The use of MONTANIDE™ IMS 1312 VG PR Adjuvant on the Immune Response of Nile tilapia (*Oreochromis niloticus*) to an Inactivated *Streptococcus agalactiae* Vaccine by Immersion Vaccination

Piyachat Phudinsai

A Thesis Submitted in Partial Fulfillment of Requirements for
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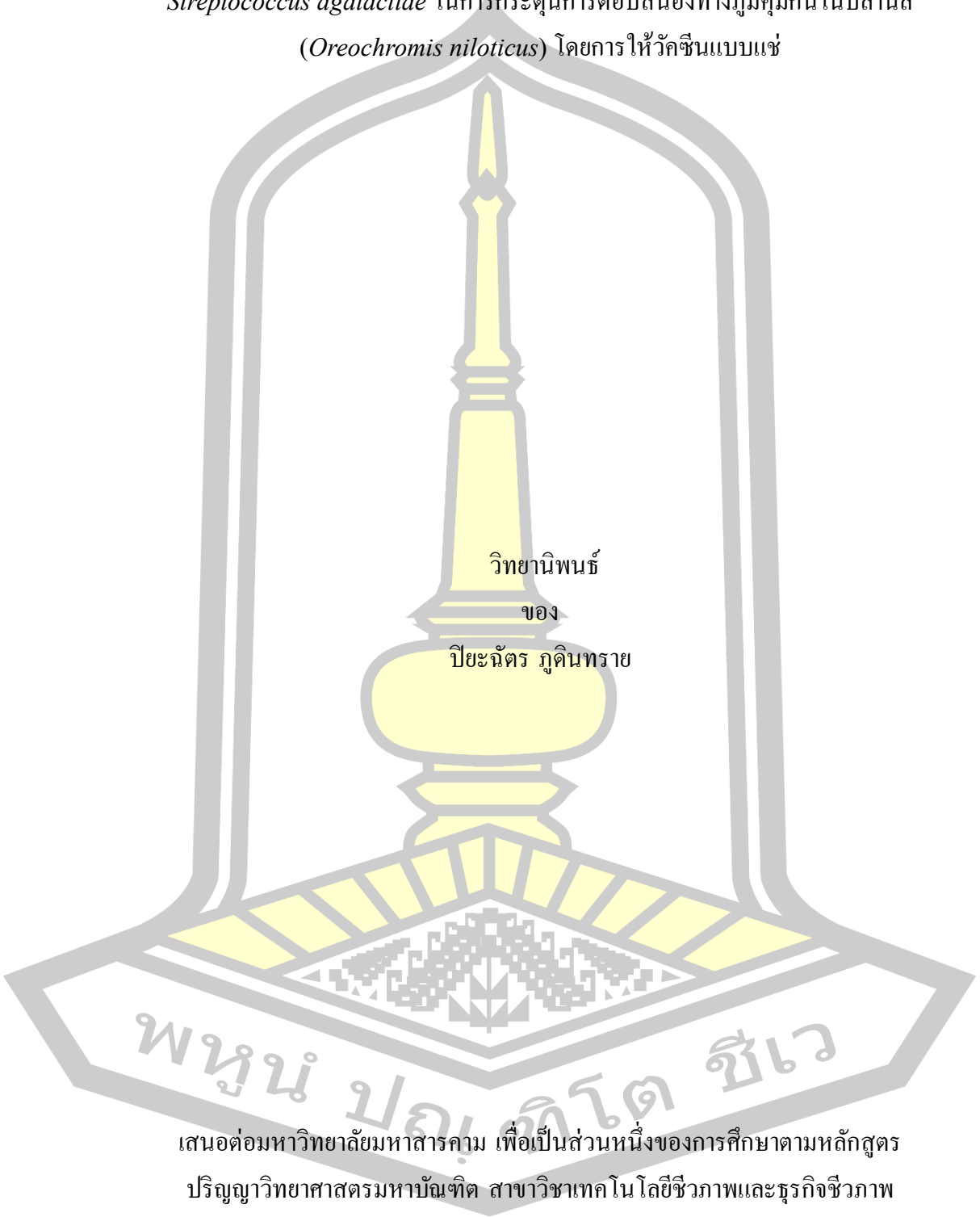
การใช้ MONTANIDE™ IMS 1312 VG PR เป็นสารเสริมฤทธิ์ในวัคซีนเชื้อตายของเชื้อ *Streptococcus agalactiae* ในการกระตุ้นการตอบสนองทางภูมิคุ้มกันในปลานิล (*Oreochromis niloticus*) โดยการให้วัคซีนแบบแช่



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ABSTRACT

Streptococcus agalactiae is a common pathogenic bacterium caused of streptococcosis, which has a negative impact on Nile tilapia aquaculture. Numerous vaccines have been recently developed to combat this disease, which are key components of global health efforts to prevent disease outbreaks. MONTANIDE™ IMS 1312 is a micro-emulsion recommended for immersion of fish. However, the data on the effectiveness of those immersion vaccines containing this aqueous adjuvant in fish are limited. The objective of this research was to explore the potential of MONTANIDE™ IMS 1312, an adjuvant for immersion vaccination, administered with an *S. agalactiae* inactivated whole-cell vaccine (SAIV) in Nile tilapia. Fishes were separated into three groups: 1) fish were vaccinated by immersion vaccination with PBS (CTRL), 2) fish were vaccinated by immersion vaccination with SAIV vaccine alone (SAIV), and 3) fish were vaccinated by immersion vaccination with SAIV containing MONTANIDE™ IMS 1312. We found that the activity of several innate immunity parameters was increased significantly ($P < 0.05$) following the immunization. As expected, the levels of specific IgM antibody were significantly increased post-vaccination, and the highest IgM antibody levels were found in the fish vaccinated with SAIV containing MONTANIDE™ IMS 1312. Analysis of the transcriptional expression of major pro-inflammatory cytokines, as well as the presence of IgM^+ B cells, revealed significant increases, suggesting that Nile tilapia were able to initiate cellular immune responses following vaccination. Taken together, our results indicate that using MONTANIDE™ IMS 1312 in combination with a SAIV can induce strong protection post *S. agalactiae* infection. Importantly, administration of an adjuvanted immersion vaccine is safe, no side effects were observed, and it does not negatively impact fish growth. In conclusion, MONTANIDE™ IMS 1312 has the potential to be used as adjuvants for immersion vaccines against streptococcosis in Nile tilapia.

Keyword : MONTANIDE™ IMS 1312, Streptococcal disease, Adjuvanted vaccine,

Vaccine development, Mucosal immune response, Group B streptococcus (GBS)

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Piyachat Phudinsai

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Chapter 1
Introduction

1.1 Research background

Nile tilapia is a commercial freshwater fish species due to its fast growth, high survivability in captivity, successful reproduction, great flesh quality, and market demand (FAO, 2020). However, highly stocking density of fish farming often results in fish stress and the spread of various diseases, leading to significant economic losses (Wangkahart *et al*., 2023). Currently, tilapia aquaculture is susceptible to infection by *S. agalactiae*, which has an adverse effect on fish farming and constitutes an important risk to Nile tilapia aquaculture in Thailand (Kannika *et al*., 2017; Yang *et al*., 2023). Consequently, chemical treatments and antibiotic drugs were widely employed to combat this pathogen. However, the use of antibiotics can lead to antimicrobial drug, resistance, and concerns about medication residues in fish. Importantly, it is critical to generate immunoprophylactic treatments that may reduce the need for chemicals and drugs. To date, vaccines remain the most effective solution for combating infectious diseases, providing immunity against specific pathogens, and enhancing animal welfwere by strengthening the immune system of fish to prevent diseases caused by pathogen infection.

Adjuvants were compounds with properties that helps enhance the immune response when used in formulation with the vaccines. They were employed to increase immune response levels, improving vaccine performance and prolonging the duration of vaccine action. Several commercial adjuvants have been developed for use alongside vaccines, and these adjuvants have been extensively tested in combination with vaccines for other economically significant fish and animals (Wangkahart *et al*., 2021; Wangkahart *et al*., 2023). Previous studies have shown that vaccinations combined with adjuvants can enhance the immune system's activity in farmed fish, making them more resistant to diseases (Wangkahart *et al*., 2023; Pholchamat *et al*., 2024). MONTANIDE™ adjuvants have been developed and applied in animal vaccines to boost the immune response generated by a specific vaccine. Importantly,

MONTANIDE™ IMS 1312, designed for use in formulation with vaccines by immersion in various aspects of several fish species.

Recently, numerous studies have explored the use of MONTANIDE™ IMS 1312 adjuvant in fish vaccines. These studies focus on immersion vaccination, demonstrating that this adjuvant effectively stimulates strong immune responses and provides high protection rates across various fish species, such as olive flounder, *Paralichthys olivaceus* (Hwang *et al*., 2017), rainbow trout, *Oncorhynchus mykiss* (Soltani *et al*., 2014; Skov *et al*., 2018; de Ruyter *et al*., 2023), starry sturgeon, *Acipenser stellutus* (Afsharipour *et al*., 2021), channel catfish, *Ictalurus punctatus*, (Lange *et al*., 2021), and zebrafish, *Danio rerio* (Solís *et al*., 2015). However, the data on the effectiveness of those immersion vaccines containing this aqueous adjuvant in Nile tilapia were limited. It is hypothesized that the effectiveness of the SAIV could be increased by including this adjuvant in the immersion route. Therefore, we aim is to assess the potential of MONTANIDE™ IMS 1312 as an adjuvant for immersion vaccine to protect Nile tilapia from *S. agalactiae* infection. The use of MONTANIDE™ IMS 1312 may provide us basic information that can be used in future research of this adjuvant on fish vaccination by immersion. Moreover, developing an adjuvanted vaccine for streptococcal disease prevention offers several advantages for tilapia aquaculture. It can enhance the fish's immune response, reduce the use of drugs and antimicrobial agents, and minimize negative environmental impacts.

1.2 Objectives

1.2.1 To develop an inactivated vaccine using an *Streptococcus agalactiae* inactivated whole-cell vaccine (SAIV) and to evaluate the vaccine safety on fish growth or bone malformation

1.2.2 To evaluate the effectiveness of the MONTANIDE™ IMS 1312 adjuvant combined with a SAIV in inducing innate immune responses in Nile tilapia through immersion vaccination

1.2.3 To evaluate the potential of the MONTANIDE™ IMS 1312 adjuvant combined with a SAIV to induce adaptive immune responses in Nile tilapia through immersion vaccination, enhancing the production of specific IgM antibodies against *S. agalactiae*

1.2.4 To evaluate the protective efficacy of the MONTANIDE™ IMS 1312 adjuvant combined with a SAIV against *S. agalactiae* in Nile tilapia through immersion vaccination

Chapter 2
Literature Review

2.1 Biology of Nile tilapia

Nile tilapia (*Oreochromis niloticus*) is an aquatic animal widely cultured and consumed worldwide because it is easy to grows (Khanjani and Sharifinia, 2021). It can live in fresh and brackish water (El-Sayed, 2006). They come live in various environmental conditions, including temperature, salinity, dissolved oxygen, and ammonia (Abd El-Hack *et al*., 2022).

Nile tilapia belongs to the family Cichlidae and the genus *Oreochromis* spp. Their body shapes were generally traditional, with a laterally compressed and deep form, resembling the Mozambique tilapia (*O. mossambicus*). Both their anal and dorsal fins have soft rays and strong spines. Notably, their pectoral and pelvic fins were larger and more anterior, enabling them to swim and maneuver with remarkable ease. These fins were also adapted for locomotion, allowing for smooth, continuous movements at low speeds. Tilapia bodies were typically characterized by vertical bars, subdued colors, and minimal color contrast. They have a limited ability to change their color in response to stress, thanks to the manipulation of skin chromatophores. Tilapia exhibit well-developed nweres and lateral lines, which were indicators of their sensory organ development. Their relatively large eyes contribute to their excellent vision skills (El-Sayed, 2006; Vajargah, 2021).

2.2 Streptococcosis diseases

Recently, aquaculture industry has been becoming rapid growth and is expected to continue expanding in the near future (Ahmad *et al*., 2021; Yue and Shen, 2022). Consequently, fish farmers employ intensive farming practices, rearing fish at high density levels to achieve greater yields. However, this high-density approach can lead to stress, weakness, and increased susceptibility to diseases, making it a significant contributor to infectious diseases in fish farming, primarily caused by

bacteria (Liao *et al*., 2020; Neto *et al*., 2023). Therefore, bacterial infections were among the most common diseases in aquaculture (Abd El-Hack *et al*., 2022). Additionally, there have been reports of infections caused by viruses, parasites, and fungi, all of which have the potential to cause significant damage to fish farming operations and result in economic losses (Rathor and Swain, 2024).

S. agalactiae (group B streptococcus; GBS) is a Gram-positive bacterium that is a major cause of infections in humans, mammals, and aquatic animals, characterized by sepsis and meningitis (Eto *et al*., 2020). It is divided into ten serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) based on capsular polysaccharides, with Group III being the most common serotype associated with diseases (Delannoy *et al*., 2013; Raabe and Shane, 2019). In addition, *S. agalactiae* is a significant pathogen in fish culture, particularly in Nile tilapia, where it causes streptococcosis. This disease often occurs during the summer, negatively impacting fish farming and leading to severe damage in Nile tilapia industry (Eissa *et al*., 2021; Phuoc *et al*., 2021). Early-stage infection in fish is characterized by erratic swimming, exophthalmia, corneal opacity, and abdominal distension (Pradeep *et al*., 2016; Owatari *et al*., 2020). Internal symptoms include septicemia, meningitis, and spleen hemorrhage. Additionally, infected fish may exhibit a darker body color, along with wounds and bleeding around the base of the gill cover and skin (Owatari *et al*., 2022; Haenen *et al*., 2023). Transmission occurs through exposure to waste from infected fish, although susceptibility varies among individuals and the symptoms differ across fish species. In fish culture, *S. agalactiae*-induced diseases were characterized by rapid morbidity and high mortality rates (Chideroli *et al*., 2017; Owatari *et al*., 2022; Yang *et al*., 2023).

2.3 Fish vaccine and type of vaccine used in fish aquaculture

Vaccine is a biological preparation designed to stimulate the immune response in fish against specific pathogens, which may include bacteria, viruses, or other microorganisms (Mondal and Thomas, 2022). Fish vaccines were developed using components derived from pathogens (Adams, 2019; Irshath *et al*., 2023). These components could be whole inactivated organisms, live attenuated forms, or purified antigens, such as proteins or polysaccharides, that represent the disease-causing organism. When vaccinated fish, it can stimulate the immune system (Mondal and Thomas, 2022; Mishra *et al*., 2023; Kumar *et al*., 2024). In general, vaccines contain pathogens that cause diseases, known as antigens (Ma *et al*., 2019). These antigens stimulate the body's immune system to produce a substance used in response, called "antibody". It is used to remove antigen from the body and can still recognize antigen as well (Yadav *et al*., 2020; Chukwuanukwu *et al*., 2022). When the body gets infected later, initiating a faster and more strong mechanism (Wangkahart *et al*., 2023). To date, fish vaccines play a crucial role in aquaculture by preventing disease outbreaks, reducing the need for antibiotics, and improving overall fish health (Mkulo *et al*., 2024).

2.3.1 Live attenuated vaccine

In aquaculture, live attenuated vaccines have been developed for prevention of fish diseases to enhance immunity and reduce disease outbreaks in fish aquaculture (Zhang *et al*., 2020). This type of vaccine is used to manage diseases caused by bacteria, viruses, and other pathogens, contributing to sustainable and healthy aquaculture practices (Mondal and Thomas, 2022). It is a vaccine prepwered from a live pathogen that has been attenuated to the point where it cannot cause disease. Additionally, live attenuated vaccines contain a weakened form of the pathogen that still retains its ability to replicate within the host but has lost its virulence (Galen *et al*., 2021; Hajra *et al*., 2021). This process often results in long-lasting immunity, sometimes with only one or two doses. The development of live attenuated vaccines typically involves passing the pathogen through different host

cells or under specific conditions that gradually reduce its virulence (Kumar *et al*., 2024). By cwerefully applying chemicals or heat at levels that do not kill the pathogen, it can stimulate the immune system and mimic a natural infection as closely as possible (Tammas *et al*., 2024). However, the substance used in the vaccine may changes into a more virulent strain (Assefa and Abunna, 2018; Wangkahart *et al*., 2023).

2.3.2 Inactivated vaccine

An inactivated vaccine, also known as a killed vaccine, is a type of vaccine in which the pathogen has been inactivated or killed through physical or chemical processes, ensuring that it cannot replicate or cause disease (Mondal and Thomas, 2022; Kumar *et al*., 2024). In fish, inactivated vaccines were used widely to prevent bacterial and viral infections in aquaculture, contributing to more sustainable fish farming (Mkulo *et al*., 2024). The advantages of this vaccine include the inability of the pathogen to mutate or cause diseases, making it a highly safe vaccine (Assefa and Abunna, 2018; Wangkahart *et al*., 2023). When an inactivated vaccine is administered to fish, the immune system recognizes the pathogen. This immune response includes the production of antibodies and the activation of immune cells that "memory" the pathogen. If the fish is later exposed to the live pathogen, its immune system can respond more rapidly and effectively, often preventing disease or reducing its severity (Tammas *et al*., 2024).

2.3.3 DNA and RNA vaccines

DNA and RNA vaccines were an advanced type of vaccine that utilize the genetic material of a pathogen to stimulate an immune response. In fish, DNA and RNA vaccines were emerging as promising tools for disease prevention in aquaculture, resulting advantages over traditional vaccine types (Priya and Kappalli, 2022; Rathor and Swain, 2024). Basically, DNA vaccines use a small, circular piece of DNA (called a plasmid) that contains genes coding for specific antigens of the pathogen (Tammas *et al*., 2024). When injected into the fish, the DNA enters cells, which then produce the pathogen's antigen, mimicking an infection and triggering an

immune response (Kim *et al*., 2000). While RNA vaccines, often in the form of messenger RNA (mRNA), deliver genetic material that codes for the pathogen's antigens. Cells in the fish then use this mRNA to produce the antigens, which prompts the immune system to recognize and respond to them (Ma *et al*., 2019). Currently, both vaccine were being extensively developed and applied in fish aquaculture because they were highly safe compwered to other vaccines (Rathor and Swain, 2024). However, using vaccine alone may result in a relatively low stimulation of fish immunity (Mondal and Thomas, 2022). Therefore, vaccines were being developed in combination with adjuvants to enhance their effectiveness (Assefa and Abunna, 2018; Wangkahart *et al*., 2023).

2.3.4 Subunit and recombinant vaccines

Subunit and recombinant vaccines were types of vaccines that contain only specific components (subunits) of a pathogen rather than the entire organism (Mondal and Thomas, 2022). These components, typically proteins or polysaccharides, were cwerefully selected for their ability to induce an immune response in the host (Ma *et al*., 2019). In fish, subunit and recombinant vaccines were increasingly used to prevent infectious diseases in aquaculture, offering a targeted and safe approach to disease control (Assefa and Abunna, 2018; Jia *et al*., 2020; Zheng *et al*., 2023). The production of these vaccines involves isolating the genes that encode the desired antigens and inserting them into a host such as bacteria, yeast, or even plants, to produce large quantities of the antigen. The antigens were then purified and formulated into a vaccine. In recombinant vaccines, the use of genetic engineering enables precise control over the antigens produced, which enhances the vaccine's effectiveness and safety. However, subunit and recombinant vaccines may produce a weaker immune response than live vaccines, as they do not replicate within the host (Lin *et al*., 2018; Ma *et al*., 2019; Mondal and Thomas, 2022).

2.4 Vaccine administration in fish

Vaccine administration in fish is a critical strategies of aquaculture health management, because it helps to prevent infectious diseases that can lead to high mortality, reduced productivity, and economic losses. Nowadays, various forms of fish vaccination were currently under development to provide the most effective disease prevention. There were three vaccination methods, each with its own advantages and disadvantages.

2.4.1 Injection vaccination

Injection is one of the most effective methods of vaccine administration in fish, providing a reliable and long-lasting immune response (Mondal and Thomas, 2022). Intraperitoneal (into the abdominal cavity) or intramuscular injections (into the muscle) were commonly used, depending on the size and species of the fish (Assefa and Abunna, 2018). Injection vaccination delivers the antigen directly into the fish, effectively stimulating the immune response. The advantage of the injection vaccination is that (1) the vaccine enhances the fish's immune system directly, resulting in a strong and effective immune response, (2) injected vaccines, especially inactivated and DNA/RNA vaccines, often provide longer-lasting immunity, which may reduce the need for booster doses, and (3) each fish receives a controlled dose of the vaccine, which enhances the consistency of the immune response across the population (Table 1). However, the main disadvantages of injection vaccination include (1) the method requires handling and injecting each fish individually, which is time-consuming and labor-intensive, especially in large-scale aquaculture, (2) injection process can cause stress and minor injuries to fish, and (3) optimal injection is usually used for larger, high-value species, as it may not be practical or economically viable for small fish or species raised in large numbers (Wangkahart *et al*., 2023; Tammas *et al*., 2024).

2.4.2 Immersion vaccination

Immersion vaccination, fish were immersed in a vaccine solution, allowing the antigen to be absorbed through the skin, gills, and mucosal surfaces (Ke *et al*., 2018; Tammas *et al*., 2024). This method is often used for smaller fish, which were easier to handle (Wangkahart *et al*., 2023). It consists of a significant number of cells and organs that were part of the contact system. These cells were interconnected with the immune system and have the capacity to produce a substantial number of antibodies, which help in preventing pathogenic infections (Bøgwald and Dalmo, 2019). Immersion vaccination can be administered in two ways: bath and dip immersion, depending on the concentration of the vaccine solution (Tammas *et al*., 2024). If a low-concentration vaccine is appropriate, the fish was immersed for extended periods, typically around one hour or more. The highly concentrated vaccination method involves dip immersion of the fish for 30 seconds (Mondal and Thomas, 2022; Tammas et al., 2024). In practice, the dip method is more popular because it is efficient, convenient, quick, causes minimal stress, and is widely used for both small fish and large quantities of fish (Tammas *et al*., 2024). However, immersion vaccination has the disadvantage of requiring a large quantity of vaccines and offering a shorter duration of immunity compwered to vaccination by injection (Table 1). The protection provided may not be long-lasting and might necessitate re-vaccination (Mondal and Thomas, 2022; Wangkahart *et al*., 2023).

2.4.3 Oral vaccination

Oral vaccination involves delivering the vaccine through the feed, making it a convenient method for vaccinating large number of fish (Radhakrishnan *et al*., 2023). Oral vaccines were typically mixed with fish feed or coated onto feed pellets (Pholchamat *et al*., 2024). Oral vaccination is ideal for large-scale aquaculture operations. Because fish were vaccinated via oral vaccination reduces stress and risk of injury, which is important for maintaining fish health and growth. It provides a convenient way to administer vaccines to fish of all sizes and in large quantities without causing stress to the fish. Moreover, oral vaccines can be easily distributed to fish in natural or farm environments, making it suitable for extensive aquaculture

industry. However, the disadvantage of oral vaccination is that the digestive system can degrade vaccines, especially protein-based vaccines, reducing their effectiveness (Table 1). To overcome this, protective coatings or encapsulation methods were often required, which can increase costs (Wangkahart *et al*., 2023; Tammas *et al*., 2024). Oral vaccines generally need to be administered multiple times to achieve sufficient immune response, which can be challenging in terms of logistics and cost (Pholchamat *et al*., 2024). In addition, if fish were stressed, infected, or have low appetite, they may not consume enough of feed to gain effective immunity. Therefore, the effectiveness of fish vaccine by oral vaccination depends on the quantity of antigen in the diet, which may necessitate a substantial vaccine dose (Assefa and Abunna, 2018; Mondal and Thomas, 2022; Wangkahart *et al*., 2023)

Table 1 Comparison of the advantages and disadvantages of vaccine administration

Administration	Advantages	Disadvantages
Injection vaccination	• The method of vaccination that is most commonly used in fish	• It necessitates advanced machinery and a significant number of highly skilled personnel.
	• Can send multiple antigens at the same time	• The vaccine could elicit a more severe reaction if it is injected into the wrong part of the fish.
	• All fish receive the same amount of antigen	• The fish were stressed.
	• Effective in stimulating the immune response	• Not suitable for small-sized fish could not respond well to this method
Immersion vaccination	• Suitable for the vaccination of large numbers of fish	• Requires large quantities of vaccine
	• Fish were less stressed	• The duration of immunity is relatively short
	• Reduce labor costs	• The period of disease prevention may not last long
	• Less risk than the injection method	• It may be necessary to repeat the vaccine a second time

Table 1 Comparison of the advantages and disadvantages of vaccine administration (Cont.)

Administration	Advantages	Disadvantages
Oral vaccination	• The vaccine can be combined with fish feed	• Need to use large quantities of antigens
	• It's an easy way, and it can be used on all sizes of fish	• Requires feeding all the fish
	• The fish don't get stressed	• It is difficult to make sure every fish gets the right amount of antigen.
	• Lower labor costs	• There's a short period of disease prevention.

2.5 The fish immune systems

The immune system of fish is a complex network designed to protect against a wide range of pathogens, including bacteria, viruses, parasites, and fungi (Kotob *et al*., 2017; Abd El-Hack *et al*., 2022). Like other vertebrates, fish possess both innate (non-specific) and adaptive (specific) immune responses. However, fish immune systems were unique in several ways due to their nature, aquatic habitats, and evolutionary adaptations (Mokhtar *et al*., 2023). Innate and adaptive immune responses were the two primary types of immunological responses. The innate immune system is the first line of defense in fish, relying on nonspecific mechanisms that respond rapidly to pathogens, while adaptive immune system provides a more targeted response and immunological memory to specific pathogens (Stosik *et al*., 2021). The fish have both innate and adaptive immune defense mechanisms. The innate immunity characteristics play a pivotal role in disease resistance and were considered the first live of immune defense. Long-lasting immunity depends on the adaptive immunity response of fish, which is frequently delayed (Rauta *et al*., 2012; Secombes and Wang, 2012).

2.5.1 The innate immunity

The innate immune system plays a major role in the immunological response, is ready to fight pathogens infection, and serves as the first line of protection against several pathogens. The physical, cellular, and humoral variables were the three main factors used to categorize the elements of the innate immune system. The first physical defense against infection is provided by the mucous surfaces of the fish scales, mucous skin surfaces, and gills (Andrés *et al*., 2022; Mokhtar *et al*., 2023). In addition, mucous plays an important role in effectively eliminating pathogens (Kong *et al*., 2022). The main immune-related cells include neutrophils, macrophages, monocytes, and cytotoxic cells (Mokhtar *et al*., 2023). One of the most significant cellular mechanisms of innate immunity is phagocytosis. Neutrophils and macrophages were the primary cells in fish that participate in phagocytosis (Cammarata *et al*., 2012). Innate immune responses begin with the identification of microbial pathogens by pattern recognition receptors (PRRs). The pathogen-associated molecular patterns (PAMPs), which the PRRs were able to detect, were conserved molecular structures that aid in the elimination of the infection by stimulating subsequent host immunity (Mokhtar *et al*., 2023). The receptors that recognize PAMPs were found in multiple locations within the cell, and they include Toll-like receptors (TLRs), Rig-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) (Abbas *et al*., 2023; Ortiz and Esteban, 2024).

The three types can be used to classify the endogenous signals that PAMPs induce such as interleukin (IL)-1, type I interferons (IFNs), IL-6, tumor necrosis factor (TNFa), and other chemokines that mediate the inflammatory response. Signals that assist in T cell activation and stimulation (Tran *et al*., 2019; Andrés *et al*., 2022). Additionally, there were signals such as transforming growth factor (TGF)-b, IFN-g, IL-4, IL-5, IL-10, and IL-12 that regulate the induction of effector activities (Sakai *et al*., 2021; Bela-Ong *et al*., 2023).

2.5.2 The adaptive immunity

The adaptive immune system of fish, it is characterized by its ability to recognize and “memory” specific pathogens (Wu *et al*., 2024). The main components of the adaptive immune system include the immunoglobulins (Igs), major histocompatibility complex (MHC), and T cell receptors (TCRs) (Abbas *et al*., 2023). The cells can broadly be divided into T-cells, which mediate cell-mediated immunity, and B-cells, which produce antibodies. The lymphocytes, which were the cell types in charge of the variety of antigen detection, specificity, and memory, were essential to the adaptive response. In addition, T-cells can be further classified as helper T-cells, which use the production of cytokines to influence other immune cells, and cytotoxicity T-cells, which eliminate infected cells directly (Mutoloki *et al*., 2014). Because infected cells might exhibit pathogen peptides and products on their cell surface, T cells were able to identify the presence of intracellular pathogens. The MHC molecules exhibit these peptides, which in turn trigger the development of pathogen-specific adaptive immunity. MHC can be broadly classified into two classes: MHC class I, which triggers $CD8^+$ cytotoxic T cell-mediated cellular immunity, and MHC class II, which triggers $CD4^+$ helper T cell-mediated humoral immunity (Zhu *et al*., 2013; Abbas *et al*., 2023).

Specifically produced by B cells, Igs were the main components of adaptive immunity. There were two types of Igs that have been found: the well-known antibody released by plasma cells, which is a crucial component controlling humoral immune responses, and the B cell receptor (BCR), a membrane-bound molecule that functions as an antigen receptor on the surface of B cells (Abbas *et al*., 2023). The Igs in mammals can be classified into five groups based on the constant region: IgM, IgD, IgG, IgA, and IgE. However, in teleost fish, they have been found to contain functioning Igs such as IgD, IgZ, and IgT (Zhu *et al*., 2013; Mutoloki *et al*., 2014).

2.5.3 The immune system organs in fish

For optimal performance of their roles, the immune response cell constituents were arranged into tissues and organs. There were two classifications for the lymphoid organs and tissues: primary (central) and secondary (peripheral). The primary lymphoid organs produce lymphocytes, which were then used by the secondary lymphoid organs and tissues (Secombes and Wang, 2012).

2.5.3.1 Head kidney

The head kidney tissue is an essential organ in the immune system of fish, contributing to hematopoiesis, immune activation, pathogen response, and the regulation of immune responses. It is central to both innate and adaptive immunity in aquatic organisms. It is responsible for the production and differentiation of blood cells, including immune cells such as macrophages, neutrophils, and lymphocytes. The head kidney is functionally equivalent to the bone marrow and spleen in mammals (Secombes and Wang 2012; Fu *et al*., 2021; Bjørgen and Koppang, 2022; Zhong and Gao, 2022). In addition to hematopoiesis, the head kidney serves as a site for the activation and differentiation of immune cells (Guo *et al*., 2024). Lymphocytes migrate to peripheral tissues or other immune organs such as the spleen or gills (Liang *et al*., 2022). The head kidney also contains antigen presenting cells that help initiate immune responses. The head kidney is one of the first sites of immune response in fish when an infection occurs (Rauta *et al*., 2012; Mokhtar *et al*., 2023). It produces various immune mediators, such as cytokines and chemokines, which regulate the immune response and attract immune cells to infection sites. These mediators were vital for coordinating both innate and adaptive immune responses (Esteban, 2023).

2.5.3.2 Thymus

The thymus is a vital organ in the immune system of fish, playing a central role in the development and maturation of T lymphocytes (T cells), which were crucial for the adaptive immune response (Nakanishi *et al*., 2015). While its structure and function were similar to that of mammals, there were some differences in how the thymus functions in fish, given their unique immune system and

environment. T cells were essential for the adaptive immune response, particularly in recognizing and responding to specific pathogens through antigen recognition (Secombes and Wang, 2012; Bjørgen and Koppang, 2022). In fish, the thymus typically consists of a bilateral structure located near the gills and heart, and it can vary in size and shape depending on the species. It is often composed of two or more lobes and is located just behind the gill arches. The thymus in fish is particularly important during early life stages, because it supports the development of a functional adaptive immune system. In fish fingerling, the thymus is crucial for establishing a diverse and functional T cell repertoire that can recognize a wide variety of pathogens (Bedekar *et al*., 2022).

2.5.3.3 Spleen

The spleen of fish is a central lymphoid organ involved in both innate and adaptive immunity. The spleen is mostly composed of lymphoid and hemapoietic cells, and it is the primary peripheral and secondary lymphoid organ in fish (Mokhtar *et al*., 2023; Sun *et al*., 2024). It is believed to be involved in the production of blood cells and the immunological response. The spleen in fish is generally small, reddish, and located close to the stomach (He *et al*., 2021; Zapata, 2024). The red pulp is responsible for blood filtration and the removal of aged or damaged red blood cells. In fish, this process is important for maintaining blood quality and responding to injury or infection (Klei *et al*., 2017). While the white pulp is composed of lymphoid tissue, including lymphocytes, macrophages, and other immune cells (Bjørgen and Koppang, 2022). The fish spleen is an important lymphoid organ that houses large populations of T and B cells. These lymphocytes were crucial for both the cellular and humoral immune response. The spleen supports antigen processing and presentation, lead to increase a specific immune response (Secombes and Wang, 2012; Bjørgen and Koppang, 2022).

2.5.3.4 Liver

The liver in fish plays a multifunctional role in metabolism, detoxification, digestion, and immune responses (Huang *et al*., 2022; Ni *et al*., 2022). It is one of the most important organs in fish, serving as a germinal center for various biochemical processes necessary for survival, growth, and adaptation to the aquatic environment (Secombes and Wang, 2012; Bjørgen and Koppang, 2022). Fish liver structure varies across fish species, but it generally consists of hepatocytes or liver cells arranged in clusters rather than the lobular structure seen in mammals. It typically has a simpler architecture, as fish livers lack the distinct lobules separated by connective tissue that were common in mammalian livers. The fish liver is often brownish or yellowish in color, influenced by diet and fat storage, and is closely associated with the gallbladder, which stores bile produced by the liver. The fish liver is central to the metabolism of carbohydrates, proteins, and lipids. It stores glycogen and lipids, which can be mobilized to meet energy demands, especially during fasting or stress (Ota and Shiojiri, 2022; Zhang *et al*., 2024).

2.6 Adjuvants for fish vaccines

Adjuvants were crucial components of fish vaccines, enhancing the immune response to the vaccine antigens, which may be less robust in aquatic species due to their distinct immune system characteristics (Tafalla *et al*., 2013; Sun *et al*., 2018; Raman *et al*., 2022). Adjuvants help to increase the effectiveness of fish vaccines by prolonging antigen exposure, promoting stronger immune responses, and reducing the number of vaccine doses needed (Wangkahart *et al*., 2023). Basically, adjuvants should be highly stable, capable of being combined with multiple vaccines without causing any side effects. It should also possess the ability to enhance an immune response when used in combination with a vaccine, rather than relying solely on the vaccine itself. Additionally, the use of an adjuvant may reduce the required amount of antigen or the number of immunization doses needed to stimulate the immune system's protective response (Tafalla *et al*., 2013; Wangkahart *et al*., 2023).

2.6.1 Types of adjuvants used in fish vaccines

2.6.1.1 Oil-based adjuvants

Freund's Incomplete Adjuvant (FIA) and Montanide were common oil-based adjuvants used in fish vaccines (Tafalla *et al.*, 2013). These adjuvants create an emulsion that slowly releases the antigen, leading to a prolonged immune response. Oil-based adjuvants stimulate both cellular and humoral immune responses in fish and were effective against a variety of pathogens, including bacteria and viruses. However, oil-based adjuvants can sometimes cause local tissue reactions at the injection site, particularly when administered in large volumes (Mutoloki *et al.*, 2010).

2.6.1.2 Aluminum salts

Aluminum hydroxide is widely used adjuvants in veterinary and human vaccines, and they also hold promise for fish vaccines. They were effective at enhancing antibody production. These adjuvants were generally safe and well stable but they may not be as effective as oil-based adjuvants for some pathogens (Tizard, 2021; Du *et al.*, 2022).

2.6.1.3 Liposome-based adjuvants

Liposomes were spherical vesicles that can encapsulate antigens, protecting them from degradation and enhancing uptake by immune cells (Li *et al.*, 2020). Liposome-based adjuvants can mimic cellular structures, making them highly effective for delivering antigens to mucosal surfaces, such as gills and skin, which were key entry points for pathogens in fish. These adjuvants were advantageous for immersion or oral vaccines, providing targeted immune stimulation with lower risk of local tissue reactions (Tafalla *et al.*, 2013).

2.6.1.4 Mineral-based adjuvants

Calcium phosphate and other mineral-based adjuvants can help stimulate both cellular and humoral immune responses. These adjuvants were known for their biocompatibility, minimizing inflammatory responses while promoting antigen presentation and immune activation (Tafalla *et al*., 2013). While oil-based adjuvants remain more popular, there is growing interest in mineral adjuvants as a safer solution, especially for fish that show sensitivity to injection reactions (Wangkahart *et al*., 2021).

2.6.1.5 Emulsion-based adjuvants

Water-in-oil (W/O) and water-in-oil-in-water (W/O/W) emulsions were a widely used type of adjuvant in vaccines designed to enhance the immune response by facilitating antigen delivery and promoting prolonged immune stimulation. These adjuvants were typically made by dispersing one liquid phase into another to form stable droplets that remain suspended, often oil-in-water (O/W) or water-in-oil (W/O) emulsions (Tafalla *et al*., 2013; Li *et al*., 2020). The stability and structure of emulsion-based adjuvants allow them to control the release of antigens, attracting immune cells to the injection site and promoting antigen uptake (Wangkahart *et al*., 2023).

2.6.1.6 MONTANIDE™ IMS

MONTANIDE™ IMS is a range of ready-to-dilute adjuvants. Those formulations were a combination of micro-emulsions, for which the size can vary from 10 to 500 nm, and an immunostimulating compound. They can contain a low amount of oil and were commercially available in preserved (PR) or sterilized (ST) grades. This range is suitable for a wide range of antigens (bacterial, viral, parasitic or subunit). This range is recommended when safety is the main concern. Those adjuvants can be proposed to induce a rapid immune response with a strong sustainability especially in case of two shots vaccination protocol. For vaccine preparation, MONTANIDE™ IMS has been designed to render stable and fluid vaccines. Vaccine formulations were obtained by an easy dilution of the aqueous

medium into MONTANIDE™ IMS, at room temperature or less, under gentle agitation (e.g. marine propeller, magnetic stirrer). The ratio of use can be adjusted from 15% to 50%, depending on the selected MONTANIDE™ IMS, and on the expected safety and efficacy balance.

The development of the micro-emulsion MONTANIDE™ IMS 1312 was done specifically for the immersion vaccination of fish (Seppic, France; Soltani *et al*., 2014) which shows that this type of vaccination stimulates positive immune responses and provides excellent protection for a variety of fish species, including olive flounder (*Paralichthys olivaceus*) (Hwang *et al*., 2017), rainbow trout (*Oncorhynchus mykiss*) (Skov *et al*., 2018), and Nile tilapia (*O. niloticus*) (Oliveira *et al*., 2022). The MONTANIDE™ IMS 1312, which is appropriate for mass vaccination through immersion, is made up of water-dispersed liquid nanoparticles that act as vaccine delivery. Adjuvants can cause long-lasting effector responses, such as the production of antibodies or cytotoxic T cell activity, to occur earlier and eliminate the need for booster shots. Aqueous adjuvants can improve antigen uptake via the fish surface, skin surfaces, and gills, which can improve the cellular and humoral immune responses of fish (O'Hagan and Singh, 2003; Soltani *et al*., 2014).

Chapter 3
Materials and Methods

3.1 Ethical statement

The animal used in the present research followed the regulation of Institute of Animals for Scientific Development (IAD) of Thailand. The protocols of the present study have been approved by Mahasarakham University ethics committee (IACUC-MSU-39/2024).

3.2 The experimental fish

Nile tilapia (weighing approximately 10 g), were purchased from a commercial farm in Roi-Et, Thailand. The experimental fish were rewered in 500-liter fiberglass tanks for 14 days for acclimatization. Fishes were fed a commercial feed twice daily at 5% body weight. Water quality was monitored and measured throughout the experiments: the temperature was 26 ± 1 °C, dissolved oxygen was 5.5 ± 0.3 mg/L, ammonia nitrogen less than 0.05 mg/L, and pH was 7.7 ± 0.1, respectively.

3.3 Bacterial strain and vaccine preparation

S. agalactiae was isolated from the diseased tilapia and used for vaccine preparation and challenge testing (Pholchamat *et al*., 2024). The *S. agalactiae* inactivated whole-cell vaccine (SAIV) was prepwered according to our previous protocol (Wangkahart *et al*., 2023). Briefly, *S. agalactiae* were inoculated into Brain Heart Infusion (BHI) broth (Difco, USA) with shaking at 180 rpm at 30 °C for 12 h. After that, the bacterial culture was inactivated by adding 2% formalin solution (v/v) at 4 °C for 48 h, and the death of bacteria determined by the absence of growth on BHI agar plates after 48 h of incubation at 30 °C. The inactivated cells were centrifuged (5,000 rpm for 10 min at 4 °C), and washed 3 times with PBS. The inactivated cells were resuspended in PBS and adjusted to a final concentration of

1×10^9 colony forming units (CFU)/mL (an absorbance of 0.67 at a wavelength of 600 nm) using spectrophotometer (Amersham Biosciences) (Wangkahart *et al*., 2022).

3.4 Adjuvants and vaccine formulation

An adjuvant for immersion, MONTANIDE™ IMS 1312 were kindly provided by SEPPIC (France). The SAIV was formulated with MONTANIDE™ IMS 1312 according to the procedure described by SEPPIC. In brief, 200 mL of SAIV (containing 1×10^8 cells/mL) dissolved in PBS or 200 mL of MONTANIDE™ IMS 1312 was mixed using the T25 easy clean digital (IKA, Germany) machine. The mixture was then diluted in 3600 mL of water. As a control, 400 mL PBS was diluted in 3600 mL of water.

3.5 Vaccine safety and side effects

To analysis whether the adjuvanted vaccine caused any adverse effects *in vivo*, an evaluation of skeletal malformations in fingerling Nile tilapia was conducted at day 14 post immersion vaccination. Clearing and staining for osteological studies of whole fishes were studied. Briefly, 10 fish from each group were fixed in 10% formalin solution for 2 days, descaled, and washed thoroughly. Fish were soaked in a solution of 3% hydrogen peroxide (H_2O_2) and 2% potassium hydroxide (KOH) in a 9:1 ratio for 2 days until pale whitish. After changing the solution, fish were soaked once again in alcian blue to dye the cartilage for 2 days, followed by soaking in 2% KOH until the backbone was visible. The hard bone was stained with alizarin red S, then the fish were soaked in glycerin solutions mixed with 0.5% KOH in ratios of 1:3, 1:1, and 3:1, each for 2 days, and finally in 100% glycerin to preserve the fish sample. Skeletal malformations were observed under a stereo microscope.

In order to evaluate the effect of the adjuvants used on fish growth, fish in each experimental group were weighed at 0, 1, 2, 3, and 4 weeks post vaccination (w.p.v.) and then calculated the specific growth rate (SGR) using the following formula: SGR; %/day) = 100 × (ln final body weight of fish − ln initial body weight of fish)/days. Where ln is the natural logarithm to the base e of a number.

3.6 Fish vaccination and blood sampling

The experiment fish was divided into 3 groups (90 fish per group), as detailed in Table 2. Fish were vaccinated by immersion for 2 min. Group 1: fish vaccinated with PBS (CTRL); Group 2: fish vaccinated with SAIV alone (SAIV); and Group 3, fish vaccinated with SAIV containing MONTANIDE™ IMS 1312. Following immunization, whole blood was collected at 1, 2, 3, and 4 w.p.v. for study of the innate and adaptive immune response from 8 fish per group.

Table 2 The experimental treatment groups and vaccination formulations

Group No.	Treatment	Abbreviation
1	Immersion with phosphate buffered saline (PBS) fish (unvaccinated fish)	CTRL
2	Immersion of with *S. agalactiae* inactivated vaccine alone	SAIV
3	Immersion of with *S. agalactiae* inactivated vaccine and MONTANIDE™ IMS 1312	SAIV + IMS 1312

3.7 Evaluation of humoral immune response

3.7.1 Analysis of innate immune response

3.7.1.1 Lysozyme activity (LZM)

The serum samples of fish was performed with some modification tested in a 96-well plate, LZM activity was determined as per method (Wangkahart *et al*., 2024). The solution containing the bacteria, *Micrococcus lysodeikticus* was added and incubated at room temperature and measured OD at 450 nm wavelengths with the iMark^{TM} microplate absorbance reader at 30 s (A1) and 180 s (A2). The lysozyme activity was calculated from the formula (A1-A2/2.5) ×1000, in unit of U/mL.

3.7.1.2 Myeloperoxidase activity (MPO)

The serum samples of fish were tested in a 96-well plate, MPO activity was evaluation as modified by the earlier method (Wangkahart *et al*., 2024). Then the PBS, TMB-Blotting Substrate Solution, and hydrogen peroxide (H_2O_2) were added. The color change reaction was stopped after 2 min by using H_2SO_4 solution, and then the OD was measured at 450 nm wavelengths by the iMark^{TM} microplate absorbance reader. The myeloperoxidase activity was calculated from the formula MPO activity = absorbance OD at 450 nm.

3.7.1.3 Catalase activity (CAT)

The serum sample of fish was prepwered and put in a 96-well plate, CAT activity was evaluation as modified by the earlier technique (Wangkahart *et al*., 2024). Then the solution containing hydrogen peroxide (H_2O_2) and PBS was added and then measured OD at 240 nm wavelengths by the iMark^{TM} microplate absorbance reader at 20 s (A1) and 80 s (A2). The catalase activity was calculated from formula A1-A2/0.0008, is in U/mL.

3.7.1.4 Superoxide dismutase activity (SOD)

The serum samples of fish were tested in a 96-well plate, SOD activity was examined by modification of the previous method (Wangkahart *et al*., 2024). The carbonate-bicarbonate buffer solution and the epinephrine solution was added. The control group of the reaction was prepwered from the solution of epinephrine dissolved in the carbonate-bicarbonate buffer. The sample was then measured for OD at 490 nm by the iMarkTM microplate absorbance reader for 30 s and 90 s. The SOD activity was calculated from the formula: percent of inhibition (%) = 100 - [(Δcontrol -Δsample)/(Δcontrol) x 100] so %inhibition x 3.75, in U/mL

3.7.2 Analysis of adaptive immune response

3.7.2.1 Enzyme-linked immunosorbent assay (ELISA)

In order to analyze the adaptive immune response post-vaccination, the IgM antibody levels against *S. agalactiae* were conducted using ELISA technique, followed by the methods as described previously (Wangkahart *et al.*, 2019). Briefly, 96-well plate were coated with 1.0×10^8 CFU/mL *S. agalactiae* in 50 µL/well coating buffer (pH 9.0, 100 mM $NaHCO_3$, 12 mM Na_2CO_3) at 37 °C for 2 h. After incubation at 37°C for 2 h, the 96-well plate was washed twice with washing buffer (1X phosphate-buffered saline with Tween® detergent (PBST)). Then, 50 µL of fish serum samples were diluted at 1:256 in PBST and added into the 96-well plate. The plates were then incubated overnight at 4°C. After that, the plates were washed once again for three times and the mouse anti-Nile tilapia IgM monoclonal antibody (Vertebrate antibodies limited, UK) was added (50 µL/well) and incubated at 37 °C for 2 h. After washing, the anti-mouse IgG labeled with horseradish peroxidase (HRP), diluted 1:2,000 in PBST was added and incubated at 37 °C for 1 h. After washing, 50 µL of TMB was added and incubated at room temperature for 20 min. Finally, 50 µL of 0.5 M H_2SO_4 was added to stop the reaction. The IgM antibody levels against *S. agalactiae* was measured using an iMark™ Microplate Reader at 450 nm.

3.8 Evaluation of the cell-mediated immune response post vaccination

3.8.1 IgM^+ B cell densities by immunohistochemical (IHC) analysis

The IHC labeling analysis was investigated by using the Power-Stain™ 1.0 Poly HRP DAB Kit (Genemed, USA). At 21 days post-challenge, the spleen tissues were sampled from three fish from each group. The tissues were fixed in 10% formalin, embedded in paraffin, sliced sections into 4.5 µm, and stained with hematoxylin. Sections were deparaffinized in xylene and rehydrated in lab grade ethanol, followed by dH_2O. Slides were then pre-treated with 1.5% H_2O_2 for 10 min to block endogenous peroxidase activity, followed by microwave treatment for antigen retrieval. Slides were washed and blocking protein by casein blocking solution for 30

min was conducted. A protein block was applied prior to the primary antibody, Nile tilapia anti-IgM, incubation for 60 min, and it was rinsed twice with washing buffer. A secondary antibody appropriate to each primary antibody was applied for 15 min, followed by a polymer and DAB Chromagen prior to counterstaining with hematoxylin, dehydration, and a coverslip. Slide analysis and image acquisition was carried out under a light microscope.

3.8.2 Gene expression analysis by quantitative real time polymerase chain reaction (RT-qPCR)

The liver, spleen, gills, and intestine were collected from four fish from each group at 1, 3, 7, and 14 days post-vaccination (d.p.v.). Total RNA was extracted using TRI reagent, cDNA synthesized and immune-related gene expression performed by RT-qPCR as described previously (Khoklang *et al*., 2024). RT-qPCR was run in the CFX Connect Real-Time PCR Detection System. The solution of master mix for the PCR reaction was prepwered from Platinum™ Taq DNA Polymerase, and SYBR green (Invitrogen) was used as detector. The gene-specific primers were listed in Table 3. The expression of each gene was initially normalized to that of β-actin, and then presented as a fold change by calculating the average expression level of the treated samples divided by that of time-matched controls. The expression level was presented as fold change, by calculating the transcription level of vaccines groups divided by that of the CTRL group.

Table 3 Primers used for RT-qPCR analysis

Gene	Accession No.	Primer	Nucleotide sequence (5′ to 3′)	Size (bp)	Annealing Temp (°C)
A housekeeping gene					
β-actin	MM003443127	Fw	ACAGGATGCAGAAGGAGATCACAG	155	60
		Rv	GTACTCCTGCTTGCTGATCCACAT		
Immune-related genes					
IL-1β	FF280564	Fw	AAGATGAATTGTGGAGCTGTGTT	175	60
		Rv	AAAAGCATCGACAGTATGTGAAAT		
IL-6	XM_019350387	Fw	ACAGAGGAGGCGGAGATG	149	60
		Rv	GCAGTGCTTCGGGATAGA		
IL-8	NM001279704	Fw	GCACTGCCGCTGCATTAAG	135	59
		Rv	GCAGTGGGAGTTGGGAAGAA		
IFN-γ	NM_001287402	Fw	AGGGTGATCTGCGGGAATACT	139	60
		Rv	GCCCAGGTAAATGGCGTTGT		
Bacteria identification gene					
S. agalactiae	CP051004.1	Fw	AGAGTTTGATCCTGGCT	192	47
		Rv	AAGGGAGGTGATCCAGCCGCA		

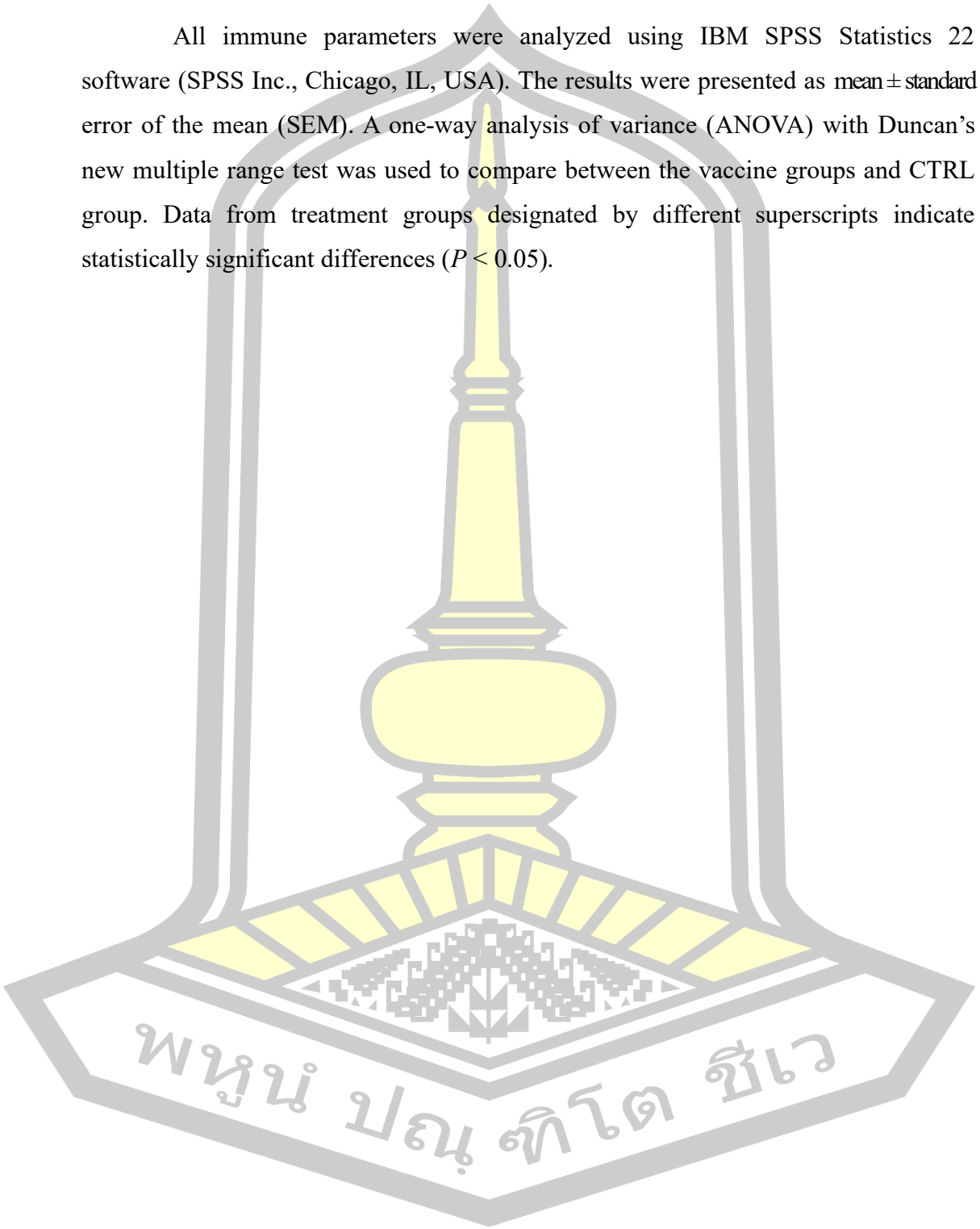
Abbreviations: β-actin: beta actin; IL-1β: interleukin 1 beta; IL-6: interleukin 6; IL-8: interleukin 8; IFNγ: interferon gamma; Fw: forward; Rv: reverse; Temp: temperature.

3.9 Disease resistance against *S. agalactiae* infection

Following immersion immunization, fish from each group were randomly separated into three replicates (15 fish/replicate) and challenged for 2 h by the immersion method as described previously (Pholchamat *et al*., 2024). Fish were challenged with 1×10^8 CFU/mL of *S. agalactiae* in a 20-L glass tank under water temperature maintaining at 32°C using a heater-controlled system (JBL Aquarium Heater, Germany). The fish were then returned into 500 L aerated fiberglass tanks and the cumulative mortality was then observed for 21 days. Dead fish were collected daily, and *S. agalactiae* was confirmed the cause of death by microbiology method. The relative percent survival (RPS) was calculated using the formula: RPS (%) = [1 - (% mortality of the vaccinated group) / (% mortality of the CTRL group)] × 100.

3.10 Statistical analysis

All immune parameters were analyzed using IBM SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA). The results were presented as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) with Duncan's new multiple range test was used to compare between the vaccine groups and CTRL group. Data from treatment groups designated by different superscripts indicate statistically significant differences ($P < 0.05$).



Chapter 4
Results

4.1 Assessment of vaccine safety and fish growth

No mortality was observed during the experimental period, and no abnormalities were seen during 14 d.p.v. Additionally, there were no signs of acute or chronic effects in any of the fish groups of vaccine safety and side effects. In addition, fish vaccinated with CTRL, SAIV alone, and SAIV+IMS1312 did not affect the skeletal malformation post vaccination (Fig. 1). There were no significant differences in fish growth in terms of SGR across all groups (Fig. 2).

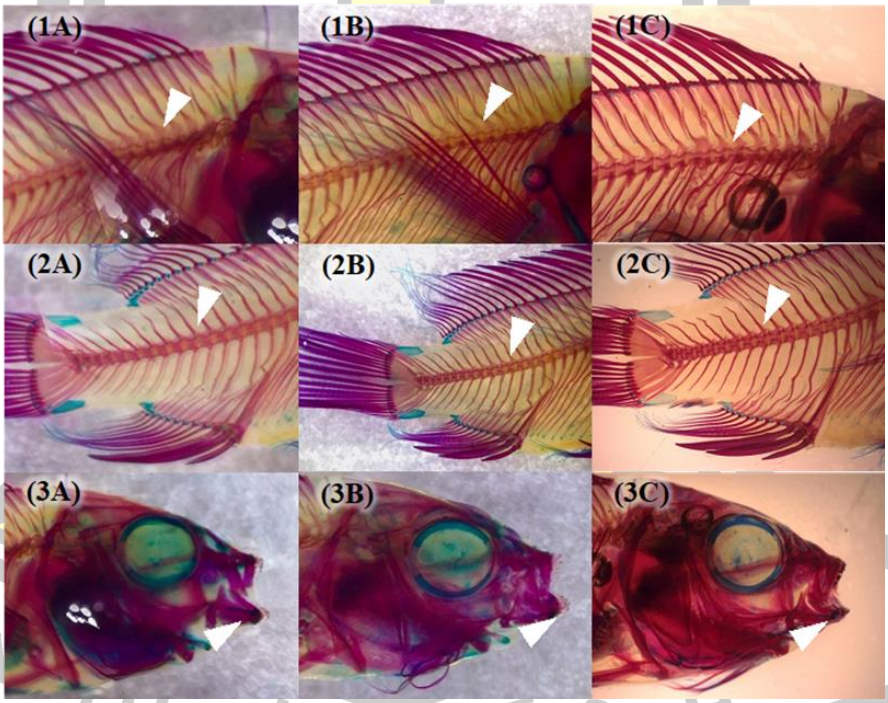


Figure 1 The skeletal malformation of the fish post-vaccination.

1–3 A) Control group (CTRL); 1–3 B) SAIV alone; 1–3C) SAIV + IMS1312: (1A, 1B, 1C) normal juveniles with normal vertebrae; (2A, 2B, 2C) normal vertebrae centrum; (3A, 3B, 3C) normal jaw and anomalous dentary. The white arrows point at the different types of skeletons.

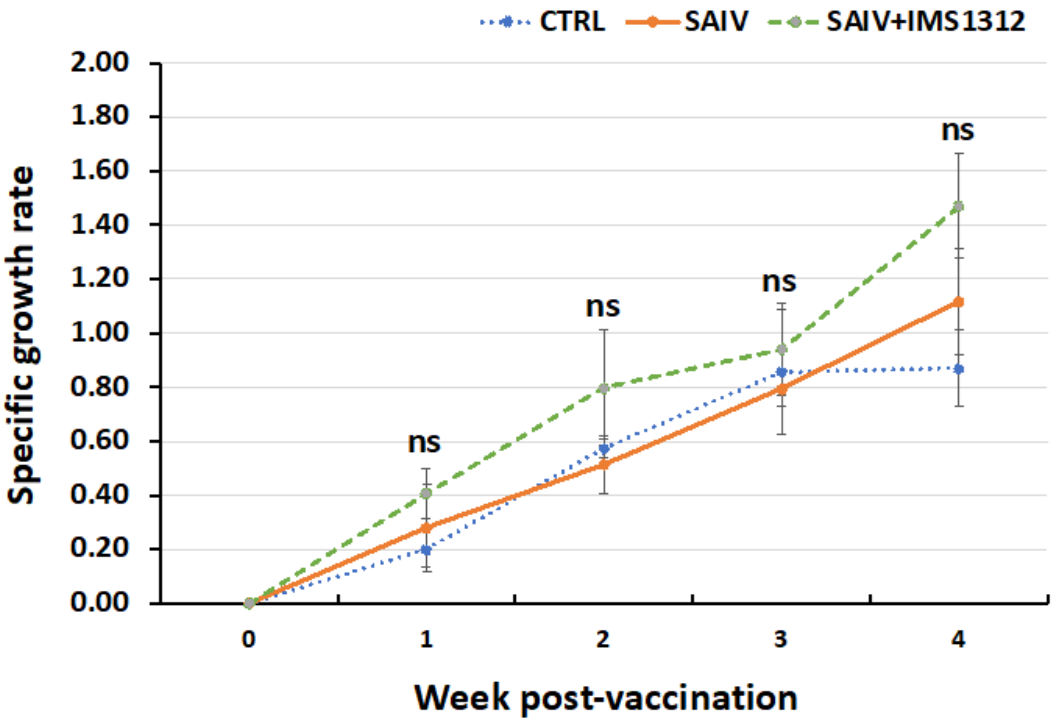


Figure 2 Growth performance of Nile tilapia after immersion vaccination for 4 weeks.

Fish were vaccinated with the SAIV alone, SAIV + IMS1312, and PBS as a control (CTRL). The results represent the mean ± SEM from 90 fish per diet at each time point. Values not statically different are indicated by "ns" ($P > 0.05$)

4.2 The humoral immune response post vaccination

4.2.1 Innate immune response and antioxidant enzyme capacity

The effects of MONTANIDE™ IMS 1312 on innate immune response were summarized in Fig. 3. The results showed that significantly higher LZM activity was observed in fish vaccinated with SAIV+IMS1312 from 1 to 3 w.p.v. compared to the CTRL group (Fig. 3A). The activity of MPO (Fig. 3B) and CAT (Fig. 3C) from the SAIV+IMS1312 group were significantly increased at 1-2 w.p.v. Moreover, the SOD activity from the SAIV+IMS1312 group was also significantly increased at 1 w.p.v. and 3 w.p.v., respectively (Fig. 3D).

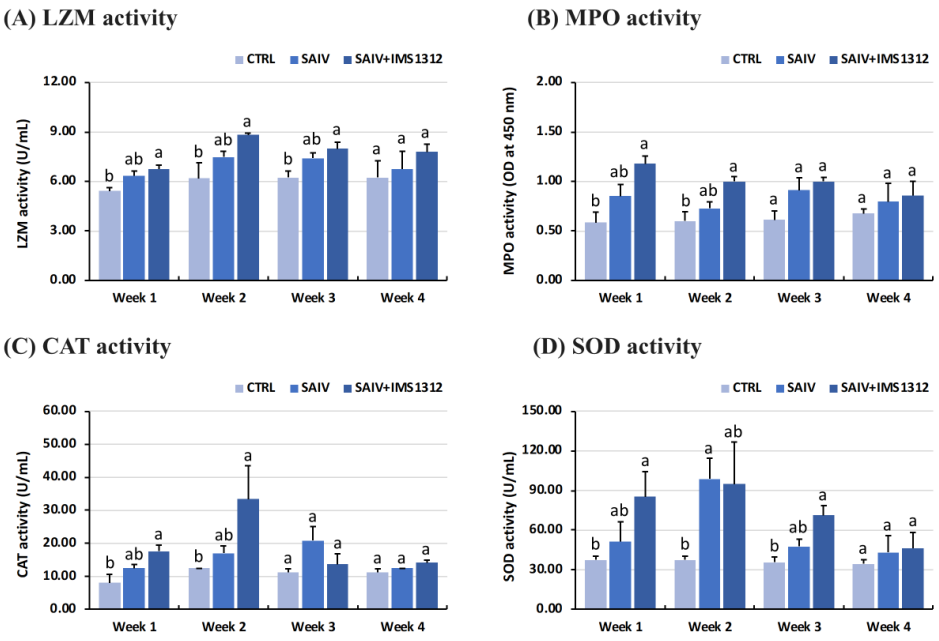


Figure 3 Serum innate immune parameters in Nile tilapia that have been vaccinated. Innate immune parameters in fish immunized with the SAIV alone, SAIV+IMS1312, or PBS as control (CTRL). Fish sera were collected at weeks 1, 2, 3, and 4. Eight biological replicates were used per group, and data expressed as the mean + SEM. (A) LZM activity, (B) MPO activity, (C) CAT activity, and (D) SOD activity. Bars with different letters indicates a significant difference ($P < 0.05$) between the experimental groups at each time point.

4.2.2 The specific IgM antibody levels against *S. agalactiae* by ELISA

The specific IgM antibody levels were shown in Fig. 4. We found that the IgM antibody level against *S. agalactiae* were significantly higher at 3-4 w.p.v. in fish vaccinated with the SAIV+IMS1312 compared with the CTRL group ($P < 0.05$). However, no significant differences were found between sera from the SAIV and CTRL groups. These data implied that adjuvanted vaccines elicited an increased humoral immune response to *S. agalactiae* than SAIV alone.

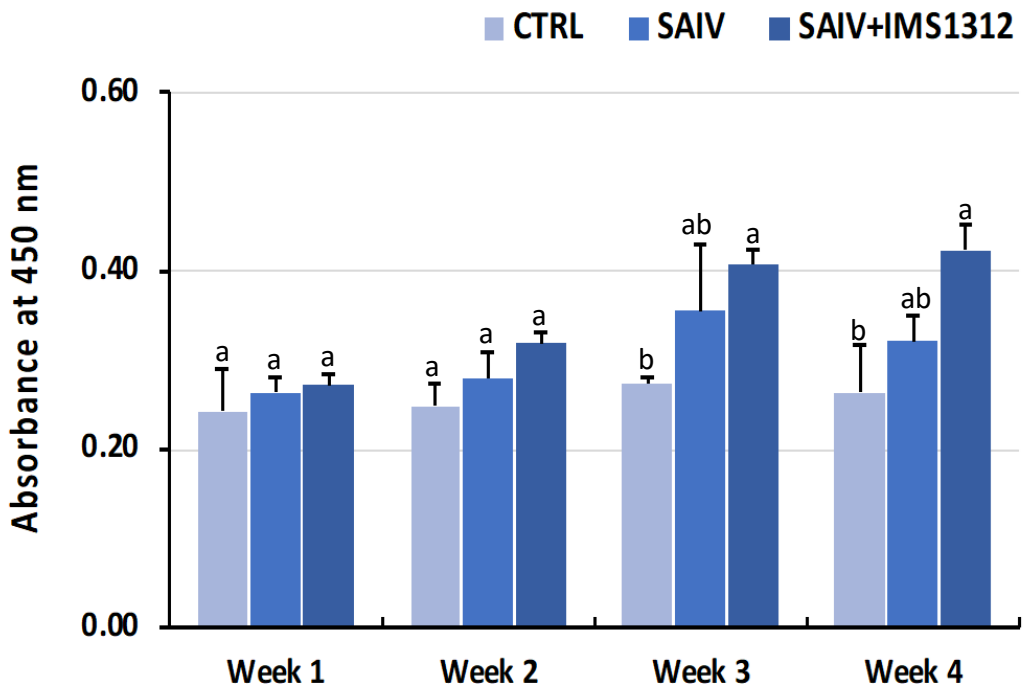


Figure 4 The specific IgM response of Nile tilapia after vaccination against *S. agalactiae*, as determined by ELISA.

Fish were vaccinated with the SAIV alone, SAIV+IMS1312, and PBS as a control (CTRL). Fish sera were collected at 1, 2, 3 and 4 w.p.v. Eight biological replicates were used per group and data expressed as the mean □ SEM. Bars with different letters indicate a significant ($P < 0.05$) difference between the different groups at each time point.

4.3 The cellular immune response post vaccination

4.3.1 Gene expression analysis by RT-qPCR

The effects of MONTANIDE™ IMS 1312 on the expression level of pro-inflammatory cytokine genes were depicted in Fig. 5. In the spleen, fish vaccinated with SAIV+IMS1312 showed a significant increase in the expression of IL-6 and IL-8 at 1 d.p.v. ($P < 0.05$). At 3 and 7 d.p.v., with the exception of IL-6, the expression levels of these cytokine genes were significantly higher in fish vaccinated with SAIV+IMS1312 relative to the CTRL. An increase in IL-8 expression was also seen at 14 d.p.v. in the SAIV+IMS1312 group. Interestingly, all cytokine gene expression were significantly increased in the intestine at 7 d.p.v., particularly fish of SAIV+IMS1312 group ($P < 0.05$). In the gills, the highest induction was also seen at 1 d.p.v. Fish vaccinated with SAIV and SAIV+IMS1312 showed higher level of IL-6 compared to the CTRL group. The expression of IL-β, IL-6, and IL-8 was significantly higher at 3 d.p.v. in both vaccine groups and returned to basal levels by day 7 to day 14 post vaccination.

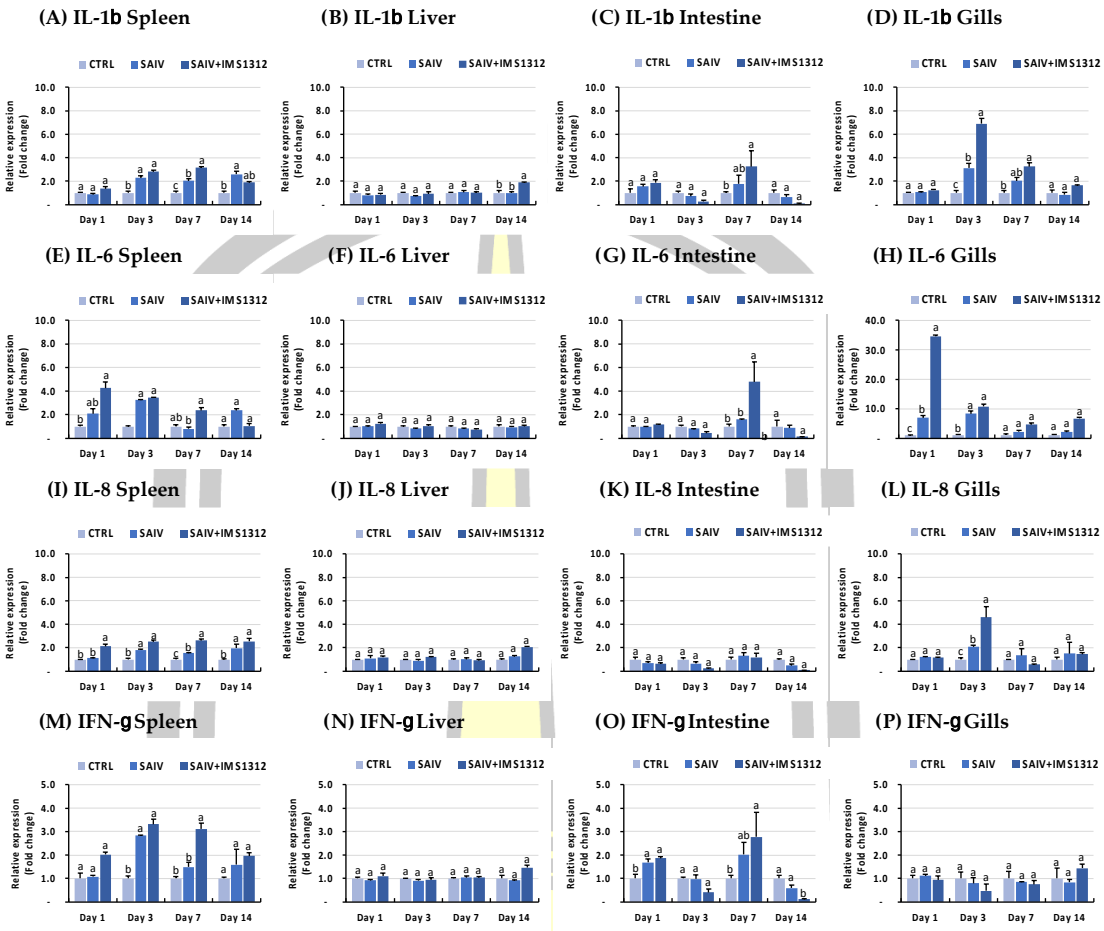


Figure 5 Gene expression of IL-1β, IL-6, IL-8, and IFN-γ was evaluated by RT-qPCR analysis.

Fish were vaccinated with the SAIV alone, SAIV+IMS1312, and PBS as a control (CTRL). Four immune-related tissues (the spleen, liver, intestine, and gills) were sampled on days 1, 3, 7, and 14 after immunization. Four biological replicates were used per group and data expressed as the mean + SEM. Bars with different letters indicate a significant ($P < 0.05$) difference between the different groups at each time point.

4.3.2 Densities of IgM^+ B cell by IHC analysis

Spleen tissue from all of the vaccinated fish groups were examined for the presence of IgM^+ B cells using IHC analysis (Table 4). After vaccination and post challenge, significant changes of the IgM^+ B cells were observed. The spleens from the SAIV+IMS1312 group had been detected more IgM^+ B cells post vaccination compared to CTRL group. Also, following the challenge, all fish exhibited a more detectable IgM^+ B cells. In the CTRL and SAIV groups, these levels reached the pre-challenge values observed in the SAIV+IMS1312 group. All challenged fish showed a higher incidence of MCC compared to unchallenged fish (Fig. 6).

Table 4 The relative immune cell densities of IgM^+ B cells and melanin-containing cells in the spleen tissue of fish that were immersed in PBS (CTRL), SAIV alone, and SAIV+IMS1312 (post-challenge) were determined by IHC staining.

Cell-type/time point	**CTRL**	**SAIV**	**SAIV+IMS1312**
IgM^+ cells			
Post-challenge	+	++	+++
Melanin containing cells (MCCs)			
Post-challenge	+	++	+++

Note: Average number of IgM+ B cells [(+) 1-10 positive cells, (++) 11-20 positive cells and (+++) ≥21 positive cells], and melanin containing cells (MCCs) [(+) 1-50 MCCs, (++) 51-100 MCCs and (+++) >100 MCCs] in the spleen.

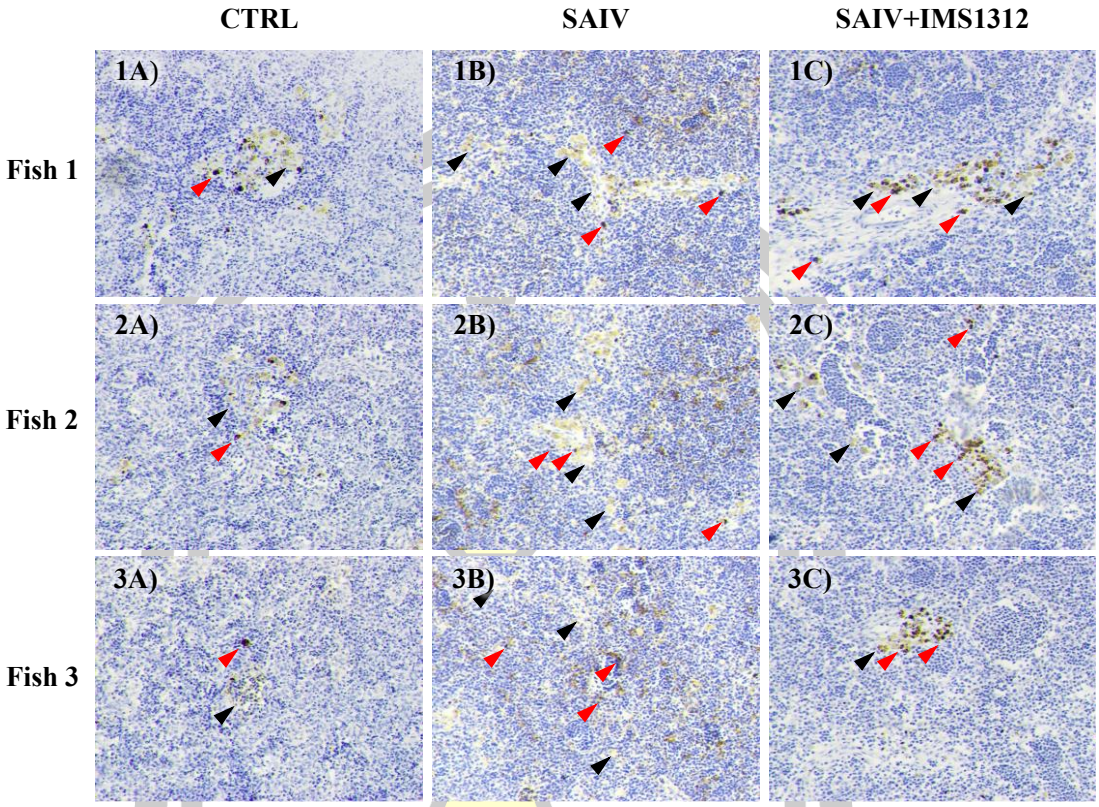


Figure 6 Immune cell densities (IgM+ B cell) of spleen from fish vaccinated with PBS (CTRL), SAIV alone, SAIV+IMS1312 (post-challenge), as assessed by immunohistochemical staining.

Photomicrographs showing the presence of IgM^+ B cells and melanin containing cells (MMCs) at 40× magnification. 1-3 A) Control group (CTRL); 1-3 B) SAIV alone; 1-3 C) SAIV+IMS1312. The black arrowheads show IgM^+ B cells and the red arrowheads show MCCs. N = 3 fish per group.

4.4 Protective efficacy of a vaccine against bacterial challenge

The effects of MONTANIDE™ IMS 1312 on the protective efficacy of Nile tilapia were presented in Fig. 7. The cumulative mortality was recorded for a period of 21 days. The survival rate of CTRL was 2.10%, whereas in the case of the SAIV and SAIV+IMS1312 groups, it was relatively higher at 37.80% and 51.10%, respectively. No significant differences were found between the SAIV+IMS1312 group and the SAIV alone group, both of which provided significantly higher protection than the CTRL group ($P < 0.05$).

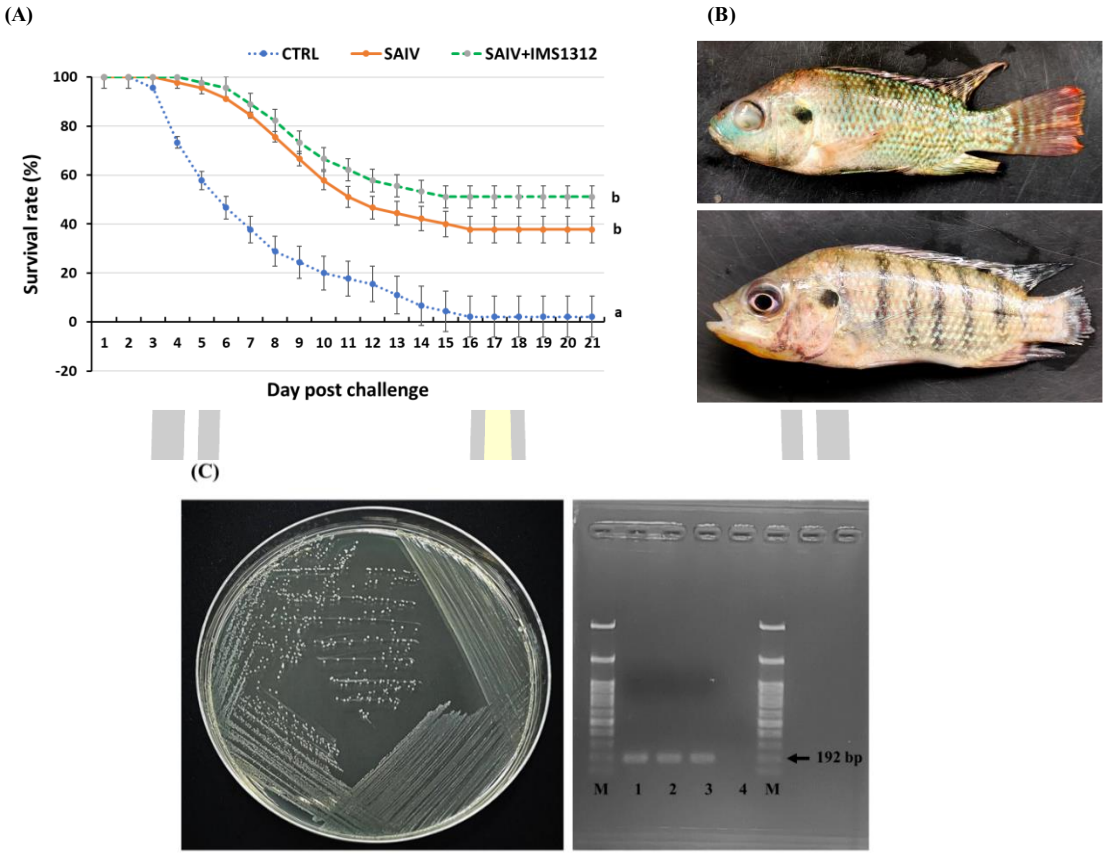


Figure 7 Protective efficacy of vaccinated Nile tilapia for 21 days post-challenge.

Fish were vaccinated with PBS as control (CTRL), SAIV alone, SAIV+IMS1312, respectively. Survival was analysed for 21 days post-challenge, when RPS values were calculated. The experiment was conducted in triplicate. Error bars represent standard deviations. (A) the survival rate of all vaccinated groups, and (B) clinical sign of streptococcosis in Nile tilapia; (C) bacterium colony and agarose gel shows PCR products of *S. agalactiae* from spleen tissues (Lane M: 1-kb molecular weight marker (BIO-HELIX, Taiwan), Lane 1: Isolates of *S. agalactiae* form CTRL, Lane 2: Isolates of *S. agalactiae* form SAIV, Lane 3: Isolates of *S. agalactiae* form SAIV+IMS1312, Lane 4: negative control) of Nile tilapia after immersion challenge with *S. agalactiae*.

Chapter 5
Discussion and Conclusions

5.1 Discussion

S. agalactiae is a significant pathogen in many fish species, particularly in Nile tilapia (Kannika *et al.*, 2017). Vaccination has become the most viable strategy for preventing infectious diseases in the aquaculture (Adams, 2019). To date, a variety of fish vaccines have been developed and demonstrated effective protection against pathogen infection. Each type of vaccine offers several advantages and applications depending on the target pathogen (Bøgwald and Dalmo, 2019). Immersion vaccination is a convenient method, fast, and stress-free vaccination, which is ideal for small fish. Compared to injection vaccination, which is more suitable for larger fish. Adjuvants also serve as vaccine delivery systems, improving vaccine efficacy and generating a more robust immunological response. MONTANIDE™ IMS 1312 is a micro-emulsion adjuvant that has been developed and is suitable for immersion vaccination. In this research, we evaluated how well SAIV combined with MONTANIDE™ IMS 1312 as an adjuvant stimulates the immune response and provides protection in Nile tilapia. To the best of our knowledge, the use of micro-emulsion adjuvant for immersion vaccination in Nile tilapia has not yet been investigated.

In this study, the safety and side effects of the adjuvanted vaccine on fish growth were investigated. It has been shown that post-vaccination with the adjuvanted vaccine has no side effect on fish growth or bone malformation. This result indicated that SAIV combined with MONTANIDE™ IMS 1312 was safe and did not impact any side effects during vaccination. However, further studies will be warranted to investigate possible side effects post-vaccination.

The innate immune response is crucial in providing the initial line of defense against invading pathogens. It plays a significant role in preventing early infection by recognizing and responding to a wide range of microorganisms. In the present study, such parameters studied included LZM, MPO, CAT, and SOD. We showed that the activity of LZM, MPO, CAT, and SOD in the group of SAIV+IMS1312 was significantly higher compared to the CTRL group ($P < 0.05$). LZM, a key component of innate immunity, is essential for inhibiting the proliferation and invasion of pathogenic microorganisms (Song *et al.*, 2021). MPO serves as a strong antimicrobial substance that contributes to the destruction of bacteria, fungi, and viruses during infections. This enzyme is abundantly present in neutrophils (Castro *et al.*, 2008; Chen *et al.*, 2019). CAT is a crucial antioxidant enzyme that breaks down H_2O_2 to prevent toxic effects. Similarly, SOD is an antioxidant enzyme that converts superoxide molecules into O_2 and H_2O_2, neutralizing toxic reactive oxygen species and protecting cells from oxidative stress (Wang *et al.*, 2016). In agreement with this finding, rainbow trout immunized with a vaccine containing the MONTANIDE™ IMS 1312 showed increased LZM activity than fish immunized with vaccine alone (Soltani *et al.*, 2014). The results demonstrate that using adjuvanted vaccines significantly enhances the innate immune response in Nile tilapia post vaccination. This enhanced the immune response contributing to better protection against *S. agalactiae* infections. Moreover, adjuvanted vaccine has the potential to improve overall health and resistance in fish by effectively boosting the immune system.

The evaluation of specific IgM antibody levels in fish serum has been a widely used method for investigating vaccine effectiveness, as it provides valuable insights into the immune response elicited by the vaccine. Importantly, IgM is a vital molecule of the humoral immune system and acts as the primary serum antibody in teleost fish. It also plays a critical role in the immune response by binding to pathogens and helping to clear them from the fish body. Additionally, IgM activates the complement system, which further enhances the destruction of these pathogens. Because IgM is the first antibody produced in response to infection or vaccination, its levels are a key bioindicator of vaccine effectiveness. Therefore, detectable IgM levels seen is essential marker for evaluating immune function and vaccine protection (Velázquez *et*

al., 2014). Our results showed that the specific IgM antibody levels of fish vaccinated with SAIV+IMS1312 were significantly higher when compared to CTRL groups at week 3 and 4. However, no significant differences were found between the SAIV+IMS1312 and SAIV groups. Importantly, the IgM levels are important indicator of adaptive immune response, which plays a critical role in providing long-term immunity against bacterial infections (Soltani *et al.*, 2014; Ramos-Espinoza *et al.*, 2020; Ke *et al.*, 2021; Queiróz *et al.*, 2024). These results were supported by the IHC analysis, showing that fish vaccinated with SAIV+IMS1312 had a significantly higher number of IgM^+ B cells compared to those in the SAGV or CTRL groups. This suggests that the SAIV+IMS1312 vaccination was effective in stimulating a production of IgM^+ B cells, which are essential for a robust adaptive immune response. Thus, the findings of this study suggest that vaccination of SAIV formulated with MONTANIDE™ IMS 1312 can boost the production of specific IgM antibodies against *S. agalactiae*.

Although the available markers for cellular immunity in fish remain limited, their investigation is essential for improving our understanding of the role cellular immunity, particularly in the context of vaccine-induced protection. Additionally, this investigation enhances the study of humoral immune responses, allowing for a more thorough evaluation of the immune processes that contribute to fish health and their defense against pathogens. In teleost fish, the spleen and liver are essential tissues for the acute phase response and actively involved in both humoral and cell-mediated immune responses (Zou and Secombes, 2016). Moreover, the gills serve as the primary mucosal surfaces and act as the first line of defense (Nguyen *et al.*, 2015), responding to invading microbes, while the intestine reacts to pathogenic bacteria in the gut lumen. The present study showed that using MONTANIDE™ IMS 1312 in combination with SAIV led to a significant induction of IL-1β, IL-6, and IL-8 expression in both mucosal tissues after vaccination. These biomarkers were used to assess the immune response in teleost fish after vaccination (Solís *et al.*, 2015). Our findings are consistent with recent research in rainbow trout (Soltani *et al.*, 2014). However, despite the various experimental conditions, no significant changes were observed in the expression levels of pro-inflammatory cytokines in the liver.

The enhanced immune responses and antioxidant enzymes activities of fish serum led to strong disease resistance against pathogen infection. In this study, we showed that fish vaccinated with SAIV+IMS1312 can significantly enhance the disease resistance against *S. agalactiae* challenge. The RPS of fish vaccinated with SAIV and SAIV+IMS1312 was 36.4% and 50.0%, respectively. Several studies in other fish species supported our findings (Soltani *et al.*, 2014; Solís *et al.*, 2015; Hwang *et al.*, 2017; Skov *et al.*, 2015; Afsharipour *et al.*, 2021; Lange *et al.*, 2021; de Ruyter *et al.*, 2023). The effectiveness of vaccination is dependent on various factors, such as the concentration of the vaccine, the size of the fish, the length of immune protection, and the nature of the challenge model, which includes the strain, dose, and route of infection. Our study showed that MONTANIDE™ IMS 1312, when combined with SAIV, produced highly effective protection, suggesting numerous potential applications in tilapia aquaculture beyond controlling streptococcal disease.

5.2 Conclusions

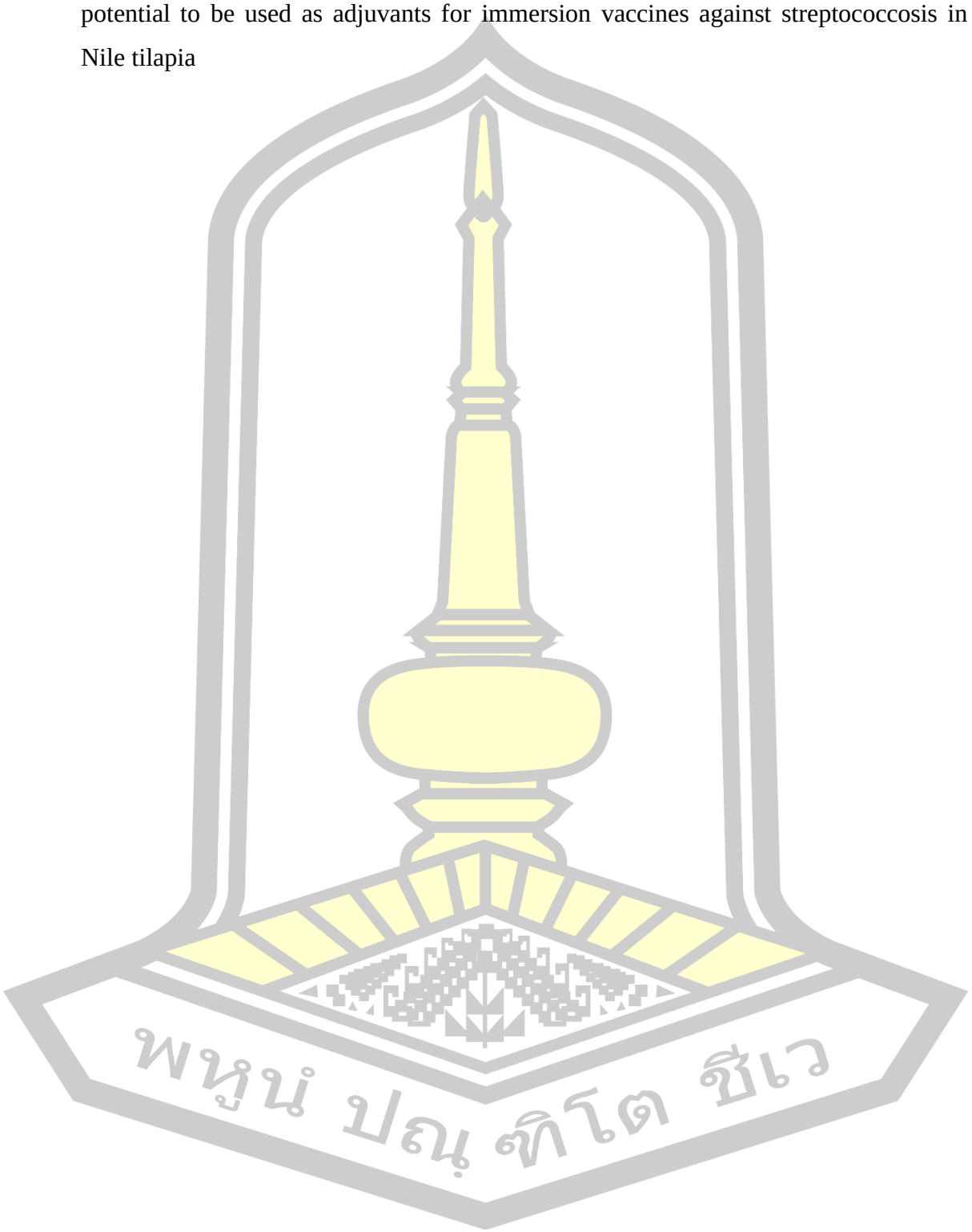
5.2.1 Adjuvanted vaccine had no side effect on fish growth or bone malformation, indicating that SAIV combined with MONTANIDE™ IMS 1312 was safe and did not impact any side effects during vaccination. However, further studies have to be investigate the side effects post-vaccination.

5.2.2 Adjuvanted vaccine has the potential to improve overall health in fish by effectively boosting the immune system. The effectiveness of MONTANIDE™ IMS 1312 adjuvant in combination with a SAIV can induce both innate immune responses of Nile tilapia by immersion vaccination.

5.2.3 Adjuvanted vaccines significantly enhances the adaptive response in Nile tilapia post vaccination, suggesting that vaccination of SAIV formulated with MONTANIDE™ IMS 1312 has the potential to improve health and can boost the production of specific IgM antibodies against *S. agalactiae*.

5.2.4 MONTANIDE™ IMS 1312, when combined with SAIV, produced highly effective protection against *S. agalactiae*, suggesting numerous potential applications in tilapia aquaculture for preventing streptococcal disease.

5.2.5 To the best of our knowledge, MONTANIDE™ IMS 1312 has the potential to be used as adjuvants for immersion vaccines against streptococcosis in Nile tilapia



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