method is quick, (taking 30-120 s to perform) and easy, permitting large numbers of fish to be readily vaccinated. However, the problems regarding the disposal of the spent vaccine could be found. It is debatable whether or not disposal should take place in the fish farm effluent (Plant and LaPatra, 2011; Kumar *et al*., 2018). Live bacterial vaccines or formalin-inactivated bacterial suspensions are the two main types of commercial immersion vaccines. For inactivated antigens, the immunization process involves either a short immersion in a concentrated antigen suspension or a longer period of exposure in a more diluted bath. The dilution of a concentrated vaccination suspension used in many inactivated immersion vaccines is ten times lower (Brudeseth *et al*., 2013). This type of vaccination is a simple and efficacious method of immunizing fish for protection against infection. Formalin inactivated bacteria and live bacterial vaccines are the immersion type of vaccines commercially available (Mondal and Thomas, 2022). This vaccine is delivered into the skin and mucosal surfaces accessible to the surrounding liquid, which contains the antigen (vaccine), which permits immune cells located in the fish skin and gills to become directly exposed to antigens. After vaccination, the immune cells can produce antibody, which

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protecting the fish from future infection. The common delivery methos of vaccine by immersion vaccination including, by dip or by bath. Dips are short period, in a high concentration of vaccine whereas baths are of a long period and in a low concentration of vaccine (Plant and LaPatra, 2011; Kumar *et al*., 2018).

2.5.3 Oral vaccination

Oral vaccines are produced by coating the feed with antigen, mixing antigen into the feed during production or bioencapsulated. Delivery of antigen in or on fish feed offers several advantages. There are easy to administer and causes no stress to the fish. However, oral vaccination does have some disadvantages. It is difficult to determine exactly how much each fish consumes and therefore the dose of antigen received and leaching and the need to protect antigens as they pass through the stomach as well as the formulation of vaccines to improve the stimulation of protective immunity. There may be problems with the degradation of the vaccine in the intestinal tract, although this may be overcome by using micro-encapsulation (Austin, 1984). These disadvantages of oral vaccination in fish are solved as by using vaccine combined with adjuvant (Quentel and Vigneulle, 1997; Plant and LaPatra, 2011; Kumar *et al*., 2018). In general, oral vaccination with antigens included in the feed is in principle the ideal method of vaccine delivery. Such products are usually coating the feed with antigen or by coating on top of the feed. The vaccine is either sprayed over the feed, mixed with the feed, or bio-encapsulated (Brudeseth *et al*., 2013; Mondal and Thomas, 2022). The efficacy of oral vaccines is dependent on antigen content in the feed resistance against gastric degradation and antigen adsorption or uptake in the gut (Brudeseth *et al*., 2013). Oral vaccination gives the least efficacy compared to the immersion and injection methods (Vinitnantharat *et al*., 1999).

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Table 2 Major bacterial fish diseases, the causative agents, main host and some of vaccine commercially availability

Diseases	Pathogen	Major Fish Host	Type of vaccine	Vaccination route
Edwardsiellosis/ Enteric septicemia	*Edwardsiella ictaluri*	Catfish species	Attenuated	IMM
Flavobacteriosis/ Columnaris	*Flavobacterium columnare*, *F. maritimus*	Salmonids Channel catfish Freshwater	Bacterin/ Attenuated	IMM
Furunculosis	*Aeromonas salmonicida*	Salmonids	Inactivated	IP or IMM
Lactococcosis	*Lactococcus garvieae*	Salmonids Sea bass/ bream	Inactivated	Oral
Streptococcosis	*S. agalactiae, S. iniae,* *S. dysgalactiae,* *S. paraberis,* *S. phocae*	Nile tilapia Asian sea bass/bream Salmonids Yellowtail Rainbow trout	Bacterin	IP, IMM, Oral
Vibriosis	*Vibrio alginolyticus,* *V. parahaemolyticus,* *V. vulnificus,* *V. anguillarum*	Salmonids Cods/ halibut Sea bass/ bream Amberjack/ yellowtail	Bacterin/ Inactivated	IP or IMM
Yersiniosis/ Enteric redmouth (ERM)	*Yersinia ruckeri*	Salmonids Rainbow trout Nile tilapia	Inactivated	IMM or Oral

Abbreviations: IP: Intraperitoneal injection; IMM: Immersion

(Modified from: Sommerset et al., 2005; Mohd-Aris et al., 2019; Ma et al., 2019)

2.6 Adjuvants

Adjuvants, as derived from the term adjuvare which means “to help”, are helper substances that can enhance the immune response to the immunogens. Adjuvants can boost and promote the immunity, prolong the production of antibodies or cytotoxic T-cell activity (Tafalla *et al*., 2014). Adjuvants have been defined as helper substances increasing the magnitude of an adaptive response to a vaccine or enhance the ability to prevent the infection. Nowadays adjuvants have been defined as

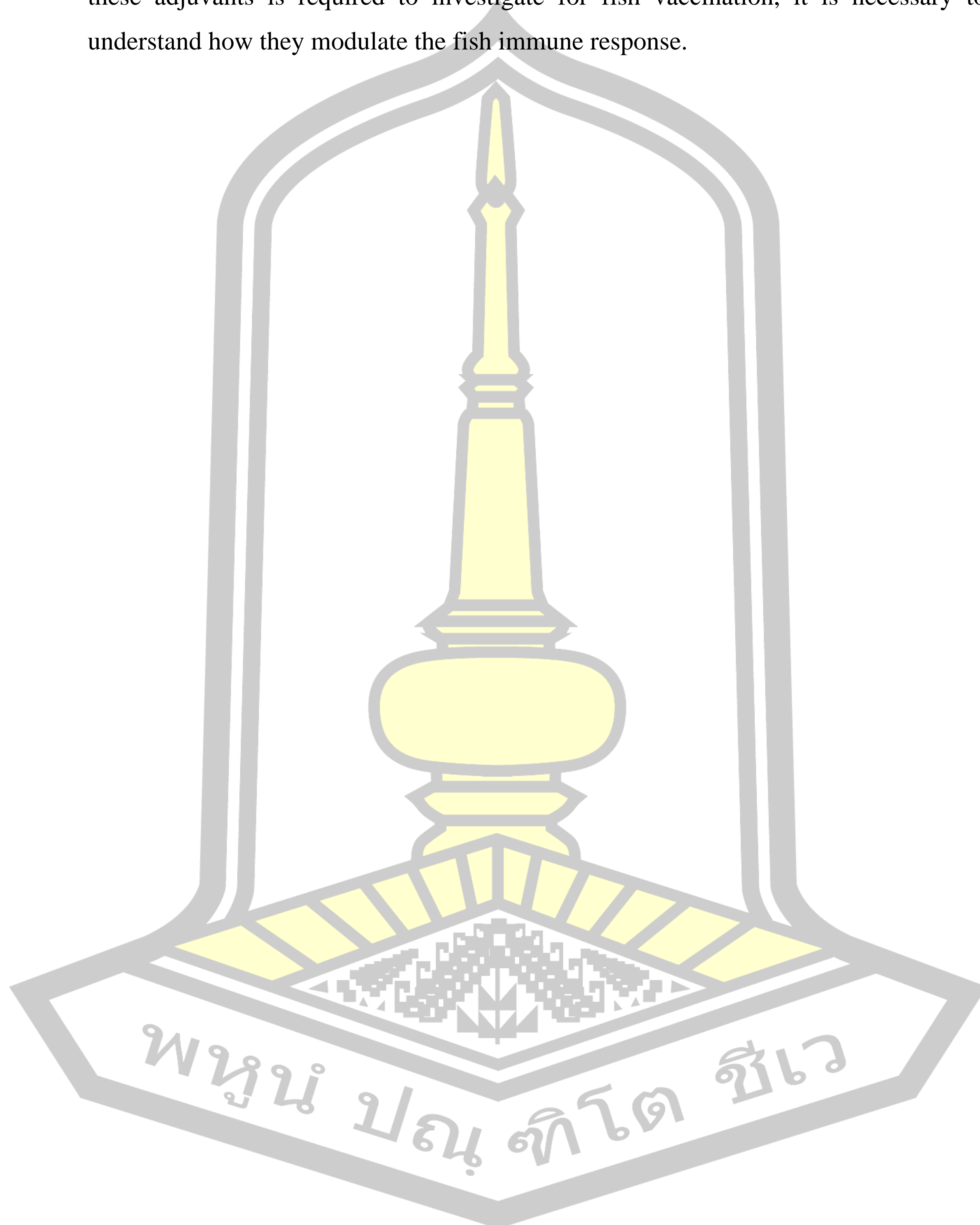
a group of structurally heterogeneous compounds and able to modulate the intrinsic immunogenicity of the antigens (Guy, 2007; Tafalla *et al*., 2013).

The various aspects of adjuvants include: i) enhancing the immune response to the antigen while at the same time prolonging the duration of immune responses, ii) modulating the breadth, affinity and specificity of antibody responses, iii) stimulating the expression of potent cellular-mediated immune responses, iv) increasing immunogenicity for weak antigens and seroconversion for low responders, v) promoting induction of mucosal immunity, vi) facilitating the reduction of antigen doses and reducing the need for booster vaccinations, and vii) reducing antigen competition in multivalent vaccines (Tafalla *et al*., 2014, Guimarães *et al*., 2015).

The purpose of an adjuvant is simply to increase the immunogenicity of an antigen and to modulate the immune response to the antigen in a desired direction (Lövgren-Bengtsson and Fossum, 2002). Montanide adjuvants have been used in both mammalian and fish vaccines. Non-metabolisable oils and surfactants also be included in various Montanide preparations (Bøgwald and Dalmo, 2012). Montanide™ adjuvants are a well-established brand of vaccine adjuvants used in animal vaccines, including aquaculture species. So far, mineral oil Montanide™ ISA 761 VG and non-mineral oil Montanide™ ISA 763A VG and Montanide™ ISA 763B VG have been developed specifically for injection vaccination in fish (SEPPIC, 2021). Also, Montanide™ IMS 1312 VG PR has been developed specifically for immersion vaccination (SEPPIC, 2021). Use of these adjuvants in fish vaccines have been shown to stimulate good immune responses and to give excellent protection in many species, as seen in Atlantic salmon (*Salmo salar*) (Thim *et al*., 2014; Hoare *et al*., 2019), rainbow trout (*Oncorhynchus mykiss*) (Soltani *et al*., 2014; Jaafar *et al*., 2015; Veenstra *et al*., 2017), orange-spotted grouper (*Epinephelus coioides*) (Nguyen *et al*., 2017), rohu (*Labeo rohita*) (Pradhan *et al*., 2018), turbo (*Scophthalmus maximus*) (Xu *et al*., 2019) and Nile tilapia (*O. niloticus*) (Zhang *et al*., 2017; Shahin *et al*., 2020).

Montanide™ GR01 is an experimental white and liquid oil adjuvant (SEPPIC, 2021), has been developed recently. It contains a gastro-resistant matrix which allows formulating veterinary oral vaccines by simple mixing with aqueous antigens. The vaccine emulsion can be poured on feed prior to be administered to fish. However,

this adjuvant has not yet been investigated for fish vaccination. The effectiveness of these adjuvants is required to investigate for fish vaccination, it is necessary to understand how they modulate the fish immune response.



Chapter 3
Methodology

Part I: Adjuvant effect of Montanide GR01 in the Streptococcosis formalin-killed vaccine of the Nile tilapia: Laboratory trial

3.1 Ethical statement

The animal experiments were approved by the Committee of Animal Experiments in Mahasarakham University. This study all animal care and experimental protocol were performed according to the guidelines of the guide for the care and use of laboratory animals, 8th edition (IACUC-MSU-08/2022).

3.2 The Experimental fish

One thousand and two hundred Nile tilapia weighed approximately 30 g were purchased from Roi-Et commercials farm in the northeastern part of Thailand. Fish were acclimatized in 5000-L fiberglass containers for 14 days. At the end of acclimatization 120 fish were divided into 1000-L fiberglass containing freshy and fully aerated tap water. During this period, fish were fed daily with a commercial diet at 3% body weight and approximately 20 % of the water volume was exchanged daily. Flow through freshwater supply system with water under natural temperature (26.7 ± 0.7 ℃), dissolved oxygen (DO) (9.35 ± 2.21 mg/L) and pH (8.4 ± 0.3) ranged on acceptable values throughout the experimental period.

3.3 The pathogenic bacterium

S. agalactiae was isolated from the liver and spleen of diseased Nile tilapia and used for testing the disease resistance properties of vaccination. The bacterium was identified as *S. agalactiae* using microbiologically standard protocols and showed specific characteristics including catalase-negative Gram-positive cocci and β-hemolytic properties. Biochemical analyses were conducted using API 20 STREP (BioMerieux, Marcy-l'Étoile, France) following the manufacturer's protocol. Finally, the bacterium was confirmed by PCR analysis using *S. agalactiae*-specific primers

based on the methods described by Martinez et al. (2001) (Martinez *et al*., 2001). The pathogenic bacterium was grown in 15 mL test tubes containing 10 mL trypticase soy broth (TSB; Difco, USA) at 37 °C for 24 h. The total of ten milliliters cultured broth were centrifuged at 3000 rpm for 15 min, and the supernatant was discarded. The bacterial pallet was washed in normal saline twice by centrifugation at 3000 rpm for 15 min. A bacterial solution was prepared in 0.85 % normal saline using a spectrophotometer at 600 nm with an absorbance of 0.67 to reach a final concentration of 1×10^9 colony forming units (CFU)/mL (Kannika *et al*., 2017). This concentration was also applied for challenge tests.

3.4 Vaccine preparation

In this study, an inactivated vaccine using formalin-killed cell (FKC) of *S. agalactiae* was developed and the efficacy of a commercial adjuvant Montanide GR01 on this vaccine was evaluated in Nile tilapia. In order to prepare inactivated vaccines, bacterial cells sub-cultured into BHI medium and harvested by centrifugation at 3,000 rpm at 4 °C for 40 min, resuspended in phosphate-buffered saline (PBS) containing 0.2% formalin, and incubated at 4°C for 20 h. Formalin-killed bacteria suspensions were washed three times by centrifugation and re-suspended in PBS. Subsequently, an aliquot of bacterial cells was used to formulate with Montanide GR01 following the emulsification protocol transferred by Seppic.

3.5 Feed-based vaccine preparation and vaccination protocols

The experiment consists of two trials (A and B). The first trial (Trial A) evaluated vaccine of different group to elicit immune response for a continuous period in Nile tilapia. This trial is to look for the vaccination protocol with highest immune response, evaluated by innate and adaptive immune parameters.

3.5.1 Trial A: Vaccination treatment

The trial was conducted using a total of 1,200 fish that was separated into 2 groups: control group and oral vaccination group. Each group experimentation was performed in 3 replicates with in 40 fish per tank, as described in Table 3.

Oral vaccination groups, fish were divided into 2 major groups (antigen only and antigen + Montanide^{TM} GR01) with 3 subgroups; group 1 (A2, A3

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and A4) and group 2 (A5, A6 and A7). The vaccination was performed for 4 weeks. Fish was vaccinated using feed-based vaccine by mixing of 200 mL of PBS containing 1 x 10^8 CFU/kg diet of FKC or incorporation of 200 mL of PBS containing 1x10^8 CFU/kg diet of FKC with Montanide™ GR01, whereas the control group (A1) was treated with equal amount of commercial feed, without bacteria throughout the experiment. In each subgroup, fish was oral vaccinated with different time point (Table 3) following:

Oral vaccination protocol 1 (A2, A5, A8): vaccination for 3 consecutive days, on day 1, 2, 3

Oral vaccination protocol 2 (A3, A6, A9): vaccination for 7 consecutive days, on day 1, 2, 3, 4, 5, 6, 7

Oral vaccination protocol 3 (A4, A7, A10): vaccination for 7 days with a 7 days interval, on day 1, 2, 3, 4, blank on day 5, 6, 7, 8, 9, 10, 11, then vaccinate once again on day 12, 13, 14

The vaccination was performed for 6 weeks including oral vaccine priming. At day 14 post ending the oral vaccination, the fish were bled for 4 times, by performing every week (week 1-4) throughout the experimental period. Serum samples obtained from Nile tilapia at different time points was assessed to test the IgM antibody level with ELISA technique as shown in Figure 1.

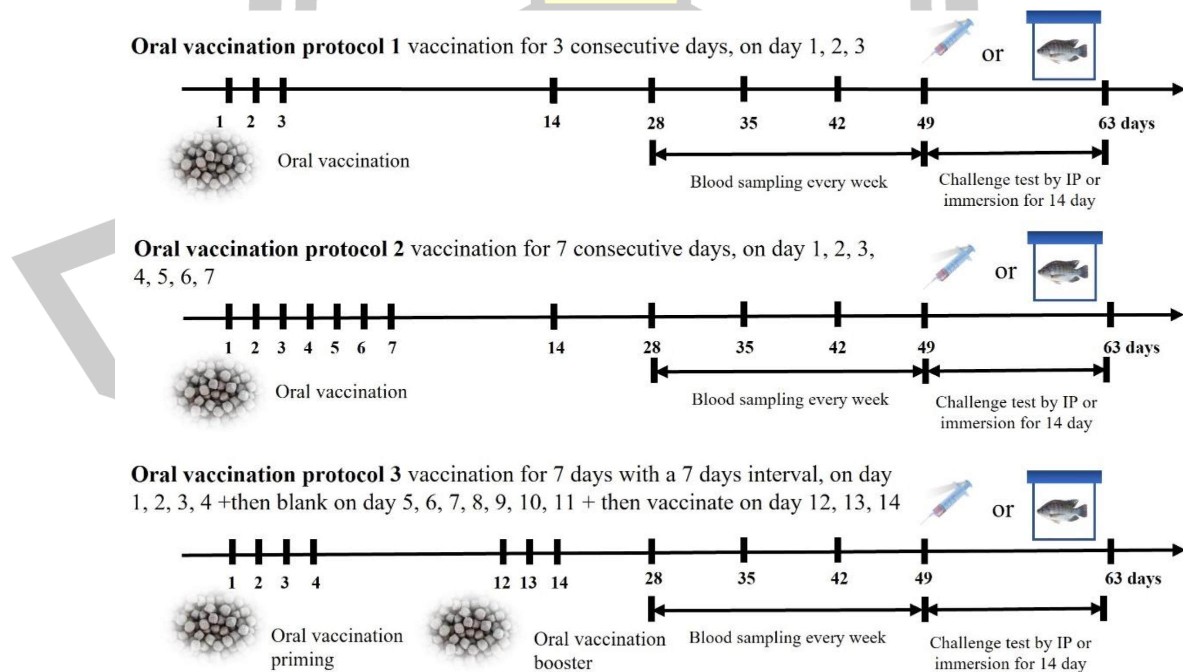

Figure 1 Schematic representation of the different trials used in this study

3.5.2 Trial B: Vaccination and challenge study

To evaluate the efficacy of the vaccination as a primary immunization, fish from each group was divided into 3 groups with 15 fish per group and then challenged with *S. agalactiae* serotype III by immersion route (section 3.6.1) or intraperitoneal injection (I.P.) route (section 3.6.2), respectively. The challenge experiment was conducted in 3 replicates. The details for challenge study of fish post vaccination were shown in Table 4.

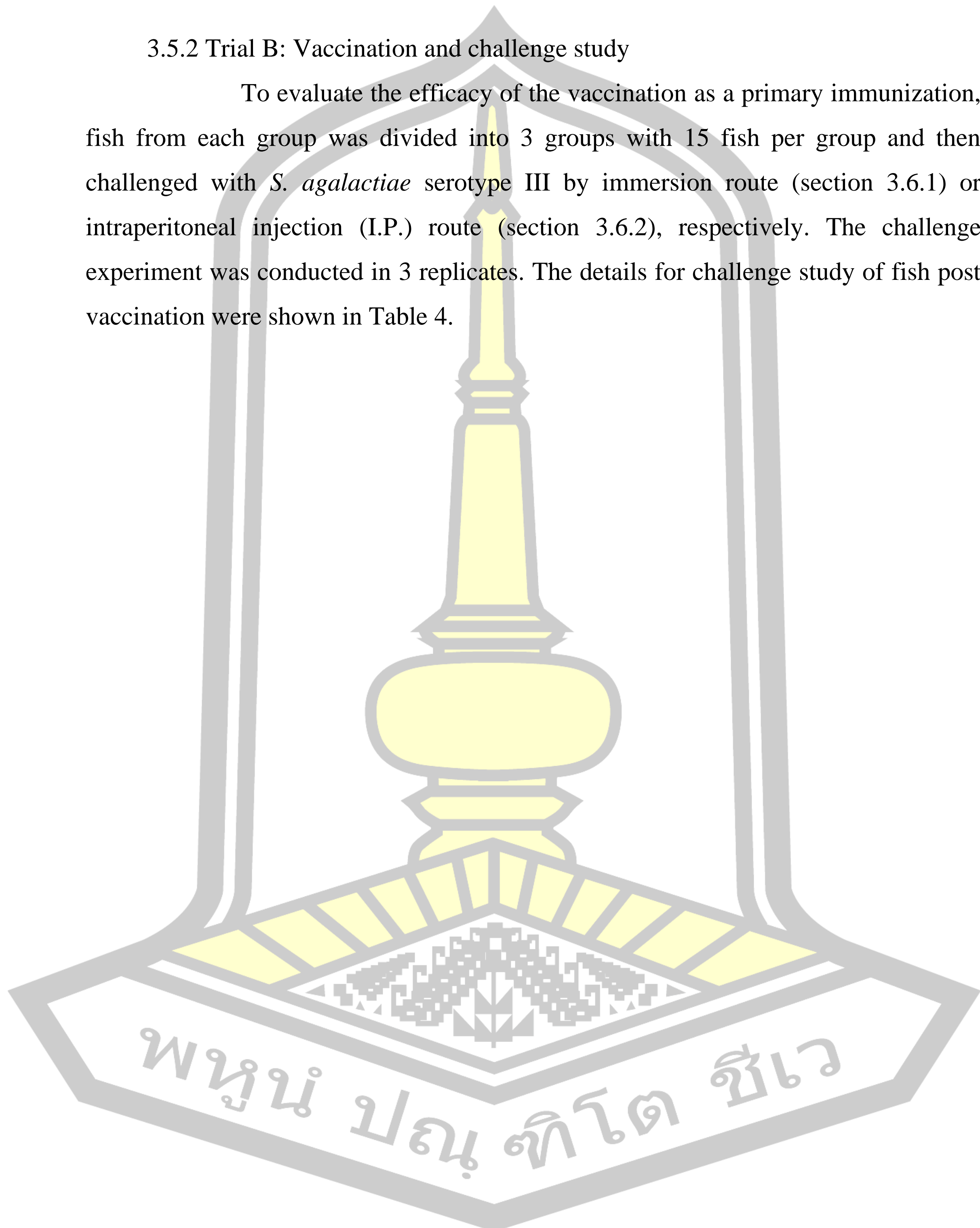

Table 3 Lab trial A - Vaccination treatment

Groups	Vaccine	Treatment protocol*	Fish no.
Oral vaccination group			
A1	Mock vaccination (control group)	Oral Vaccination with commercial feed	N = 120
A2		Oral Vaccination Protocol 1	N = 120
A3	FKC	Oral Vaccination Protocol 2	N = 120
A4		Oral Vaccination Protocol 3	N = 120
A5		Oral Vaccination Protocol 1	N = 120
A6	FKC + Montanide GR01 Adjuvant (20 % w/w; high dose)	Oral Vaccination Protocol 2	N = 120
A7		Oral Vaccination Protocol 3	N = 120
A8		Oral Vaccination Protocol 1	N = 120
A9	FKC + Montanide GR01 Adjuvant (2 % w/w; low dose)	Oral Vaccination Protocol 2	N = 120
A10		Oral Vaccination Protocol 3	N= 120
			Total N = 1,200

Table 4 Lab trial B - Vaccination treatment

Groups	Vaccination	Challenge Protocol	Fish no.
Oral vaccination group			
A1	Mock oral vaccination (control group)	Immersion challenge	N= 45
		IP challenge	N= 45
A2	Oral vaccination with antigen only : Protocol 1	Immersion challenge	N= 45
		IP challenge	N= 45
A3	Oral vaccination with antigen only : Protocol 2	Immersion challenge	N= 45
		IP challenge	N= 45
A4	Oral vaccination with antigen only : Protocol 3	Immersion challenge	N= 45
		IP challenge	N= 45
A5	Oral vaccination with antigen + Montanide GR01 Adjuvant (20 %) : Protocol 1	Immersion challenge	N= 45
		IP challenge	N= 45
A6	Oral vaccination with antigen + Montanide GR01 Adjuvant (20 %) : Protocol 2	Immersion challenge	N= 45
		IP challenge	N= 45
A7	Oral vaccination with antigen + Montanide GR01 Adjuvant (20 %) : Protocol 3	Immersion challenge	N= 45
		IP challenge	N= 45

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Table 4 Lab trial B - Vaccination treatment (continue)

Groups	Vaccination	Challenge Protocol	Fish no.
A8	Oral vaccination with antigen + Montanide GR01 Adjuvant (2 %) : Protocol 1	Immersion challenge IP challenge	N = 45 N = 45
A9	Oral vaccination with antigen + Montanide GR01 Adjuvant (2 %) : Protocol 2	Immersion challenge IP challenge	N = 45 N = 45
A10	Oral vaccination with antigen + Montanide GR01 Adjuvant (2 %) : Protocol 3	Immersion challenge IP challenge	N = 45 N = 45
			Total N = 900

3.6 Challenge tests

3.6.1 Challenge by immersion route

In this experiment, fish from all oral vaccination groups including control group were challenged with *S. agalactiae* by an immersion method. All fish in each tank for all treatments was separately immersed by transferring fish into a 20-L glass tank containing 1×10^8 CFU/mL of *S. agalactiae* for 2 h. During this period, the water temperature was stabilized at 32 °C using a heater-2.6 controlled system (JBL Aquarium Heater, Germany). This protocol induced a mortality rate of approximately 40–60% in Nile tilapia (Srisapoome and Areechon, 2017). The fish in each tank was then placed back into their previous containers. Mortality and abnormal behaviors of fish in each treatment was recorded daily. Abnormal fish that show clinical signs of streptococcosis was dissected, and bacteria was isolated to confirm infection using the loop isolation method by steaking fluid contents from liver and trunk kidney on TSA plates. The cumulative mortality was monitored for 14 days post-challenge. Relative percent of survival (RPS) was determined in accordance to Amend (1981) method with formula: RPS = (1- % mortality in vaccine group/% mortality in control group) × 100.

3.6.2 Challenge by intraperitoneal injection route

Fish from all oral vaccination groups including control group were intraperitoneally injected with 200 μL PBS containing a lethal dose of 1×10^8 CFU/mL of *S. agalactiae*. Fish was returned to their containers for the same procedures as described in section 3.6.1.

Part II: Adjuvant effect of Montanide GR01 in the Streptococcosis formalin-killed vaccine of the Nile tilapia: On-farm trial

3.7 Study site and experimental unit preparation

This experiment was established at the Ubol Ratana Dam located in Nong Kung Soen sub-district, Phu Wiang district, Khon Kaen province, Thailand. Four thousand fingerling Nile tilapia (5 g) were acclimatized in nine ($3 \times 3 \times 2.5$ m^3) cages with 1,000 fish per cage for a week prior to the beginning of the experiment. During this period, the fish were fed twice a day with commercial diet at 3% body weight.

3.8 Vaccine preparation and experimental feed preparation

In this experiment, FKC was prepared at laboratory of Mahasarakham University as described in section 2.4. The ready-to-use FKC (1×10^8 CFU/mL) were preserved in 4 °C containers and delivered to the fish farms. At the farm, the FKC were kept in a refrigerator at 4 °C. The experimental feed-based vaccine were freshly prepared by the farmers. Each 200-mL FKC (Group 1; G1) or FKC incorporated Montanide GR01 (Group 2; G2) were top-dressed to 1 kg of the commercial feed to make 2 different formulas for tested feed-based vaccine. The control feed-based vaccine (Control group; CG) were also prepared using 200 mL of PBS.

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3.9 Experimental designs, feeding and vaccination

The vaccination trial was carried out for a period of 16 weeks (5 months). A completely randomized design (CRD) of 3 treatments with 3 replicates was employed. A day before the feed-based vaccine administration, the fish were not fed to ensure maximum oral feed-vaccine uptake. The experimental groups consist of group 1 (cages 1, 2 and 3) which were given a normal commercial feed without the vaccine as

control unvaccinated group. Group 2 consisting of cages 4, 5 and 6 were fed the feed-based vaccine while group 3 consisting of cages 7, 8 and 9 were fed the feed-based vaccine cooperating Montanide GR01 as adjuvant.

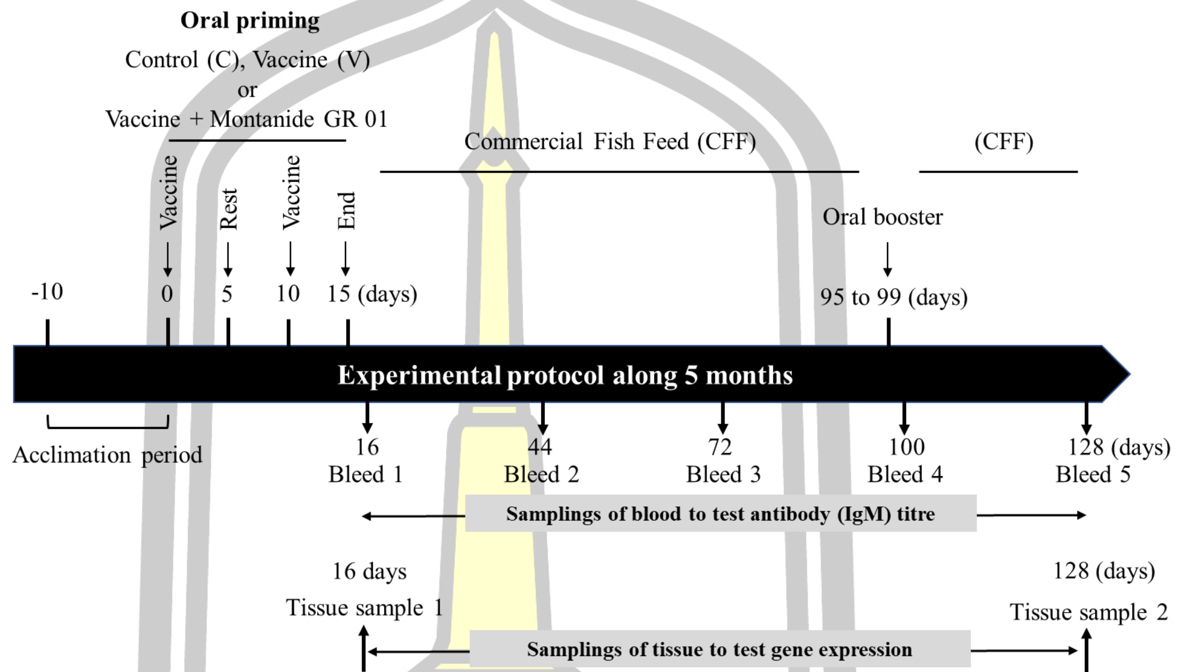


Figure 2 Simplified diagram showing the Nile tilapia trial groups, timing of priming (Day 0-15) and booster (Day 95-99) vaccine administrations

The experimental design showing the trial groups, timing of priming and booster vaccine administrations is shown in Fig. 3.2. Oral delivery of experimental groups (G1, G2 and CG) were performed on day 0 (twice a day; morning and evening), 5 days vaccine, 5 days rest and 5 days vaccine to achieve a final concentration at least 1x10^8 CFU/g over 15 days in each period and on day 95-99 the fish were boosted once more with the same administration of feed-based vaccine in each group. Commercial fish feed (CFF) was used as vector. After ending the oral vaccine priming on day 15, the fish were bled for 5 times, on day 1 (bleed 1) and then performed every 4 weeks (bleed 2-5) throughout the 16 weeks of the experimental period. Serum samples obtained from Nile tilapia at different time points were assessed to test the IgM antibody level with ELISA technique (section 3.4.1). The spleen, liver and intestine were sampled at day 128 to study the immune-relevant gene expression by real-time PCR.

Besides, three experimental groups of Nile tilapia were separately fed diets for 5 months until fish reached market size. During the trial, 20 fish in each case were randomly collected every 14 days (2 weeks) to determine growth parameters, such as body weight and average daily weight gain (ADG), and a feed conversion ratio (FCR) were calculated and recorded at the end of the experiment. Survival rates for each group were noted daily by the farmers. The cumulative percent mortality for each treatment was calculated every day until the end of the experiment. Moreover, average total weight per cage and total feed consumed was calculated. All fish mortalities and abnormal signs were recorded throughout the whole period of experimentation. Fish showing clinical signs of disease were selected for diagnostic examination. The growth parameters were calculated using the formulated:

Average daily gain (ADG; %) = (final body weight – initial body weight)/ duration (128 days)………………………………………………………………… (1)

Weight gain (WG; g) = final body weight – initial body weight………….. (2)

Feed conversion ratio (FCR) = Apparent feed intake/weight gain……….. (3)

Survival rate (%) = 100 - (initial number of fish / final number of fish) × 100……………………………………………………………………………… (4)

3.10 Immune parameters

3.10.1 Non-specific immune response parameters

3.10.1.1 Lysozyme (LZM) activity

LZM activity in fish serum was measured by using the turbidimetric method according to the method of (Suarabh and Sahoo, 2008). Briefly, 25 µL aliquots of serum was added to 1 mL suspension of *Micrococcus lysodeikticus* (0.2 mg/mL in 0.05 M PBS, pH 7.4), and the absorbance was measured at 450 nm after 30 s and 180 s using an ELISA reader. LZM activity were calculated using the formulated: LZM activity (Unit/mL) = $[(OD_{450 \text{ at } 30 \text{ s}} - OD_{450 \text{ at } 180 \text{ s}})/2.5] \times 1{,}000$

3.10.1.2 Glutathione peroxidase (GPx) activity

GPx activity was determined by recording the rate of NADPH oxidation at 405 nm using iMark™ microplate absorbance reader (Wangkahart *et al*., 2022). Briefly, 20 µL of serum were made in a final volume of 115 µL containing solution 1 (50 mM phosphate buffer pH 7.0; 0.1 % Triton X), solution 2 (24 µmol

glutathione; 12 U glutathione reductase; 4.8 µmol µ-nicotinamide-adenine dinucleotide (NAD^+)) and solution 3 (H_2O_2 diluted 100X). 20 µL of solution 1, 20 µL of solution 2 and 35 µL of solution 3 were added in 96 well-plate. Samples were incubated at room temperature for 3 minutes and the absorbance was measured at 405 nm using microplate reader (VersaMax™, Molecular devices). GPx activity was calculated using the formulated: GPx activity (U/mL) = $OD_{405}/0.00622$

3.10.1.3 Glutathione reductase (GRD) activity

GRD activity was determined by recording the rate of NADPH oxidation. Briefly, the solution containing 50 µL of Tris-EDTA (TE) buffer, 20 µL dH_2O containing 18 mM glutathione (GSSG) were mixed and added in 96 well-plate. 50 µL of fish serum and 50 µL NADPH (3 mM) were added. The absorbance was measured at 450 nm using microplate reader (VersaMax™, Molecular Devices). GRD activity was calculated using the formulated: GRD activity (U/mL) = $(OD_{450}/60) \times 1{,}000$

3.10.1.4 Catalase (CAT) activity

CAT activity was evaluated by the enzyme's ability to convert hydrogen peroxide (H_2O_2) into water and molecular oxygen (Casetta *et al*., 2022). 10 µL of serum was added into the 96 well plate, 100 µL of a reactive mixture (50 µL of PBS buffer and 50 µL of H_2O_2) were then added. The absorbance was measured at 240 nm after 20 s and 60 s using microplate reader (VersaMax™, Molecular Devices). Catalase activity were calculated using the formulated: CAT activity (Unit/mL) = $(A_1\text{-}A_2) / 0.0008$

3.10.1.5 Myeloperoxidase (MPO) activity

MPO activity was measured as described previously (Wangkahart *et al*., 2022). 20 µL of serum was added into the 96 well plate, then 80 µL of PBS, 35 µL TMB-Blotting Substrate Solution and 35 µL hydrogen peroxide (H_2O_2) were added. The color change reaction was stopped after 1 minute by using H_2SO_4 solution. The absorbance was measured at 450 nm using microplate reader (VersaMax™, Molecular Devices). Myeloperoxidase activity were calculated using the formulated: MPO activity = Absorbance OD at 450 nm

3.11 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA 96-well microplates were coated with antigen diluted to 20 µg/mL in coating buffer (carbonate bicarbonate buffer) and 50 µL was added to each well incubated for 2 h at 37 °C. The plates were washed twice with washing buffer (PBS containing 1% w/v skimmed milk). Tilapia serum samples were diluted in washing buffer and 50 µL of sample solution was added to each well incubated overnight at 4 °C. The plates were washed three times with washing buffer. Then, 50 µL of anti-tilapia IgM and 200 µL of PBS containing 5 % (w/v) skimmed milk powder was added to each well and the plate incubated at 37 °C for 2 h. After washing with wash buffer, the secondary antibody (anti-mouse IgG labelled with horseradish peroxidase (1:2000 in 1X PBS, 1% milk) was added (50 µL/well) and incubated for 1 h at 37°C. Plates was washed twice with washing buffer and developed by adding 50 µL /well of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) Liquid Substrate, Supersensitive, for ELISA (Sigma) and incubated for 15-30 minute. The reaction was stopped by adding 50 µL/well of 0.5 M sulfuric acid (H_2SO_4). The absorbance of the plates read using a microplate reader at 450 nm.

3.12 Cell-mediated immune response

In order to evaluate cell-mediated immune response after vaccination, immune related genes expression by real-time quantitative PCR (RT-qPCR) were examined as described previously (Wangkahart *et al*., 2019). Briefly, RT-qPCR analyses were performed in a CFX96 system (BioRad, Hercules, CA) using Taq Master Mix. The primers (Table 5) were designed based on the genome sequence of GenBank (Tomaś *et al*., 2021). The cycling conditions were as follows: initial denaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s followed by a melting curve. The results are presented as the fold change of gene expression in the all treated samples compared with control samples.

Table 5 Sequences of forward (F) and reverse (R) primers used in this study

Gene	Accession no.	Primer	Nucleotide sequence 5' – 3'	Annealing T (°C)
β-act	XM_003443127	Fw	ACAGGATGCAGAAGGAGATCACAG	60
		Rv	GTACTCCTGCTTGCTGATCCACAT	
IgM	KJ_676389	Fw	GGATGACGAGGAAGCAGACT	59
		Rv	CATCATCCCTTTGCCACTGG	
COX-2	XM_003445052	Fw	TGCTGAAAGAGGTCCACCCATACT	60
		Rv	CGCTCAGATGCTGCACGTAGTC	
TNF-α	NM_001279533	Fw	AGGGTGATCTGCGGGAATACT	60
		Rv	GCCCAGGTAAATGGCGTTGT	
IL-6	XM_019350387.2	Fw	ACAGAGGAGGCGGAGATG	60
		Rv	GCAGTGCTTCGGGATAGA	
IL-8	NM_001279704	Fw	GCACTGCCGCTGCATTAAG	58
		Rv	GCAGTGGGAGTTGGGAAGAA	
IFN-γ	NM_001287402.1	Fw	GAAACTTCTGCAGGGATTGG	60
		Rv	CTCTGGATCTTGATTTCGGG	
SOD	XM_003440807	Fw	CATGCCTTCGGAGACAACAC	55
		Rv	ACCTTCTCGTGGATCACCAT	
GPx	NM_001279711	Fw	GGTGGATGTGAATGGAAAGG	60
		Rv	CTTGTAAGGTTCCCCGTCAG	
CAT	JF_801726.1	Fw	AGCTCTTCATCCAGAAACGC	55
		Rv	GACGTCAGGCGTCACATCTT	

Abbreviations: β-act: β-actin, IgM: immunoglobulin M; COX-2; clyclooxygenase 2; TNF-α: tumor necrosis factor alpha; IL-6: inteleukin 6: IL-8: interleukin 8; IFN-γ: interferon gamma; SOD: superoxide dismutase 1; GPx: glutathione peroxidase; CAT: catalase.Fw: forward, Rv: reverse.

3.13 Water quality analysis

Throughout the experiment, water qualities were evaluated including dissolved oxygen (DO), temperature and pH using a YSI 550A Dissolved Oxygen Instrument and a YSI pH100A, respectively.

3.14 Statistical and data analysis

The data of each parameter was expressed as the mean + standard error (SEM). One way-ANOVA and LSD post hoc tests were used to analyse all of the data using IBM SPSS statistics 22 software (SPSS Inc., Chicago, IL, USA). Differences between vaccinated and control group for each time point were considered statistically significant at $P<0.05$.

Chapter 4
Results

Part I: Evaluation of Montanide GR01 for oral vaccination in Nile tilapia (*Oreochromis niloticus*): Laboratory trial

4.1 Water quality parameters

During the experimental period, physio-chemical water qualities were measured by a YSI 550A Dissolved Oxygen Instrument and a YSI pH100A. All water parameters were within optimal levels for fish survival (Table 6).

Table 6 Water quality conditions during laboratory trials indicated by maximal (Max), minimal (Min) and average values (Mean ± SD)

Water parameter	**Unit**	**Max**	**Min**	**Mean ± SD**
pH	-	8.74	8.10	8.4 ± 0.3
Temperature	°C	27.1	26.5	26.7 ± 0.7
Dissolved oxygen	mg/L	10.9	7.78	9.35 ± 2.21
Conductivity	µS/cm	97.0	90.2	93.2 ± 6.7

4.2 Innate immune response analysis

4.2.1 Lysozyme (LZM) activity

The LZM activity in sera of vaccinated fish are presented in Table 7. The oral vaccination protocol 1, fish vaccinated with FKC vaccine + Montanide GR01 20 % showed higher level of LZM activity than control group at 1, 2, 4 w.p.v. ($P<0.05$). The vaccinated fish showed highest level of LZM activity at 4 w.p.v. and significant differences were obtained between vaccinated and control group ($P<0.05$).

The oral vaccination protocol 2, fish vaccinated with FKC vaccine + Montanide GR01 2 % showed higher level of LZM activity than control group and significant differences were obtained between the vaccinated group with Montanide GR01 2% and control group ($P<0.05$).

Oral vaccination protocol 3, fish vaccinated with FKC vaccine + Montanide GR01 2% showed higher level of LZM activity than control group (P<0.05). The maximum activity was observed at 5 w.p.v. and significant differences were obtained between the FKC vaccine + Montanide GR01 2% group and control group at 2, 3, 4, 5 w.p.v. (P<0.05). At 4 and 5 w.p.v. the vaccinated group with Montanide GR01 2% and 20% were higher level of LZM activity than control group and significant differences were obtained between the vaccinated group with Montanide GR01 and control group (P<0.05).

Table 7 Lysozyme activity (LZM) in the serum of Nile tilapia vaccinated with different oral vaccination protocols for 5 weeks

Time	CTRL	FKC	FKC + GR01 20%	FKC + GR01 2%	Pooled SEM	P-value
Protocol 1						
Week 1	1.20^{a}	1.68^{b}	2.80^{c}	1.52^{ab}	0.10	0.002
Week 2	0.96^{a}	1.36^{ab}	2.00^{bc}	2.48^{c}	0.19	0.001
Week 3	0.88	0.88	1.44	1.04	0.18	0.209
Week 4	1.12^{a}	1.92^{b}	1.84^{b}	2.08^{b}	0.13	0.001
Week 5	2.00	2.08	2.08	2.24	0.25	0.963
Protocol 2						
Week 1	1.20^{a}	1.52^{a}	2.24^{b}	1.60^{a}	0.17	0.021
Week 2	0.96	1.12	1.28	1.44	0.16	0.247
Week 3	0.88^{a}	2.56^{ab}	3.12^{ab}	3.84^{b}	0.68	0.088
Week 4	1.12^{a}	1.20^{a}	1.52^{a}	2.16^{b}	0.18	0.004
Week 5	2.00^{a}	1.92^{a}	2.56^{ab}	3.76^{b}	0.39	0.090
Protocol 3						
Week 1	1.20	1.84	1.76	1.76	0.16	0.124
Week 2	0.96^{a}	1.28^{ab}	2.00^{b}	2.24^{bc}	0.25	0.020
Week 3	0.88^{a}	3.84^{b}	2.24^{ab}	3.44^{b}	0.60	0.039
Week 4	1.12^{a}	1.52^{ab}	2.08^{bc}	2.56^{c}	0.24	0.005
Week 5	2.00^{a}	3.76^{b}	4.56^{b}	5.37^{b}	0.57	0.013

Abbreviation: CTRL: control group; FKC: oral vaccination with FKC only; FKC + GR01 20%: oral vaccination with FKC + Montanide GR01 20%; FKC + GR01 2%: oral vaccination with FKC + Montanide GR01 2%; LZM: lysozyme.

Values represent the mean of three replicates.Values followed by different letters in the same row are significantly different (P<0.05).

4.2.2 Myeloperoxidase (MPO) activity

Oral vaccination protocol 1, MPO activity in sera of vaccinated Nile tilapia are presented in Table 8. The fish vaccinated with FKC vaccine + Montanide GR01 2% showed the highest level of MPO activity. The maximum activity was observed at 5 w.p.v. and significant differences were obtained between the FKC vaccine + GR01 2% group and control group ($P<0.05$).

Oral vaccination protocol 2, the vaccinated fish group with FKC vaccine + Montanide GR01 showed higher level of MPO activity than control group ($P<0.05$). The maximum activity was observed at 5 w.p.v. and significant differences were obtained between vaccinated group and control group ($P<0.05$).

Oral vaccination protocol 3, fish vaccinated with FKC vaccine + Montanide GR01 20% showed higher level of MPO activity than control group ($P<0.05$). The maximum activity was observed at 1 w.p.v. and significant differences were obtained between the FKC vaccine + GR01 20% group and control group ($P<0.05$).

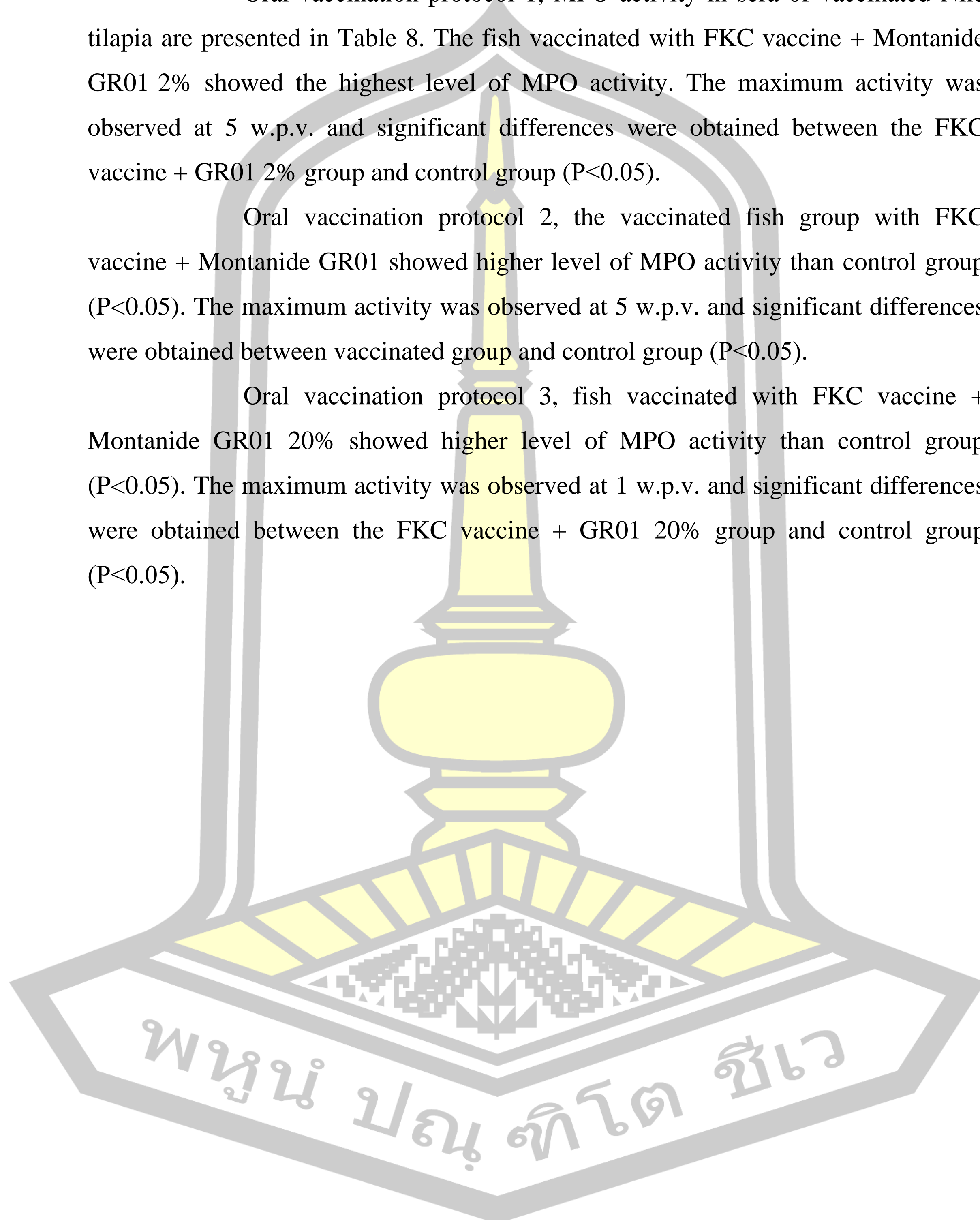


Table 8 Myeloperoxidase (MPO) activity in the serum of Nile tilapia vaccinated with different oral vaccination protocols for 5 weeks

Time	CTRL	FKC	FKC + GR01 20%	FKC + GR01 2%	Pooled SEM	P-value
Protocol 1						
Week 1	1.00[a]	1.49[b]	1.24[ab]	1.25[ab]	0.09	0.020
Week 2	1.30[b]	1.02[a]	1.09[ab]	0.93[a]	0.08	0.040
Week 3	1.28	1.46	1.42	1.33	0.07	0.302
Week 4	0.87[a]	1.13[ab]	1.48[c]	1.30[bc]	0.08	0.001
Week 5	1.49[a]	1.45[a]	1.55[a]	1.89[b]	0.07	0.002
Protocol 2						
Week 1	1.00	1.12	1.20	1.15	0.14	0.786
Week 2	1.30	1.10	1.10	1.28	0.10	0.332
Week 3	1.28	1.42	1.33	1.34	0.11	0.830
Week 4	0.87	0.97	1.27	1.25	0.10	0.085
Week 5	1.49[a]	1.44[ab]	1.88[b]	1.58[b]	0.06	0.021
Protocol 3						
Week 1	1.00[a]	1.25[a]	1.78[b]	1.39[ab]	0.13	0.009
Week 2	1.30	0.96	1.32	1.13	0.11	0.133
Week 3	1.28	1.56	1.48	1.76	0.15	0.407
Week 4	0.87	1.15	1.22	1.23	0.11	0.145
Week 5	1.49	1.44	1.88	1.58	0.10	0.056

Abbreviation: CTRL: control group; FKC: oral vaccination with FKC only; FKC + GR01 20%: oral vaccination with FKC + Montanide GR01 20%; FKC + GR01 2%: oral vaccination with FKC + Montanide GR01 2%; MPO: myeloperoxidase.

Values represent the mean of three replicates. Values followed by different letters in the same row are significantly different ($P<0.05$).

4.2.3 Catalase (CAT) activity

The CAT activities in sera of the vaccinated Nile tilapia are presented in Table 9. The oral vaccination protocol 1, fish vaccinated with FKC vaccine + Montanide GR01 showed higher level of CAT activity than control fish group at 2, 3, 4, 5 w.p.v. and significant differences were obtained between FKC vaccine + Montanide GR01 group and control group ($P<0.05$).

Oral vaccination protocol 2, the vaccinated fish group showed higher level of CAT activity than control group (CTRL) at 1, 2, 3, 4, 5 w.p.v. and significant differences were obtained between the vaccinated group with Montanide GR01 and control group. The vaccinated fish with FKC + Montanide GR01 showed higher level of CAT activity than vaccine group (FKC only) at 2, 3, 4, 5 w.p.v. and significant

differences were obtained between FKC vaccine + Montanide GR01 group and FKC vaccine group (P<0.05).

Oral vaccination protocol 3, the vaccinated fish showed higher level of CAT activity than control group (CTRL) and significant differences were obtained between vaccinated group and control group (Table 9) at 1, 2, 3, 4 and 5 w.p.v. The vaccinated group with FKC vaccine + Montanide GR01 showed higher level of CAT activity than vaccine group (FKC only) and significant differences were obtained between FKC vaccine + Montanide GR01 group and vaccine only group at 4 and 5 w.p.v. the vaccinated group with FKC + Montanide GR01 2% showed higher level of CAT activity than vaccinated group with FKC + Montanide GR01 20% and significant differences were obtained between FKC vaccine + Montanide GR01 2% group and FKC vaccine + Montanide GR01 20% group at 3 w.p.v. (P<0.05).

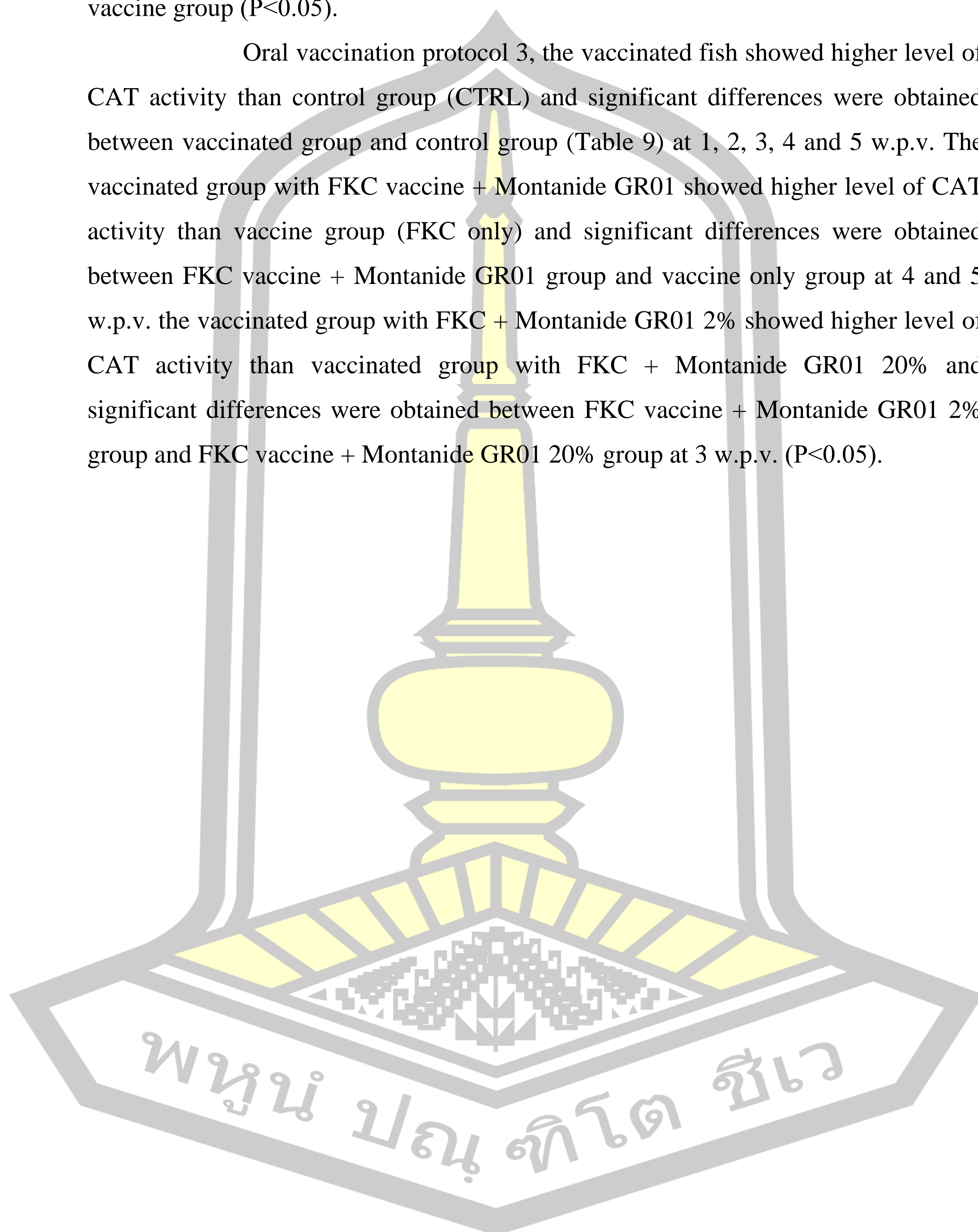


Table 9 Catalase (CAT) activity in the serum of Nile tilapia vaccinated with different oral vaccination protocols for 5 weeks

Time	CTRL	FKC	FKC + GR01 20 %	FKC + GR01 2 %	Pooled SEM	P-value
Protocol 1						
Week 1	3.97[a]	4.69[a]	5.82[ab]	6.74[b]	0.52	0.026
Week 2	4.06[a]	5.12[ab]	7.13[b]	7.21[b]	0.64	0.020
Week 3	4.06[a]	5.49[ab]	7.34[bc]	7.74[c]	0.64	0.007
Week 4	4.66[a]	5.28[a]	7.40[b]	7.43[c]	0.43	0.001
Week 5	5.18[a]	7.07[ab]	8.17[b]	8.61[b]	0.66	0.020
Protocol 2						
Week 1	3.97[a]	5.91[b]	6.14[b]	6.88[c]	0.15	0.000
Week 2	4.06[a]	6.85[b]	8.28[c]	9.09[d]	0.18	0.000
Week 3	4.06[a]	6.89[b]	7.93[c]	9.21[d]	0.23	0.000
Week 4	4.66[a]	7.48[b]	8.60[c]	9.03[c]	0.27	0.000
Week 5	5.18[a]	7.37[b]	9.03[c]	9.59[c]	0.29	0.000
Protocol 3						
Week 1	3.97[a]	5.98[b]	7.10[b]	7.83[b]	0.53	0.003
Week 2	4.06[a]	8.84[bc]	8.25[b]	9.78[c]	0.38	0.000
Week 3	4.06[a]	9.61[b]	11.21[c]	13.54[d]	0.14	0.000
Week 4	4.66[a]	8.59[b]	11.80[c]	12.03[c]	0.39	0.000
Week 5	5.18[a]	9.96[b]	13.85[c]	14.55[c]	0.54	0.000

Abbreviation: CTRL: control group; FKC: oral vaccination with FKC only; FKC + GR01 20%: oral vaccination with FKC + Montanide GR01 20%; FKC + GR01 2%: oral vaccination with FKC + Montanide GR01 2%; CAT: catalase.

Values represent the mean of three replicates.Values followed by different letters in the same row are significantly different (P<0.05).

4.2.4 Glutathione peroxidase (GPx) activity

The GPx activities in sera of the vaccinated Nile tilapia are presented in Table 10. Oral vaccination protocol 1, the vaccinated fish group with FKC vaccine showed the highest level of GPx activity at 4, 5 w.p.v. and significant differences were obtained between vaccinated group and control group (P<0.05).

Oral vaccination protocol 2, fish vaccinated with FKC vaccine + Montanide GR01 20% showed higher level of GPx activity than control group (CTRL) at 3 w.p.v. The vaccinated fish group with FKC vaccine and Montanide GR01 showed higher level of GPx activity than control group (CTRL) at 5 w.p.v.and significant differences were obtained between the vaccinated group and control group (P<0.05).

Oral vaccination protocol 3, the vaccinated group with FKC vaccine + Montanide GR01 20% showed higher level of GPx activity than vaccine group (FKC only) and significant differences were obtained between FKC vaccine + Montanide GR01 20% group and vaccine only group at 3 w.p.v. The vaccinated fish group showed higher level of GPx activity than control group (CTRL) and significant differences were obtained between vaccinated group and control group at 5 w.p.v. (P<0.05).

Table 10 Glutathione peroxidase (GPx) activity in the serum of Nile tilapia vaccinated with different oral vaccination protocols for 5 weeks

Time	CTRL	FKC	FKC + GR01 20%	FKC + GR01 2%	Pooled SEM	P-value
Protocol 1						
Week 1	23.99	27.11	20.80	27.75	1.60	0.071
Week 2	25.31	31.42	31.42	29.52	1.68	0.081
Week 3	24.82[a]	29.52[ab]	26.43[a]	31.67[b]	1.46	0.032
Week 4	25.02[a]	31.16[b]	33.51[b]	37.20[c]	2.01	0.002
Week 5	24.86[a]	44.85[b]	70.29[c]	50.77[b]	2.63	0.000
Protocol 2						
Week 1	23.99	26.56	27.11	24.05	1.35	0.297
Week 2	25.31	27.08	25.52	29.65	1.50	0.242
Week 3	24.82	23.60	24.98	28.94[b]	1.10	0.030
Week 4	25.02	27.65	28.91	28.04	2.04	0.632
Week 5	24.86[a]	49.49[c]	47.49[bc]	57.72[c]	2.47	0.000
Protocol 3						
Week 1	23.99	25.47	26.78	28.45	1.51	0.289
Week 2	25.31	24.18	25.79	27.81	1.95	0.649
Week 3	24.82[a]	24.37[a]	30.84[b]	27.14[ab]	1.42	0.034
Week 4	25.02	30.03	28.10	27.40	1.26	0.102
Week 5	24.86[a]	48.78[b]	48.91[b]	53.09[b]	2.17	0.001

Abbreviation: CTRL: control group; FKC: oral vaccination with FKC only; FKC + GR01 20 %: oral vaccination with FKC + Montanide GR01 20%; FKC + GR01 2%: oral vaccination with FKC + Montanide GR01 2%; GPx: glutathione peroxidase. Values represent the mean of three replicates.Values followed by different letters in the same row are significantly different (P<0.05).

4.2.5 Glutathione reductase (GRD) activity

The GRD activities in sera of the vaccinated Nile tilapia are presented in Table 11. Oral vaccination protocol 1, the vaccinated fish group with FKC vaccine showed highest level of GRD activity at 4, 5 w.p.v. and significant differences were obtained between vaccinated group and control group (P<0.05).

Oral vaccination protocol 2, fish vaccinated with FKC vaccine + Montanide GR01 showed higher level of GRD activity than control group (CTRL) at 4 and 5 w.p.v. but non-significant differences were obtained between the vaccinated group and control group (P<0.05).

Oral vaccination protocol 3, the fish vaccinated with FKC vaccine + Montanide GR01 20% showed higher level of GRD activity than control group (CTRL) and significant differences were obtained between FKC vaccine + Montanide GR01 20% group and control group at 4 w.p.v. The vaccinated fish group showed higher level of GRD activity than control group (CTRL) and significant differences were obtained between vaccinated group and control group at 5 w.p.v. (P<0.05).

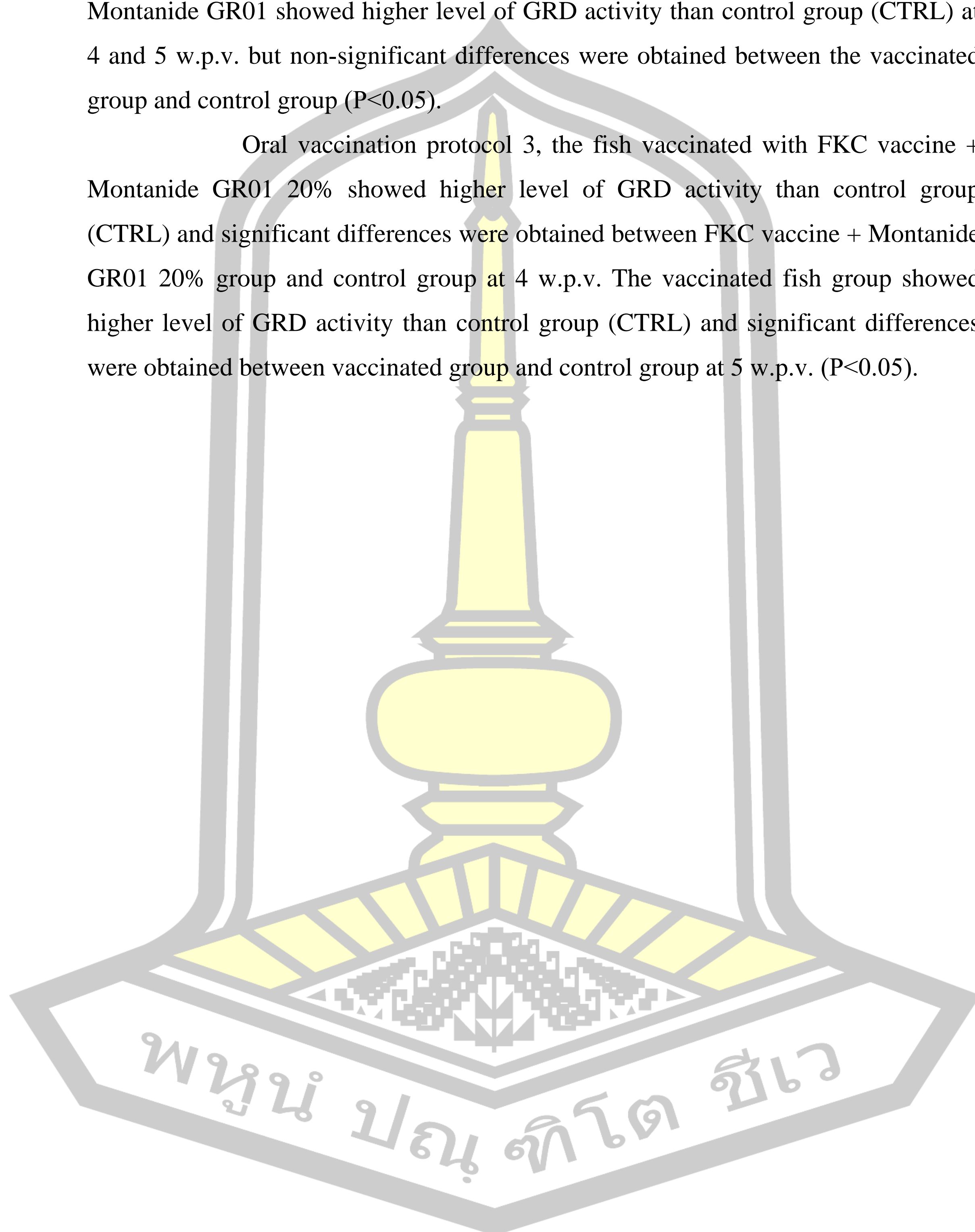


Table 11 Glutathione reductase (GRD) activity in the serum of Nile tilapia vaccinated with oral vaccination protocols for 5 weeks

Time	CTRL	FKC	FKC + GR01 20%	FKC + GR01 2%	Pooled SEM	P-value
Protocol 1						
Week 1	3.36[a]	4.18[b]	3.19[a]	4.38[b]	0.18	0.004
Week 2	3.70[a]	4.93[ab]	6.00[b]	3.76[a]	0.35	0.013
Week 3	3.43	4.38	3.54	3.78	0.34	0.500
Week 4	2.66[a]	4.32[c]	3.73[b]	3.27[b]	0.19	0.000
Week 5	2.87[a]	5.53[b]	5.29[b]	6.03[b]	0.47	0.001
Protocol 2						
Week 1	3.36	3.27	4.01	3.93	0.32	0.291
Week 2	3.70	5.00	4.54	4.94	0.39	0.199
Week 3	3.43	3.25	3.80	3.17	0.20	0.282
Week 4	2.66	3.02	3.41	3.47	0.21	0.070
Week 5	2.87	7.30	5.07	5.47	0.56	0.818
Protocol 3						
Week 1	3.36	3.79	3.65	4.65	0.41	0.285
Week 2	3.70	3.45	3.52	4.23	0.36	0.516
Week 3	3.43	4.00	4.86	3.74	0.27	0.057
Week 4	2.66[a]	3.65[bc]	4.27[c]	3.20[a]	0.22	0.001
Week 5	2.87[a]	4.48[b]	6.84[c]	5.42[bc]	0.42	0.000

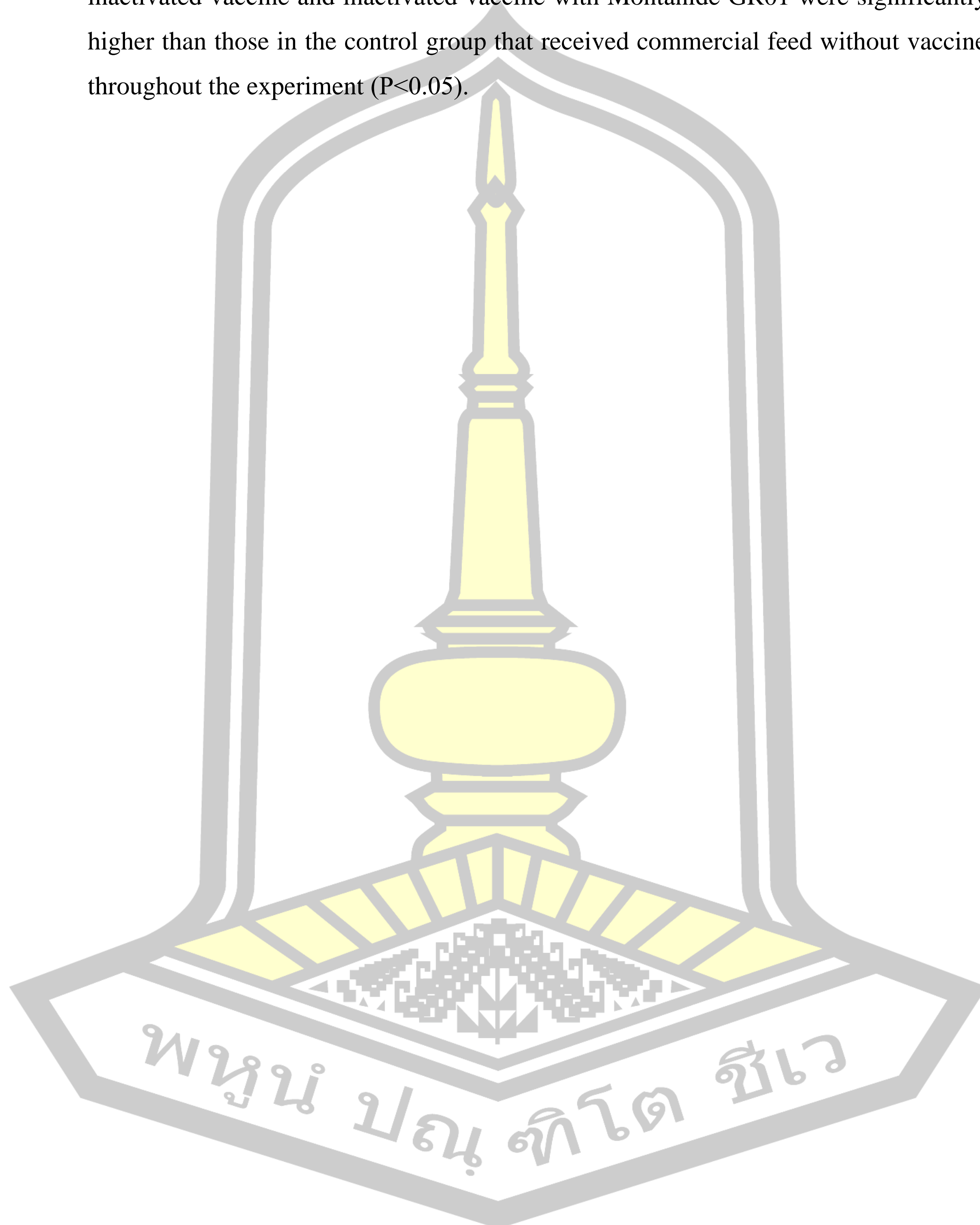
Abbreviation: CTRL: control group; FKC: oral vaccination with FKC only; FKC + GR01 20%: oral vaccination with FKC + Montanide GR01 20%; FKC + GR01 2%: oral vaccination with FKC + Montanide GR01 2%; GRD: glutathione reductase. Values represent the mean of three replicates. Values followed by different letters in the same row are significantly different (P<0.05).

4.3 Adaptive immune response analysis

4.3.1 Specific IgM antibody response analysis

After vaccination, the specific antibody (IgM) levels in Nile tilapia sera are presented in Fig. 3. Oral vaccination protocol 1-3, the analysis showed that the highest IgM antibody levels were found in sera from the FKC + GR01 2% group followed by the FKC + GR01 20% group and FKC only group at each of the examined time points, with all sample significantly higher compared with CTRL group sera (P<0.05). However, the antibody levels from the FKC + GR01 2% group showed non-significantly higher compared with the FKC + GR01 20% group. In addition, the antibody levels in sera from the FKC + GR01 2% group increased from week 1 to week 5 (P<0.05).

Overall, the serum antibody responses in the fish vaccinated with inactivated vaccine and inactivated vaccine with Montanide GR01 were significantly higher than those in the control group that received commercial feed without vaccine throughout the experiment ($P<0.05$).



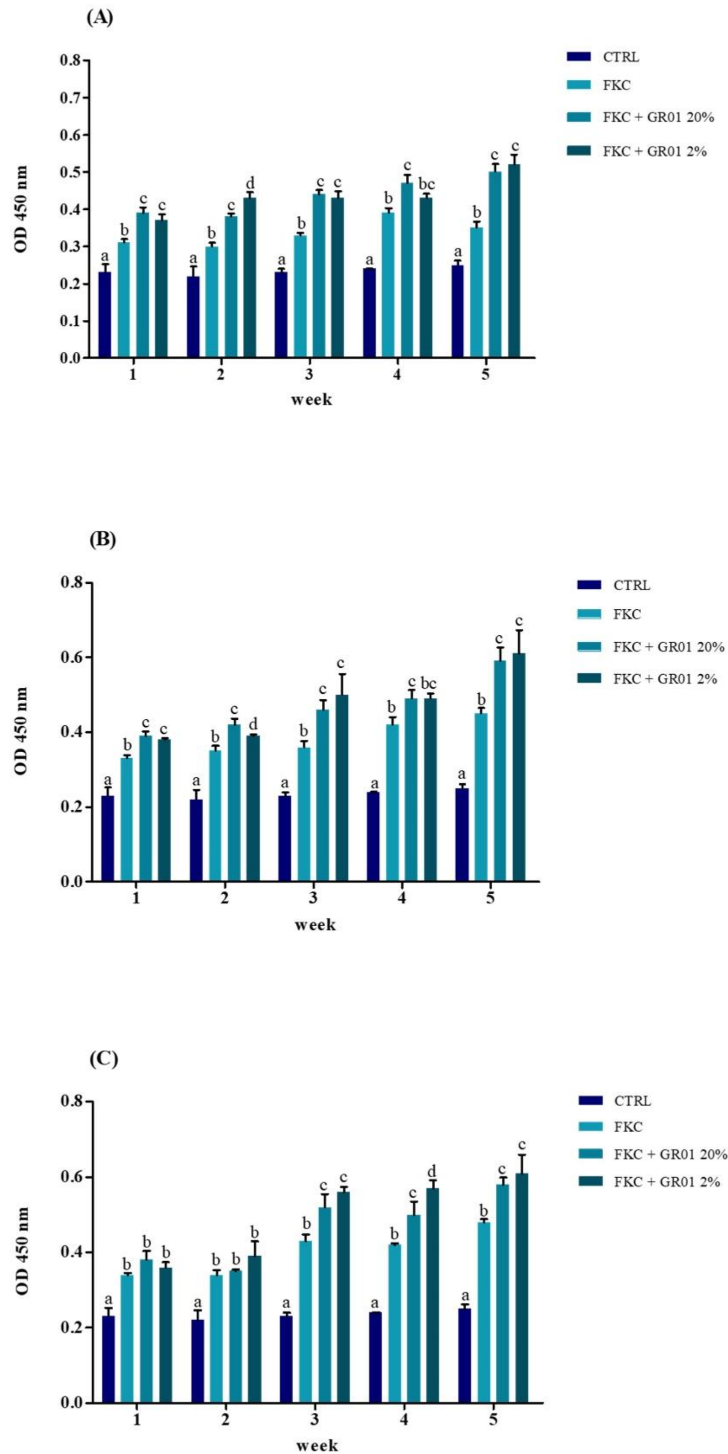


Figure 3 Specific antibody (IgM) levels in sera of Nile tilapia vaccinated with the inactivated vaccine at 1, 2, 3, 4, 5 weeks post vaccination

(A) Oral vaccination protocol 1. (B) Oral vaccination protocol 2. (C) Oral vaccination protocol 3. Specific IgM antibody titers were determined by ELISA. Statistical significance was analyzed between vaccinated and control Nile tilapia ($P<0.05$).

4.4 Protective efficacy of vaccine against *S. agalactiae*

4.4.1 Protective efficacy of vaccination against *S. agalactiae* by injection challenge

The protective of the FKC vaccine against *S. agalactiae* is presented in Fig. 4.2. At 1, 2, 3, 4, and 5 w.p.v., fish were challenged with 1.0×10^8 CFU/mL of *S. agalactiae*. As shown in Fig. 4, significant higher survival rates of all vaccinated groups than the control group were observed.

Oral vaccination protocol 1, the RPS of vaccinated groups was 56% in FKC vaccine only group, the fish vaccinated with Montanide GR01 2% group (FKC + GR01 2%) was 72.00% and reached the highest level of fish vaccinated with Montanide GR01 20% group (FKC + GR01 20%) was 76.00% respectively.

Oral vaccination protocol 2, the RPS value of fish vaccinated groups was 68.00% in FKC vaccine only group, the vaccinated with Montanide GR01 2% group (FKC + GR01 2%) was 80.00% and reached the highest level in FKC vaccine adjuvanted with Montanide GR01 20% group (FKC + GR01 20%) was 84.00% respectively.

Oral vaccination protocol 3, the RPS value of fish vaccinated groups was 40.54% in FKC vaccine only group, the vaccinated with Montanide GR01 20% group (FKC + GR01 20%) was 78.38% and reached the highest level in FKC vaccine vaccinated with Montanide GR01 2% group (FKC + GR01 2%) was 81.08% respectively (Fig. 4C). These results suggested that FKC vaccine accompanied with Montanide GR01 displayed an excellent protective effect in Nile tilapia.

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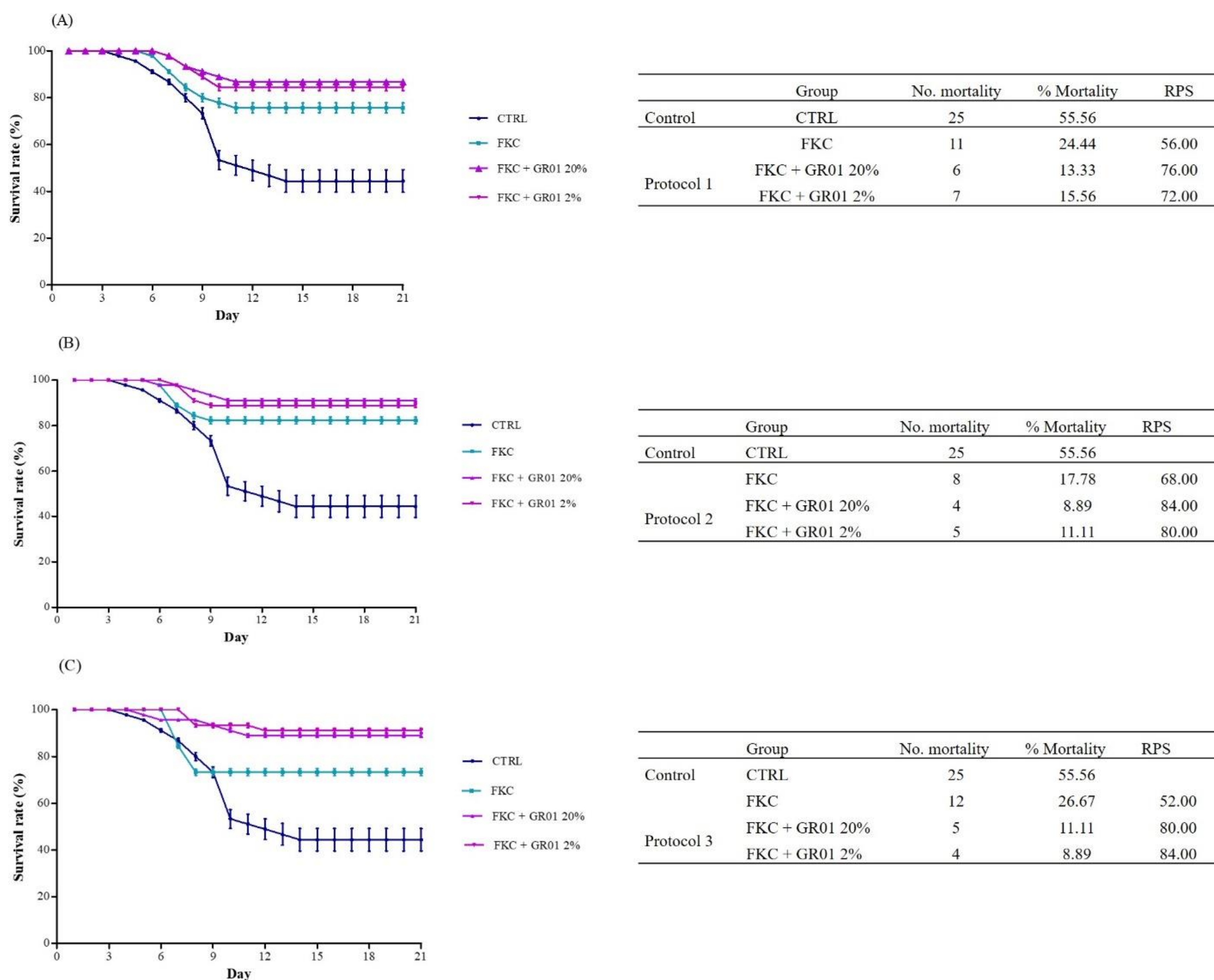


	Group	No. mortality	% Mortality	RPS
Control	CTRL	25	55.56	
Protocol 1	FKC	11	24.44	56.00
	FKC + GR01 20%	6	13.33	76.00
	FKC + GR01 2%	7	15.56	72.00

	Group	No. mortality	% Mortality	RPS
Control	CTRL	25	55.56	
Protocol 2	FKC	8	17.78	68.00
	FKC + GR01 20%	4	8.89	84.00
	FKC + GR01 2%	5	11.11	80.00

	Group	No. mortality	% Mortality	RPS
Control	CTRL	25	55.56	
Protocol 3	FKC	12	26.67	52.00
	FKC + GR01 20%	5	11.11	80.00
	FKC + GR01 2%	4	8.89	84.00

Figure 4 Survival rate of vaccinated Nile tilapia challenged with *S. agalactiae* for long immunity duration

(A) Oral vaccination protocol 1. (B) Oral vaccination protocol 2. (C) Oral vaccination protocol 3. Fish in vaccinated group and control group were intraperitoneally injection challenged with 1.0×10^8 CFU/fish of *S. agalactiae*. Fish mortality was recorded for 21 days. The experiment was conducted in triplicate. Error bars represented standard deviations.

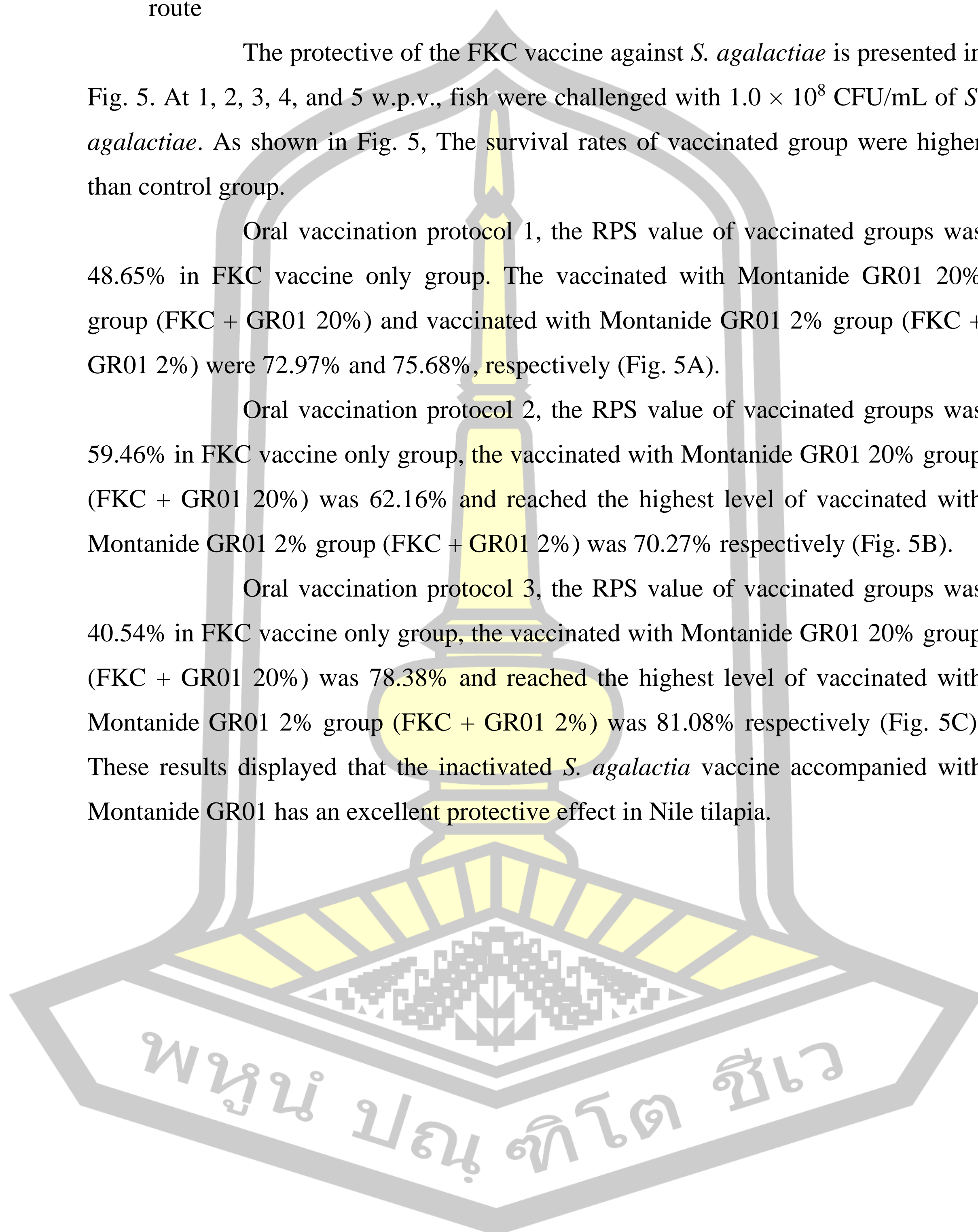
4.4.2 Protective efficacy of vaccination against *S. agalactiae* by immersion route

The protective of the FKC vaccine against *S. agalactiae* is presented in Fig. 5. At 1, 2, 3, 4, and 5 w.p.v., fish were challenged with 1.0×10^8 CFU/mL of *S. agalactiae*. As shown in Fig. 5, The survival rates of vaccinated group were higher than control group.

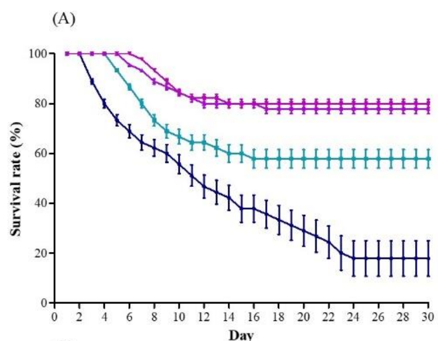
Oral vaccination protocol 1, the RPS value of vaccinated groups was 48.65% in FKC vaccine only group. The vaccinated with Montanide GR01 20% group (FKC + GR01 20%) and vaccinated with Montanide GR01 2% group (FKC + GR01 2%) were 72.97% and 75.68%, respectively (Fig. 5A).

Oral vaccination protocol 2, the RPS value of vaccinated groups was 59.46% in FKC vaccine only group, the vaccinated with Montanide GR01 20% group (FKC + GR01 20%) was 62.16% and reached the highest level of vaccinated with Montanide GR01 2% group (FKC + GR01 2%) was 70.27% respectively (Fig. 5B).

Oral vaccination protocol 3, the RPS value of vaccinated groups was 40.54% in FKC vaccine only group, the vaccinated with Montanide GR01 20% group (FKC + GR01 20%) was 78.38% and reached the highest level of vaccinated with Montanide GR01 2% group (FKC + GR01 2%) was 81.08% respectively (Fig. 5C). These results displayed that the inactivated *S. agalactia* vaccine accompanied with Montanide GR01 has an excellent protective effect in Nile tilapia.

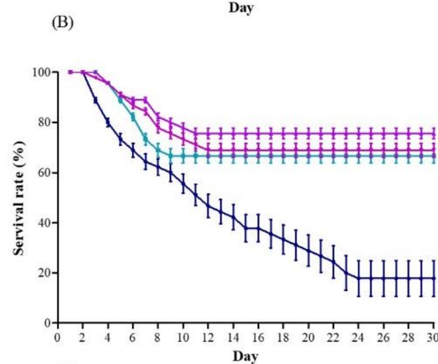


(A)



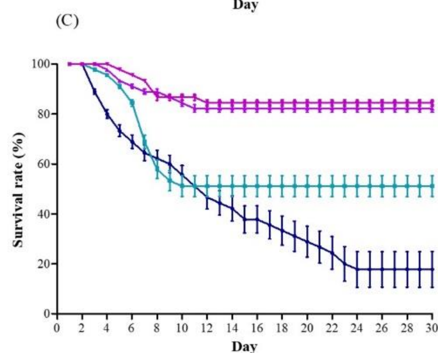
	Group	No. mortality	% Mortality	RPS
Control	CTRL	37	82.22	
Protocol 1	FKC	19	42.22	48.65
	FKC + GR01 20%	10	22.22	72.97
	FKC + GR01 2%	9	20.00	75.68

(B)



	Group	No. mortality	% Mortality	RPS
Control	CTRL	37	82.22	
Protocol 2	FKC	15	33.33	59.46
	FKC + GR01 20%	11	24.44	70.27
	FKC + GR01 2%	14	31.11	62.16

(C)



	Group	No. mortality	% Mortality	RPS
Control	CTRL	37	82.22	
Protocol 3	FKC	22	48.89	40.54
	FKC + GR01 20%	8	17.78	78.38
	FKC + GR01 2%	7	15.58	81.08

Figure 5 Survival rate of vaccinated Nile tilapia challenged with *S. agalactiae* for long immunity duration

(A) Oral vaccination protocol 1. (B) Oral vaccination protocol 2. (C) Oral vaccination protocol 3. Fish in vaccinated group and control group were immersion challenged with 1.0 × 10^8 CFU/fish of *S. agalactiae*. Fish mortality was recorded for 21 days. The experiment was conducted in triplicate. Error bars represented standard deviations.

Part II: Evaluation of Montanide GR01 for oral vaccination in Nile tilapia (*Oreochromis niloticus*): On-farm trial

4.5 Commercial feed and proximate analysis for vaccine formulation

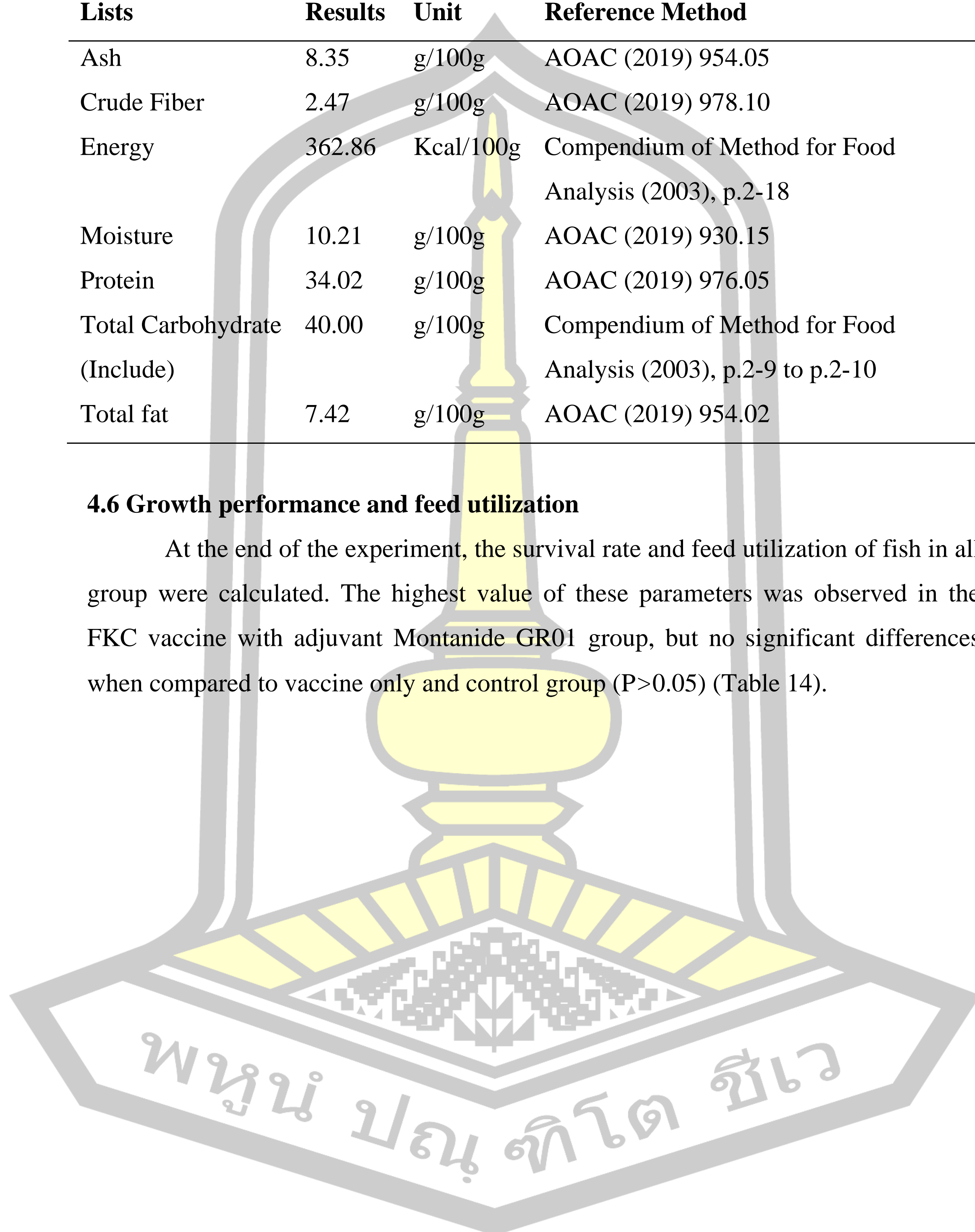
The proximate analysis of the commercial diets used in this study were measured, including the ash, crude fiber, energy, moisture, protein, total carbohydrate, and total fat. Based on NRC (2011), diets for small fish (feeding at day 1-16) were analyzed and found to contain approximately ash 8.86 g/100g, crude fiber was 4.94 g/100g, energy 357.33 Kcal/100g, moisture 9.67 g/100g, protein 33.07 g/100g, total carbohydrate 42.11 g/100g and total fat 6.29 g/100g (Table 12). While diets for large fish (feeding at day 17-128) were also analyzed and found to contain approximately ash 8.35 g/100g, crude fiber 2.47 g/100g, energy 362.86 Kcal/100g, moisture 10.21 g/100g, protein 34.02 g/100g, total carbohydrate 40.0 g/100g ant total fat 7.42 g/100g (Table 13).

Table 12 Proximate analysis of the commercial feed for small fish (Day 1-16)

Lists	Results	Unit	Reference Method
Ash	8.86	g/100g	AOAC (2019) 942.05
Crude Fiber		g/100g	AOAC (2019) 978.10
Energy	357.33	Kcal/100g	Compendium of Method for Food Analysis (2003), p. 2-18
Moisture	9.67	g/100	AOAC (2019) 930.15
Protein	33.07	g/100g	AOAC (2019) 976.05
Total Carbohydrate (Include)	42.11	g/100g	Compendium of Method for Food Analysis (2003), p.2-9 to p.2-10
Total fat	6.29	g/100g	AOAC (2019) 954.02

Table 13 Proximate analysis of the commercial feed for large fish (Day 17-128)

Lists	Results	Unit	Reference Method
Ash	8.35	g/100g	AOAC (2019) 954.05
Crude Fiber	2.47	g/100g	AOAC (2019) 978.10
Energy	362.86	Kcal/100g	Compendium of Method for Food Analysis (2003), p.2-18
Moisture	10.21	g/100g	AOAC (2019) 930.15
Protein	34.02	g/100g	AOAC (2019) 976.05
Total Carbohydrate (Include)	40.00	g/100g	Compendium of Method for Food Analysis (2003), p.2-9 to p.2-10
Total fat	7.42	g/100g	AOAC (2019) 954.02



4.6 Growth performance and feed utilization

At the end of the experiment, the survival rate and feed utilization of fish in all group were calculated. The highest value of these parameters was observed in the FKC vaccine with adjuvant Montanide GR01 group, but no significant differences when compared to vaccine only and control group ($P>0.05$) (Table 14).

Table 14 Growth performance and feed utilization of Nile tilapia fed with different vaccination for 5 months

Treatment	IW (g)	Survival fish (number of fish)	SR (%)	AWF (kg/fish)	ADG (g/day)	FB (kg)	TFC (kg)	FCR
CTRL	25.85	1,036 ± 8.75	72.73 ± 0.62	0.993 ± 0.007	5.37 ± 0.06	1043 ± 15.48	1510	1.45 ± 0.01
FKC	25.85	1,023.93 ± 16.07	71.59 ± 1.04	0.997 ± 0.003	5.39 ± 0.02	1027.33 ± 27.84	1510	1.47 ± 0.02
FKC + GR01	25.85	1,062.67 ± 10.47	74.05 ± 0.73	1.000 ± 0.001	5.41 ± 0.00	1062 ± 18.15	1510	1.42 ± 0.01

Abbreviation: CTRL: control group; FKC: oral vaccination with FKC only; FKC + GR01: oral vaccination with FKC + Montanide GR01; IW: initial weight; SR: survived rate; AWF: average weight per fish; ADG: average daily gain; FB: final biomass ; TFC: total feed consumed; FCR: feed conversion ratio.

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4.7 Water quality

During the experimental period, physio-chemical water qualities were measured by a YSI 550A Dissolved Oxygen Instrument and a YSI pH100A. All water parameters were within optimal levels for fish survival (Table 15).

Table 15 Water quality conditions during farm trials (Mean ± SD)

Water parameter	Unit	Max	Min	Mean ± SD
pH	-	9.10	7.50	7.6 ± 0.59
Temperature	°C	33.6	26.2	30.7 ± 2.6
Dissolved oxygen	mg/L	13.6	4.66	8.0 ± 2.9
Conductivity	µS/cm	104.8	85.2	94.5 ± 6.1

Abbreviations: Max: maximum; Min: minimum

4.8 Innate immune response analysis

4.8.1 Lysozyme (LZM) activity

The LZM activity was higher in fish vaccinated with FKC vaccine formulated with Montanide GR01 group followed by the FKC vaccine only. The result showed that the higher level of LZM activity was found at 2 and 3 month post-vaccination (m.p.v.). Fish vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher compared with control group ($P<0.05$) at 2 and 3 m.p.v. (Table 16).

4.8.2 Myeloperoxidase (MPO) activity

The MPO activity was higher in fish vaccinated with FKC vaccine formulated with Montanide GR01 group followed by the FKC vaccine only. The result showed that the highest level of MPO activity was found at 1 m.p.v. and then declined to lower level at 2, 3, 4, 5 m.p.v. Fish vaccinated with FKC vaccine formulated with Montanide GR01 showed significantly higher with control group ($P<0.05$) at 1 and 2 m.p.v. Fish vaccinated with FKC vaccine only group showed significantly higher with control group ($P<0.05$) at 1, 2, 3 and 5 m.p.v. (Table 16).

4.8.3 Glutathione peroxidase (GPx) activity

The GPx activity was higher in fish vaccinated with FKC vaccine formulated with Montanide GR01 group followed by the FKC vaccine only. The result showed that the high level of GPx activity was found at 1, 2, 3 m.p.v. and declined to lower level at 4, 5 m.p.v. Fish vaccinated with FKC vaccine formulated with Montanide GR01 and the FKC vaccine only showed significantly higher compared with control group ($P<0.05$) at 1, 2, 3, 5 m.p.v. (Table 16).

4.8.4 Glutathione reductase (GRD) activity

The GRD activity was higher in fish vaccinated with FKC vaccine formulated with Montanide GR01 group followed by the FKC vaccine only. The result showed that the high level of GRD activity was found at 1, 2, 3 m.p.v. and declined to lower level at 4, 5 m.p.v. Fish vaccinated with FKC vaccine formulated with Montanide GR01 group and the FKC vaccine only showed significantly higher compared with control group ($P<0.05$) at 2 m.p.v. Fish vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher compared with control group ($P<0.05$) at 2, 3, 5 m.p.v. (Table 16).

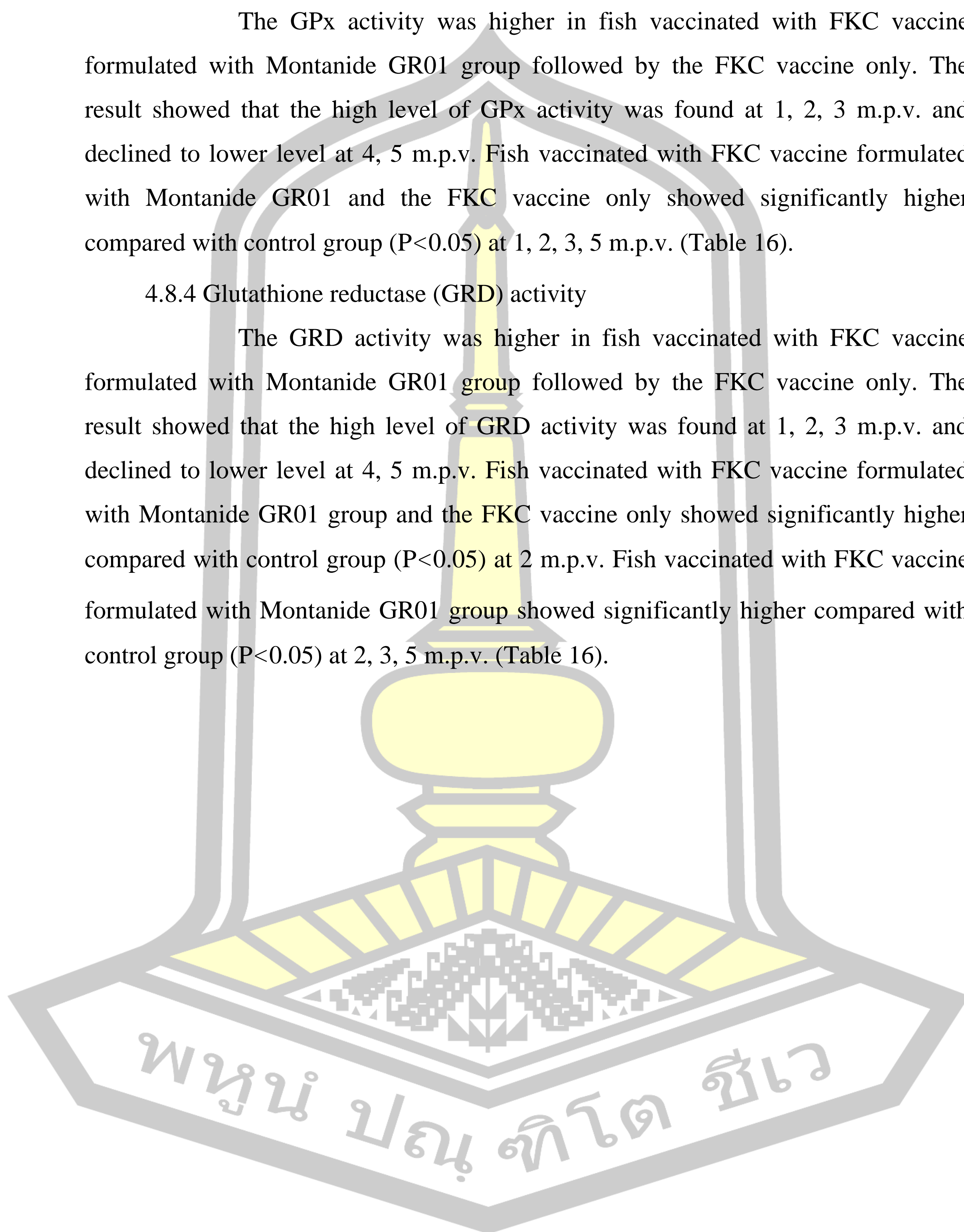


Table 16 Non-specific immune indexes in the serum of Nile tilapia vaccinated experimental protocol for 5 months

Time	CTRL	FKC	FKC + GR01	Pooled SEM	P-value
LZM (U/mL)					
Month 1	2.56	3.12	3.48	0.27	0.114
Month 2	2.32^{a}	3.28^{b}	3.44^{b}	0.16	0.001
Month 3	3.04^{a}	3.52^{a}	5.84^{b}	0.25	0.000
Month 4	3.12	3.20	3.36	0.31	0.865
Month 5	3.36	3.36	3.44	0.29	0.977
MPO (OD at 450 nm)					
Month 1	2.93	3.01	2.60	0.16	0.240
Month 2	2.22	2.77	2.80	0.32	0.125
Month 3	1.31	2.29^{b}	2.49^{b}	0.28	0.012
Month 4	1.95	2.12^{a}	2.46^{b}	0.10	0.008
Month 5	1.28	1.88^{b}	1.64^{ab}	0.17	0.045
GPX (U/mL)					
Month 1	26.84	36.06	36.55	1.63	0.135
Month 2	36.00	38.61	39.55	1.73	0.368
Month 3	31.61	33.42	40.71^{b}	1.37	0.001
Month 4	16.81	19.07^{b}	16.61	0.71	0.068
Month 5	24.22	26.42	23.16	1.95	0.515
GRD (U/mL)					
Month 1	5.14	6.56	6.21	0.89	0.368
Month 2	6.62	7.04^{a}	7.89^{b}	0.34	0.010
Month 3	6.35	6.62^{ab}	8.29^{b}	0.71	0.076
Month 4	2.83	3.86^{b}	2.84^{a}	0.22	0.002
Month 5	3.85^{a}	4.62^{ab}	5.80^{b}	0.64	0.098

Abbreviation: CTRL: control group; FKC: oral vaccination with FKC only; FKC + GR01: oral vaccination with FKC + Montanide GR01; LZM: lysozyme; MPO: myeloperoxidase; GPx: glutathione peroxidase; GRD: glutathione reductase.

Values represent the mean of three replicates. Values followed by different letters in the same row are significantly different ($P<0.05$).

4.9 Adaptive immune response analysis

4.9.1 Specific IgM antibody response analysis by ELISA technique

During 1-5 m.p.v., the specific IgM antibody levels in sera were measured by ELISA. The analysis showed that all vaccinated groups produced detectable specific IgM antibodies in the sera over this time course (Fig. 6). However, the highest antibody levels were found in sera from fish vaccinated with FKC vaccine

formulated with Montanide GR01 group followed by the fish vaccinated with FKC vaccine only group at each time points examined. The IgM antibody levels increased from 1-5 m.p.v. in fish vaccinated with FKC vaccine formulated with Montanide GR01, and significantly higher compared with control group (P<0.05) at 2, 3, 4, 5 m.p.v.. These results show that the highest IgM antibody levels against *S. agalactiae* was found in fish vaccinated with FKC vaccine formulated with Montanide GR01, implying that Montanide GR01 can assist the humoral immune response of fish post vaccination.

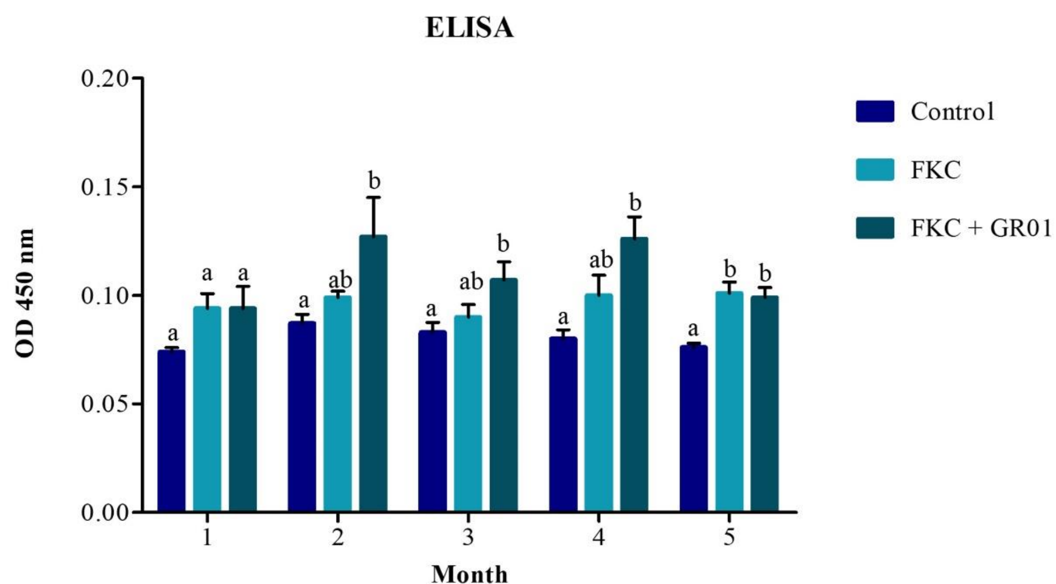


Figure 6 Specific IgM antibody levels against *S. agalactiae* in the sera of vaccinated Nile tilapia, as determined by ELISA

Fish vaccinated with commercial diet and used as control, or vaccinated with FKC vaccine only, and FKC vaccine formulated with Montanide GR01 at 1, 2, 3, 4 and 5 m.p.v., sera were collected from vaccinated fish (N=5). Data represent as mean + SEM. Bars with different letters denote significant differences (P<0.05).

4.9.2 Gene expression analysis in the spleen

The expression of immune-related genes was examined by RT-qPCR analysis in the spleen, liver and intestine at 5 m.p.v. (Fig 7). The genes included IgM, COX-2, TNF-α, IL-6, IL-8, IFN-γ, SOD, GPx and CAT. Fish vaccinated with FKC vaccine formulated with Montanide GR01 showed significantly higher gene

expression of IgM, COX-2, TNF-α and GPx compared with fish vaccinated with FKC vaccine only. Fish vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher expression of SOD, IgM, COX-2, TNF-α, IL-6, IL-8, GPx and CAT in the spleen compared with fish vaccinated with commercial diet (control group) (P<0.05).

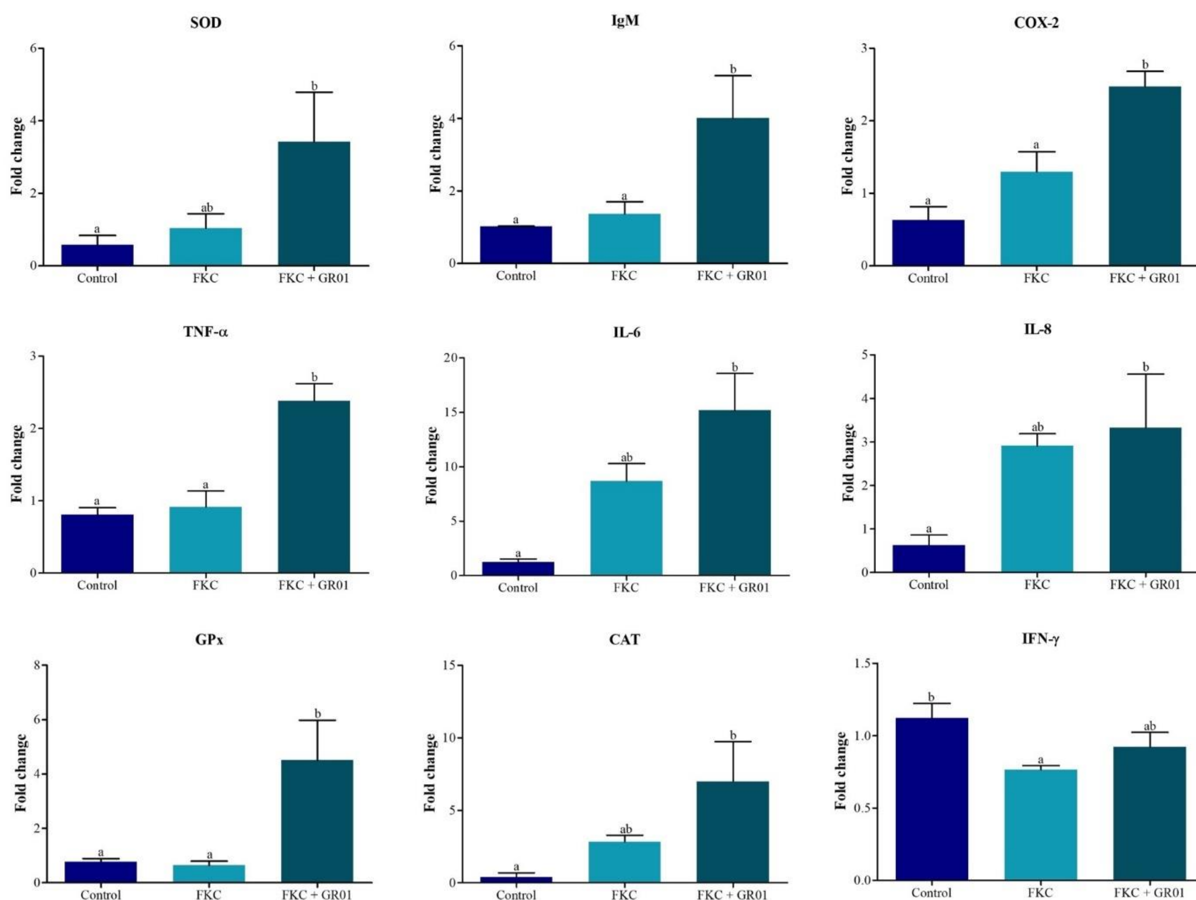


Figure 7 Transcript expression analysis of immune-related genes in the spleen

Fish vaccinated with commercial diet and used as control, or vaccinated with FKC vaccine only, and FKC vaccine formulated with Montanide GR01. The spleen of tilapia was sampled at 5 m.p.v. The mRNA level of each immune-related gene was normalized to that of β-actin, and a fold change was calculated by dividing the values of the vaccinated tissues by those of control fish. For each gene, the mRNA level of the CTRL group was set as 1. Data are presented as mean + SEM (N=3). Bars with different letters denote significant differences (P<0.05).

4.9.3 Gene expression analysis in liver

Fish vaccinated with FKC vaccine formulated with Montanide GR01 showed significantly higher gene expression of SOD, TNF-α, IL-6 and CAT in the liver compared with control group (Fig. 8). Fish vaccinated with FKC vaccine only group showed significantly higher gene expression of COX-2 compared with control group or fish vaccinated with FKC vaccine formulated with Montanide GR01 group (P<0.05).

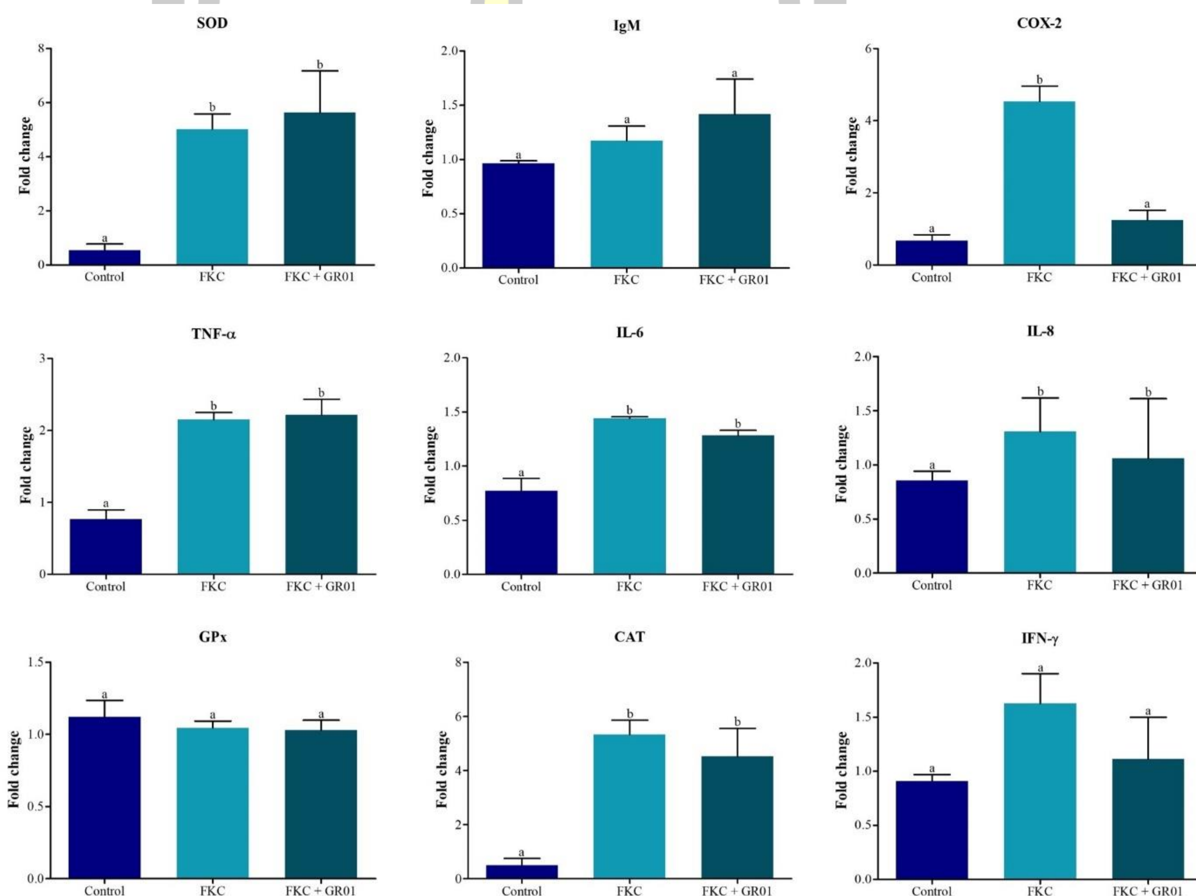


Figure 8 Transcript expression analysis of immune-related genes in the liver
Fish vaccinated with commercial diet and used as control, or vaccinated with FKC vaccine only, and FKC vaccine formulated with Montanide GR01. The liver of tilapia was sampled at 5 m.p.v. The mRNA level of each immune-related gene was normalized to that of β-actin, and a fold change was calculated by dividing the values of the vaccinated tissues by those of control fish. For each gene, the mRNA level of the CTRL group was set as 1. Data are presented as mean + SEM (N=3). Bars with different letters denote significant differences (P<0.05).

4.9.4 Gene expression analysis in intestine

Fish vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher gene expression of GPX, CAT and IFN-γ in the intestine compared with control group (Fig. 9). Fish vaccinated with FKC vaccine only showed significantly highest gene expression of TNF-α, IL-6 and IL-8 (P<0.05).

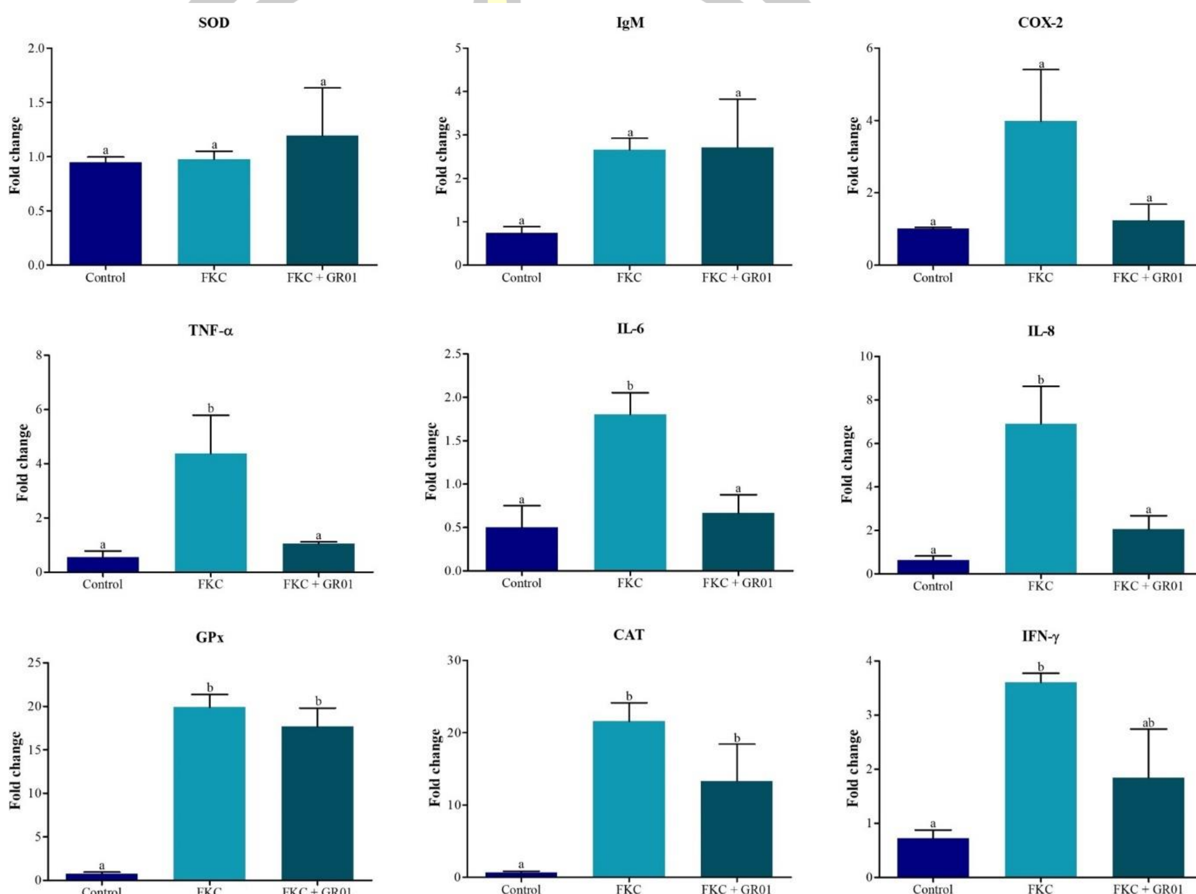


Figure 9 Transcript expression analysis of immune-related genes in the intestine
Fish vaccinated with commercial diet and used as control or vaccinated with FKC vaccine only, and FKC vaccine formulated with Montanide GR01. The intestine of tilapia was sampled at 5 m.p.v. The mRNA level of each immune-related gene was normalized to that of β-actin, and a fold change was calculated by dividing the values of the vaccinated tissues by those of control fish. For each gene, the mRNA level of the CTRL group was set as 1. Data are presented as mean + SEM (N=3). Bars with different letters denote significant differences (P<0.05).

Chapter 5
Discussions and Conclusions

An emerging infectious disease caused by *S. agalactiae* has adversely affecting Nile tilapia aquaculture. Streptococcosis is one of the most common diseases in freshwater and marine fish culture (Munang'andu *et al*., 2016). Many Southeast Asia countries such as Thailand, Malaysia, Indonesia and Vietnam have reported *S. agalactia* infection (Kayansamruaj *et al*., 2020, Syuhada *et al*., 2020). The majority of vaccines developed against *S. agalactiae* in Nile tilapia were whole cell inactivated vaccines. Methods for inactivating vaccines include heating, ultraviolet light and chemical methods such as using formalin, solvents and detergents (Ramos-Espinoza *et al*., 2020). Mineral oil adjuvants registered under the trademark Montanides have been optimized to improve vaccine formulation efficacy and stability while reducing side effects. These adjuvants are based on mineral oil, non-mineral oil, or a combination of the two, as well as those derived from specific surfactant chemistry employing mannitol oleate and can be used to create various types of emulsions, water-in-oil (W/O), oil-in-water (O/W), or water-in-oil-water (W/O/W), for use in both mammals and fish (Tafalla *et al*., 2013). In this study, we assessed the immune response and protective efficacy of formalin-killed cell (FKC) vaccine only or FKC vaccine mixed with a new adjuvant Montanide^{TM} GR01 in Nile tilapia. To the best of our knowledge, this is the first report of the use of new adjuvant for oral vaccination in fish.

Innate immunity is the first immune mechanism that protects the host from non-specific infection by other organisms (Rauta *et al*., 2012). In this study, the innate immune response parameters studied included LZM, GPx, GRD, CAT, SOD and MPO activity. LZM activity is a key indicator of innate immunity and is found in all living organisms. LZM has been shown to have lytic activity against Gram-positive and Gram-negative bacteria. It is also known to be opsonic in nature, activating the complement system and phagocytosis and plays an important role in innate immune system defense molecule that mediates protection against microbial invasion. LZM activity in Nile tilapia was found in plasma, liver, skin, and mucus (Saurabh and

Sahoo, 2008). MPO is a conspicuous enzyme in neutrophils of many fish species and uses hydrogen peroxide to oxidase several substrates, which have been reported to be toxic for microorganisms (Castro *et al*., 2008; Yu *et al*., 2020). CAT is one of the most important antioxidant enzymes and present in almost all aerobic organisms (Moretti *et al*., 2017; Nandi *et al*., 2019). CAT breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water (Nandi *et al*., 2019). GPx is responsible for reducing lipid hydroperoxides to their corresponding alcohols as well as reducing free hydrogen peroxide to water. SOD catalyzes the dismutation (or partitioning) of O_2^- into either oxygen (O_2) or hydrogen peroxide (H_2O_2) (Cazenave *et al*., 2006). GRD is essential for cellular antioxidant protection as well as metabolic pathway adjustment. In a NADPH-dependent reaction, GRD catalyzes the reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH) (Wang *et al*., 2018). In our study, the activity of LZM, MPO, CAT, SOD, GPx and GRD in the groups of fish vaccinated with FKC vaccine formulated with Montanide GR01 20% or fish vaccinated with FKC vaccine formulated with Montanide GR01 2% groups were significantly higher than those in the FKC vaccine only or control groups ($P<0.05$) in laboratory trial. Meanwhile, on-farm trial, the activity of LZM, MPO, SOD, GPx and GRD of fish vaccinated with FKC vaccine formulated with Montanide GR01 group was significantly higher than those in the fish vaccinated with FKC vaccine or control group ($P<0.05$). Moreover, fish vaccinated with FKC vaccine group was significantly higher than control group ($P<0.05$).

In laboratory trial, the specific antibody (IgM) levels in Nile tilapia sera were measured by ELISA. The results of all vaccinated fish by oral vaccination showed that the higher antibody IgM levels were found at each time points examined, when compared with control group ($P<0.05$). However, no significantly difference between fish vaccinated with FKC vaccine formulated with Montanide GR01 2% and 20% group. Moreover, the antibody IgM levels in sera from fish vaccinated with FKC vaccine formulated with Montanide GR01 2% group increased from week 1 to week 5. Overall, the antibody IgM level in all vaccinated fish groups were significantly higher than those in the control group. While on farm trial, the analysis showed that all vaccinated groups produced detectable specific IgM antibodies over this time

course examined. However, the higher IgM antibody levels was found in fish vaccinated with FKC vaccine formulated with Montanide GR01 group followed by the fish vaccinated with FKC vaccine only group at each of time points. The IgM antibody levels increased from 1 m.p.v. to 5 m.p.v. in adjuvanted vaccine group, and revealed significantly higher than the control group ($P<0.05$) at 2, 3, 4, 5 m.p.v.. These results indicate that enhanced production of specific antibody (IgM) against *S. agalactiae* occurs when vaccine was administered with Montanide GR01, implying that this adjuvant can assist the humoral immune response. Jaafar *et al*. (2019) found that no significant differences in IgM antibody level at 52 days post-vaccination between control, one time dip vaccination and one time oral vaccination. Samples taken 45 days after boostering showed a slight but significant increase in antibody levels in all groups that had received booster vaccination, which the exception of fish that had received one time dip vaccination and one time oral vaccination, while control and one time vaccinated fish showed no change of IgM antibody levels. Overall, all groups had higher IgM antibody levels than before the challenge (Jaafar *et al*., 2019).

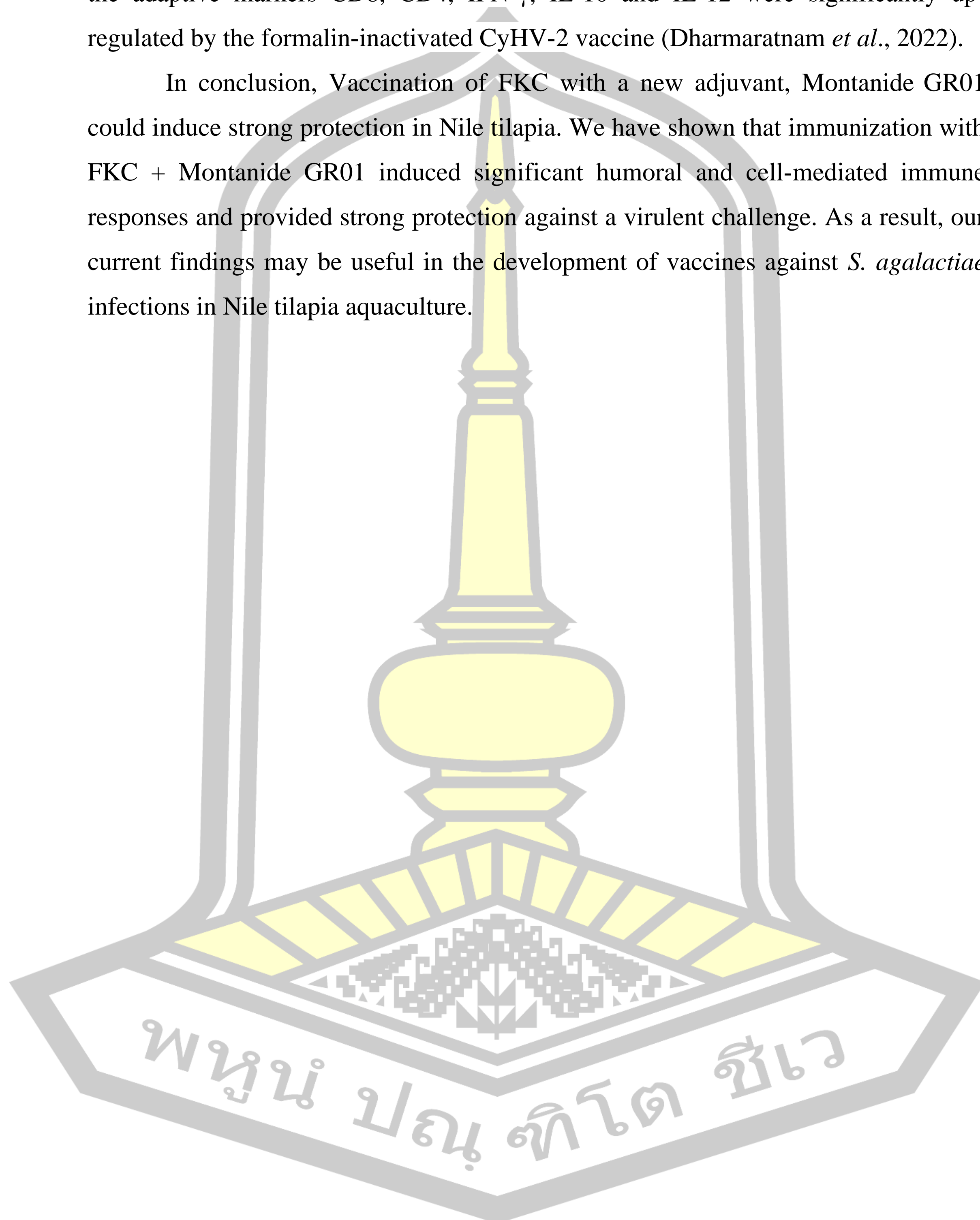
In this study, we found that the survival rates of vaccinated groups were significantly higher than control group post injection and immersion challenge. The results of this study were similar to previous studies, for example, Li *et al*. (2015) developed a potential live attenuated *S. agalactiae* vaccine. At 15 days, the RPS of fish immunized with YM001 (1.0×10^8 CFU/fish) via injection, immersion, and oral administration were 96.88, 67.22 and 71.81%, respectively. At 30 days, the RPS were 93.61, 60.56 and 53.16% respectively. When compared to the control group, the level of protective antibody elicited by oral immunization was significantly higher ($P<0.01$). Ke *et al*. (2017) developed an inactivated vaccine for Nile tilapia using *S. agalactiae* with LrrG protein encapsulated in PLGA microparticles showed good immune protection and RPS (77.54%) by oral administration.

IgM is a biological molecule in all vertebrates and is capable of eliciting effective specific humoral antibody responses against a variety of antigens. The intensity of this response, however, has been shown to differ between teleost species and under different environmental conditions (Whyte, 2007). TNF-α is a cytokine that plays a key role in systemic inflammation and immune cell regulation. It is primarily

produced by activated macrophages in membrane or secreted form (Alvarez-Pellitero, 2008). In higher vertebrates, the pro-inflammatory cytokines IL-6 is important mediators of inflammatory reactions and orchestrators of the immune system (Hong *et al*., 2013). COX-2 has been identified and characterized as an important moderator in a variety of fish physiologic and pathologic settings, including immunity, ovulation, and adipogenesis. It has been identified as an enzyme that catalyzes the formation of bioactive anti-inflammatory lipids (Wang *et al*., 2016; Eggestøl *et al*., 2020). The vertebrate antiviral system is defined by interferons (IFNs). IFN-γ activate specific signaling pathways that result in the activation of innate immune defenses against viral infection (Groeger *et al*., 2010). The enzymatic mechanisms involved in reactive oxygen species detoxification include SOD, CAT, and GPx. GPx is a key enzyme that protects living organisms from oxidative damage (Zou and Secombes, 2011). Excess ROS can be removed by SOD and CAT, thus protecting fish from stress-induced oxidative damage (Do *et al*., 2019). In this study, fish vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher gene expression of COX-2, TNF-α and GPx compared with fish vaccinated with FKC vaccine only. Fish vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher gene expression of SOD, COX-2, TNF-α, IL-6, GPx and CAT in the spleen compared with fish vaccinated with commercial diet (control group). Moreover, in the liver, fish vaccinated with FKC vaccine only and fish vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher gene expression of SOD, TNF-α, IL-6 and CAT compared with control group. Fish vaccinated with FKC vaccine only group showed significantly higher gene expression of COX-2 compared with control group and fish vaccinated with FKC vaccine formulated with Montanide GR01 group. While in the intestine, fish vaccinated with FKC vaccine only and vaccinated with FKC vaccine formulated with Montanide GR01 group showed significantly higher gene expression of GPX, CAT and IFN-γ compared with control group. The expression analysis performed by Yin *et al*. (2019) revealed that both secretory IgM and membrane-bound IgM were highly expressed in the head kidney, spleen, and mucosal tissues such as the intestine and gill in Nile tilapia post vaccination and challenge. Previous research focused on developing an inactivated vaccine for the cyprinid herpesvirus (CyHV-2) and testing

its immunogenicity in the host. The gene expression in spleen and kidney tissues of the adaptive markers CD8, CD4, IFN-γ, IL-10 and IL-12 were significantly up-regulated by the formalin-inactivated CyHV-2 vaccine (Dharmaratnam *et al*., 2022).

In conclusion, Vaccination of FKC with a new adjuvant, Montanide GR01 could induce strong protection in Nile tilapia. We have shown that immunization with FKC + Montanide GR01 induced significant humoral and cell-mediated immune responses and provided strong protection against a virulent challenge. As a result, our current findings may be useful in the development of vaccines against *S. agalactiae* infections in Nile tilapia aquaculture.



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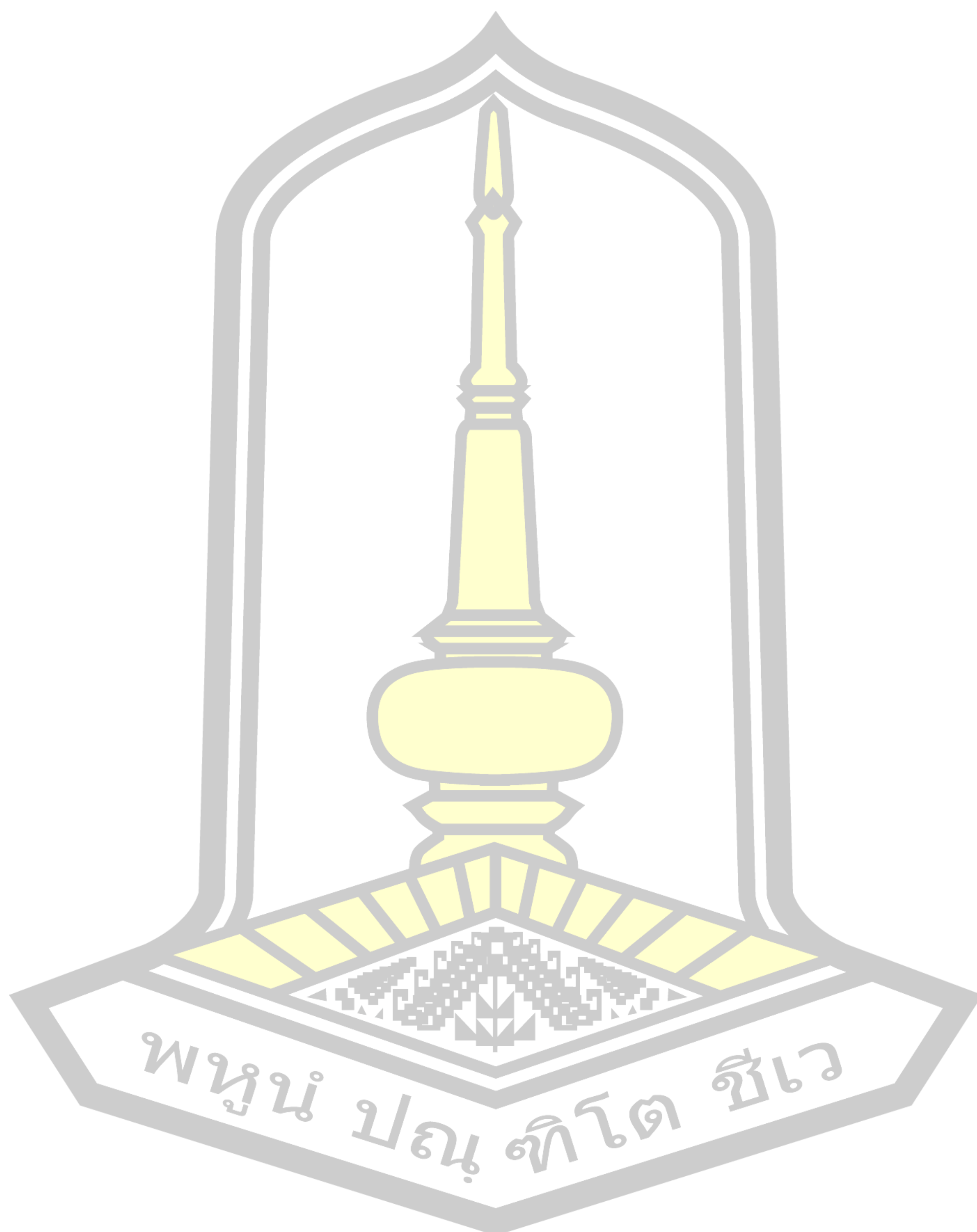

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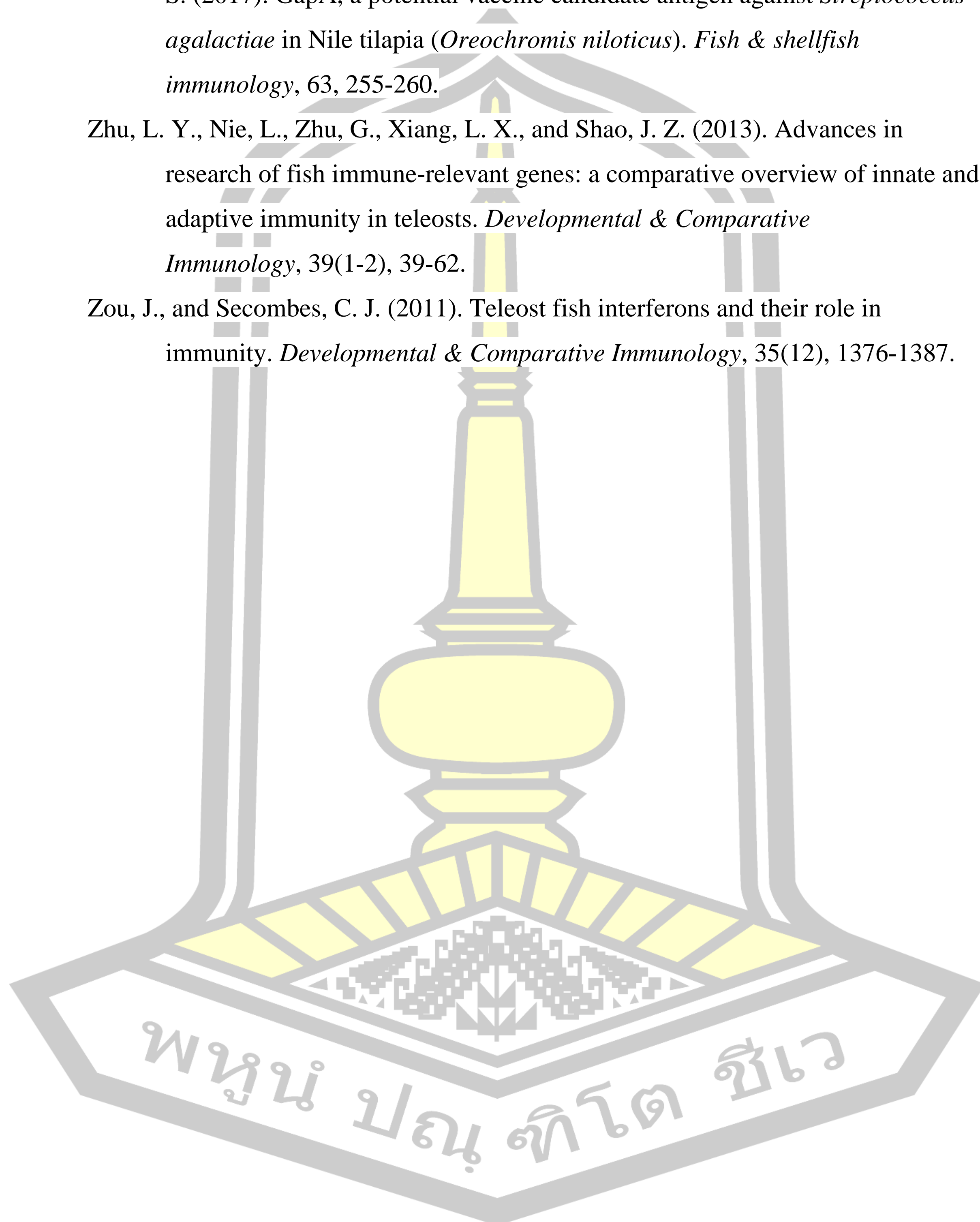

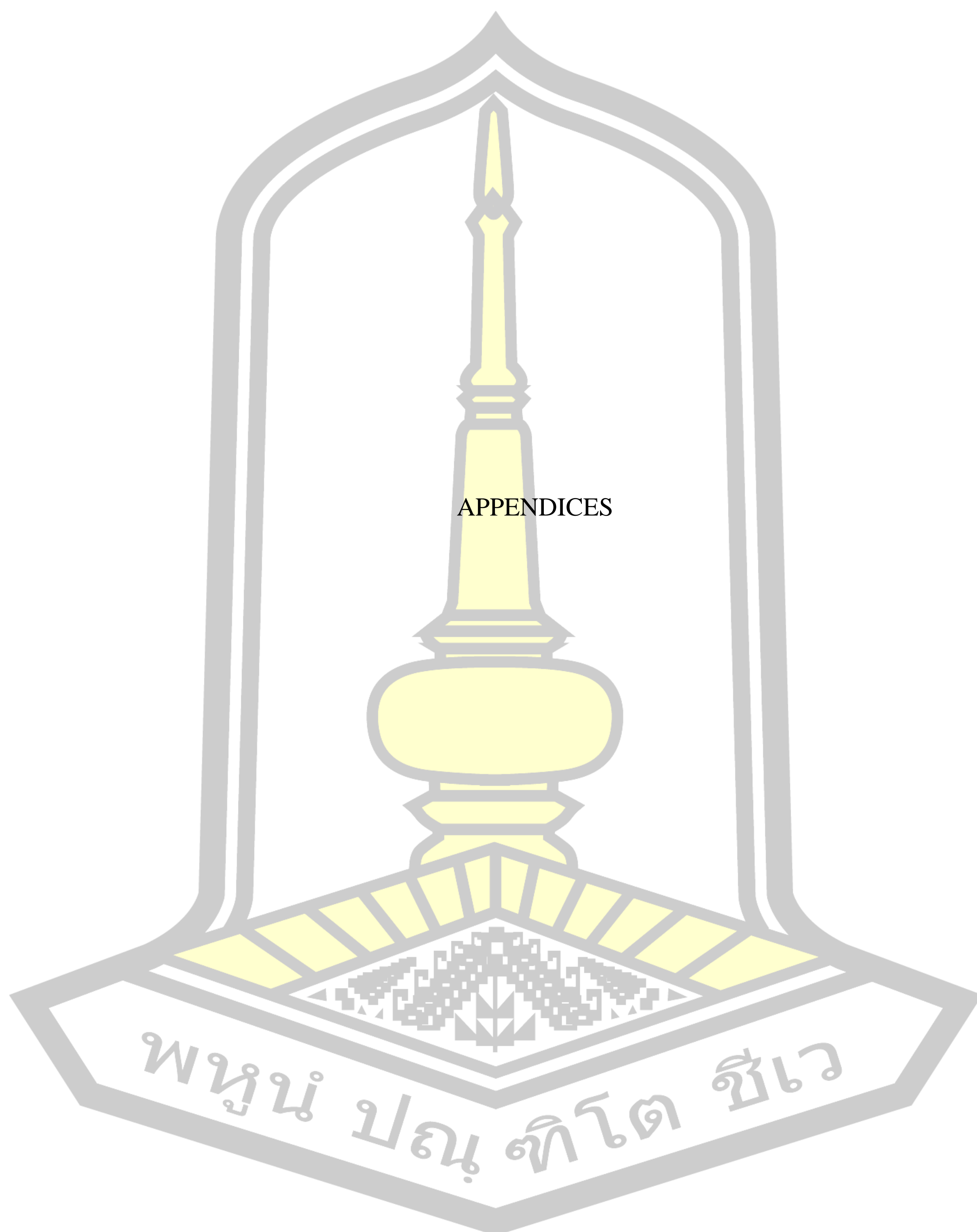

APPENDICES

PBS (Phosphate Buffered Saline) 1X, pH 7.4 preparation

NaCl	8.00 g
KCl	0.20 g
Na_2PO_4	1.44 g
KH_2PO_4	0.245 g
Distilled water	1,000 mL

Adjust solution to desired pH (typically ≈ 7.4). Autoclave 121 °C, 15 Ib, 15 minutes and store at 4 °C.

Brain heat infusion broth (BHI broth, pH 7.4)

Calf Brain, infusion form	12.50 g
Beef heart, infusion form	5.00 g
Peptone	10.00 g
NaCl	5.00 g
Na_2HPO_4	2.50 g
Dextrose	2.00 g
Distilled water	1,000 mL

Autoclave 121 °C, 15 Ib, 15 minutes and store at 4 °C.

Brain heart infusion agar (BHI agar, pH 7.4)

Brain heat infusion broth (BHI broth, pH 7.4)

Calf Brain, infusion form	12.50 g
Beef heart, infusion form	5.00 g
Peptone	10.00 g
NaCl	5.00 g
Na_2HPO_4	2.50 g
Dextrose	2.00 g
Agar	15.00 g
Distilled water	1,000 mL

Autoclave 121 °C, 15 Ib, 15 minutes and store at 4 °C.

Reagents for cDNA synthesis preparation

1. Mix for cDNA

5X buffer	8.00 µL
25 mM dNTP	2.60 µL
Reverse transcriptase	1.00 µL
Sample	29.40 µL

2. 25mM dNTP

100 mM dCTP	100.00 µL
100 mM dATP	100.00 µL
100 mM dTTP	100.00 µL
100 mM dGTP	100.00 µL

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Reagents for real-time PCR preparation

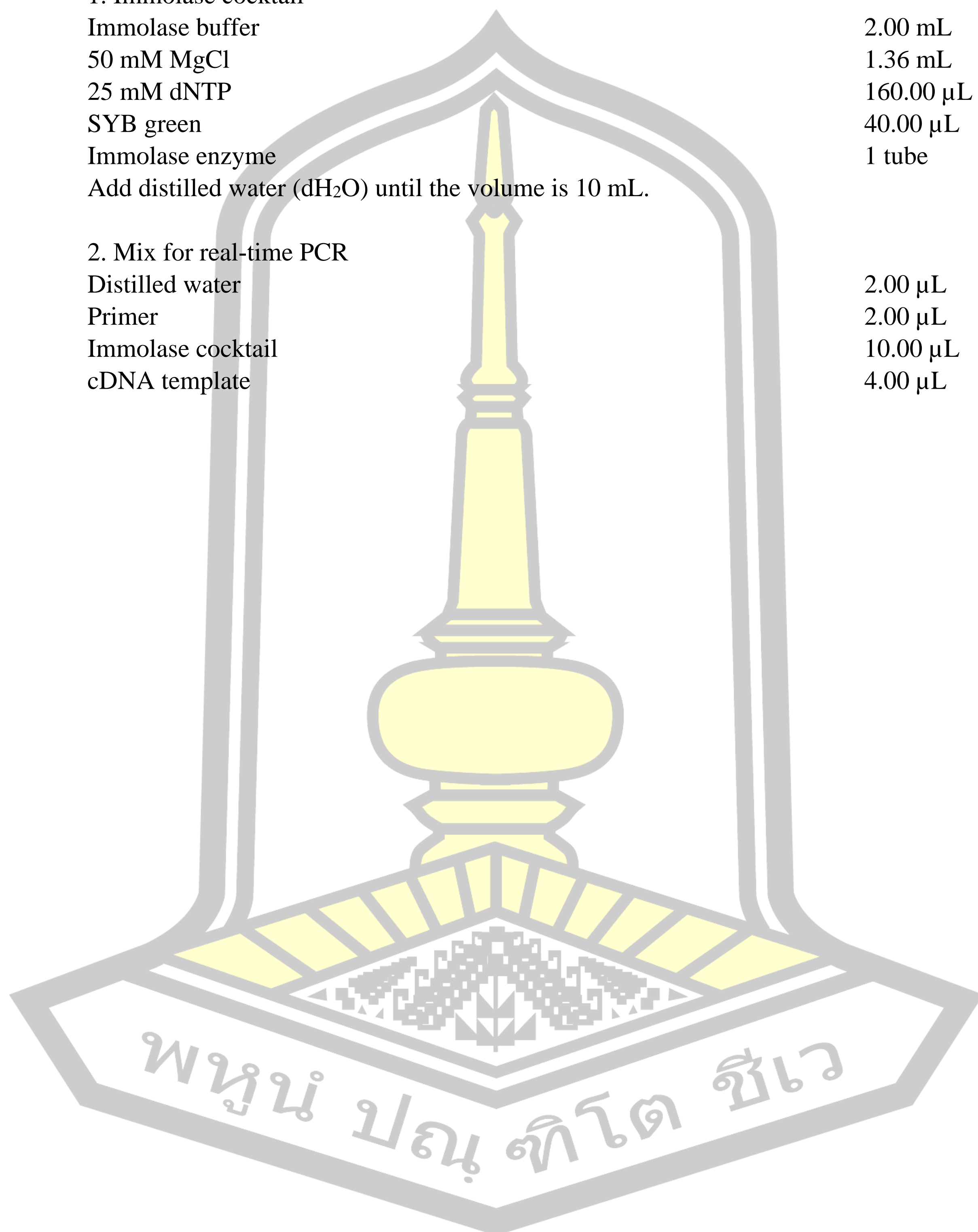
1. Immolase cocktail

Immolase buffer	2.00 mL
50 mM MgCl	1.36 mL
25 mM dNTP	160.00 µL
SYB green	40.00 µL
Immolase enzyme	1 tube

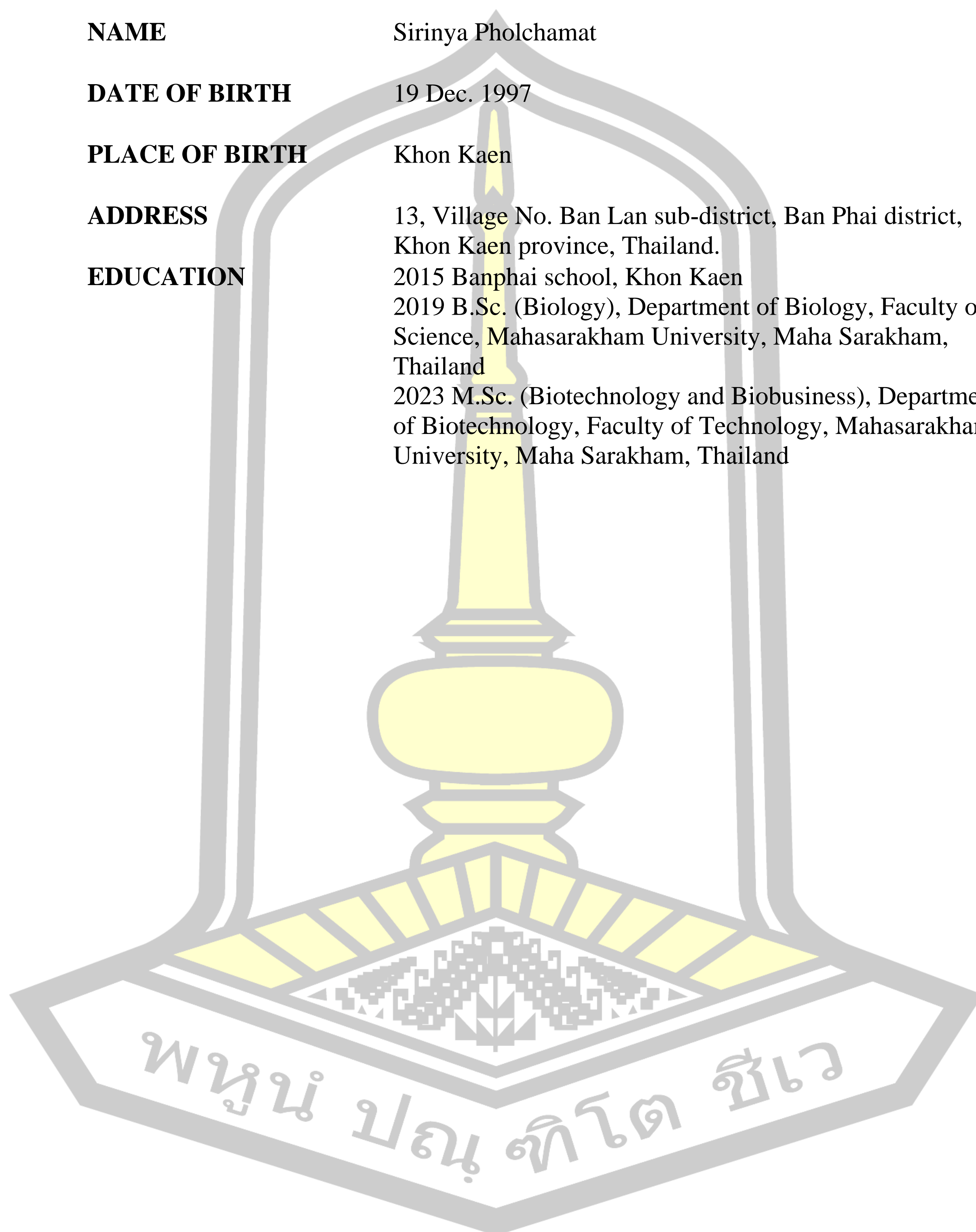
Add distilled water (dH_2O) until the volume is 10 mL.

2. Mix for real-time PCR

Distilled water	2.00 µL
Primer	2.00 µL
Immolase cocktail	10.00 µL
cDNA template	4.00 µL



BIOGRAPHY



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