MOLECULAR CHARACTERIZATION AND ADJUVANT EFFECT OF INTERLEUKIN-11 IN THE STREPTOCOCCOSIS FOMALIN-KILLED VACCINE OF THE NILE TILAPIA (*Oreochromis niloticus*)

Phitcharat Sunthamala

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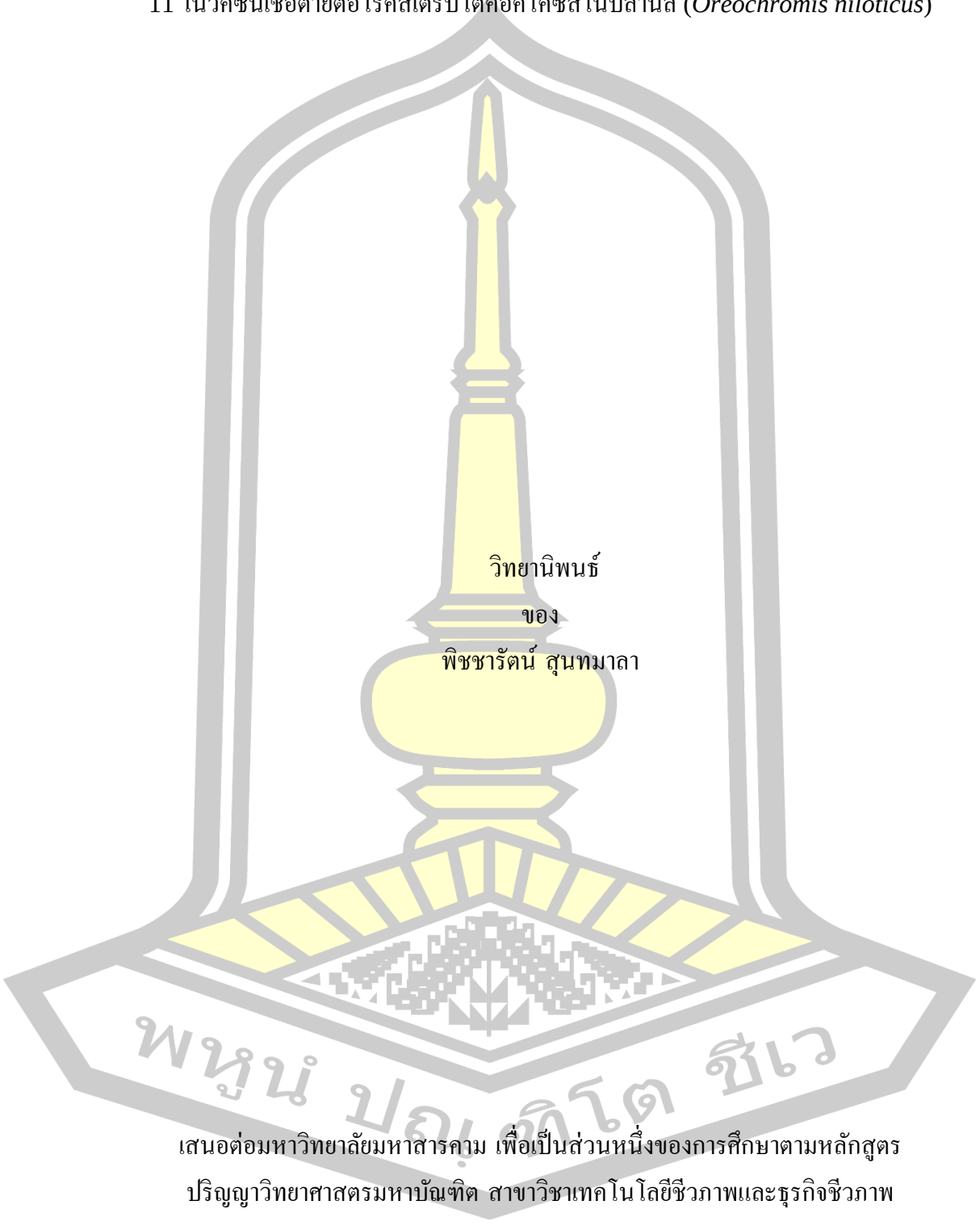
การศึกษาคุณลักษณะทางอณูวิทยาและประสิทธิภาพของการเป็นแอดจูแวนท์ของยีน Interleukin-11 ในวัคซีนเชื้อตายต่อโรคสเตรปโตคอคโคซีสในปลานิล (*Oreochromis niloticus*)



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ABSTRACT

Interleukin-11 is an active signaling molecule in the host immune response to infection and the cytokine network. In this study, IL-11a and IL-11b were studied from the Nile tilapia (*Oreochromis niloticus*). The open reading frame of Nile tilapia IL-11a and IL-11b contained 717 and 603 bp, with a calculated molecular mass of 27.4 and 22.9 kDa, and a theoretical p*I* of 9.02 and 8.73, respectively. Both IL-11a and IL-11b consisted of a potential N-linked glycosylation site and a cysteine residue, and their genes are organized similarly, with 5 exons and 4 introns according to phylogenetic analysis, the IL-11a and IL-11b from Nile tilapia were categorically isolated from other IL-11 molecules and classified with those from other species. The tissue distribution of IL-11a in healthy fish was highly expression in spleen, liver, intestine and trunk kidney, while IL-11b showed higher expression levels in the spleen and tail. At 6 hours after infection by *Streptococcus agalactiae*, IL-11b was significantly up-regulated in the gills, intestines, spleen, and liver while IL-11a was significantly up-regulated at the same time but only in the spleen and liver. To provide new insights into the role of IL-11 in fish immune responses, a recombinant protein of IL-11 (rIL-11) was produced in bacterial system. In addition, IL-11a and IL-11b were up-regulated expression in Peripheral blood leukocytes (PBLs) by Lipopolysaccharides (LPS), Polyinosinic:polycytidylic acid (Poly I:C) and rIL-11 stimulation. Moreover, adjuvant effect of rIL-11 in the streptococcosis formalin-killed vaccine of the Nile tilapia was investigated. After the vaccination, the specific IgM antibody level of fish vaccinated with formalin killed cell (FKC) combined with rIL-11 was increased from 1 to 2 week post vaccinated and peak at 2 w.p.v. compared with control group ($P<0.05$). Lysozyme activity (LZM), myeloperoxidase activity, glutathione peroxidase activity and glutathione reductase activity in the fish vaccinated with FKC combined with rIL-11 group was also significantly higher than that of the FKC and control groups ($P<0.05$). The expression of the innate and

adaptive immune gene was up-regulated at early time point examined. Overall, our findings suggested that rIL-11 could be used as vaccine adjuvant, and elicited the potential immune responses post vaccination against *S. agalactiae* infection. This study suggests that rIL-11 could be a potent vaccine adjuvant for Nile tilapia aquaculture.

Keyword : Interleukin-11, Nile Tilapia, Streptococcus agalactiae, Immune Response, Gene Expression

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Introduction

1.1 Introduction

Streptococcus agalactiae, is a Gram-positive bacterium in a wide range of hosts (Garcia *et al*., 2008). Up to the present time, *S. agalactiae* has been isolated from several animal species, including humans and fish (Evans *et al*., 2009). Among fish species, Nile tilapia (*Oreochromis niloticus*) is very sensitive to *S. agalactiae*, so continuous outbreaks of infection have been reported in Nile tilapia aquaculture throughout the world. Nile tilapia is an important fish for the economy, especially in Thailand. However, infectious diseases caused by *S. agalactiae* have been severe in recent years, resulting in great economic losses. Vaccination as a means of controlling diseases is one of the most significant and successful health practices within the aquaculture industry, and has helped to reduce the use of environmental unfriendly antibiotics to control this bacterial disease. Inactivated whole cell vaccines have been widely applied and used successful against bacterial diseases in fish for many years. Formalin-killed cell (FKC) vaccine, which is generally used in aquaculture, is effective against extracellular pathogen in fish. The advantage of inactivated bacterial vaccines is the safety offered by formalin treatment, which is a reliable and cheap way to kill the pathogen (Austin, 2012). FKC vaccine is known to be a strong inducer of humoral immunity in fish (Tu *et al*., 2010). However, the disadvantage of this type of vaccine is that it may require an adjuvant to elicit an adequate immune response.

Cytokines are key regulators of the immune system in all vertebrates. Cytokines drive inflammatory signals that regulate the capacity of resident and newly arrived phagocytes to destroy the invading pathogen (Wang and Secombes, 2013). In higher vertebrates, many cytokines have been proved to be used as adjuvants to enhance protective immunity and improve the efficacy of vaccines, and several cytokines had been reported to be efficient adjuvants for vaccines to obtain satisfactory immune responses. Interleukin (IL)-11 belongs to the IL-6 family that serves to stimulate hematopoietic progenitor cells and exerts a series of important

immunomodulatory effects (Wang *et al*., 2005). IL-11 is produced by many cell types throughout the body and is also an anti-inflammatory cytokine that inhibits the production of proinflammatory cytokines from lipopolysaccharde (LPS) stimulated macrophages (Trepicchio *et al*., 1997). To date, identification of IL-11 gene in fish have been reported in Atlantic halibut (Øvergard *et al*., 2011), rainbow trout (Wang *et al*., 2005), cobia (Tran *et al*., 2019), and Japanese flounder (Santos *et al*., 2008). However, IL-11 has not been cloned and characterized in Nile tilapia. Therefore, this research aims to characterize the structure of IL-11 of Nile tilapia and to investigate the distribution and expression pattern of IL-11 in various tissues and highlight its role during bacterial infection. Also, another goal of this research is to produce a recombinant protein of IL-11 (rIL-11) and investigate its potential role to be a highly effective adjuvant by adding rIL-11 to *S. agalactiae* FKC vaccine. After vaccination, both cell-mediated and humoral immunity responses triggered by rIL-11 were studied using gene expression analysis and measurement of antibody titer by ELISA. In addition, challenge test was performed of vaccinated Nile tilapia and evaluated the effectivity of FKC + rIL-11 vaccine by calculating survival rate of infected fish.

1.2 Objectives

1.2.1 To clone and characterize of IL-11a and IL-11b genes in Nile tilapia

1.2.2 To investigate the distribution and expression pattern of IL-11a and IL-11b in various tissues of healthy Nile tilapia and highlight its role during *S. agalactiae* infection

1.2.3 To produce a recombinant protein of IL-11 (rIL-11) and investigate its potential role to be a highly effective immunostimulant in peripheral blood leucocytes (PBL): *in vitro* study

1.2.4 To investigate its potential role to be a highly effective adjuvant by adding rIL-11 to *S. agalactiae* FKC vaccine: *in vivo* study

1.3 Scope of research

In this study, molecular cloning and characterization of IL-11a and IL-11b in Nile tilapia were investigated. A recombinant protein of IL-11 (rIL-11) was produced in bacterial system and its potential role to be a highly effective immunostimulant *in*

vitro and *in vivo* was investigated. The effectiveness of rIL-11 as adjuvant by adding rIL-11 to *S. agalactiae* FKC vaccine in Nile, both cell-mediated and humoral immune responses triggered by the rIL-11 was evaluated using innate and adaptive immune parameters. In addition, challenge test of vaccinated fish was performed and the effectivity of FKC + rIL-11 vaccine was tested by calculating survival rate of infected fish.

1.4 Expected benefits and research utilization

To the best of this research, molecular characterization and expression analysis of IL-11a and IL-11b in Nile tilapia *in vitro* and *in vivo* were reported for the first time and shed new insight into the function of IL-6 family in fish. The rIL-11 produced may act as adjuvant and may effectively stimulate the immune response against *S. agalactiae* in Nile tilapia. The results of this study may provide basic information that can be used in future research on adjuvant effect of recombinant cytokine in teleost.

Chapter 2
Literature review

2.1 Nile tilapia

Nile tilapia (*Oreochromis niloticus*) is one of the commercial fish species and has increased year by year to provide staple protein source for world demand of fish products (FAO, 2017). This fish species can be grown quickly over a range of temperatures and does not require stringent water quality. Since the 1950s, production of this species has been increasing, in particular in Asia. It is a freshwater fish species for aquaculture and wild stock fisheries. Moreover, among commercial fish species, tilapia aquaculture has one of highest economic impacts worldwide (Kumar and Engle, 2016). It is now recorded to be present in at least 70 different countries globally. Total world production reached 1.5 million tones (MT) in 2003 and was recorded in 2014 as being over 3.6 MT with a total export value around USD 5 billion. Thailand is by far the main producer of farmed Nile tilapia. By 2021 annual production had risen to nearly 97,000 tones.

2.2 The immunity of fish

The fish immune system is physiologically similar to that in higher vertebrates, although there are some differences. Thus, the fish immune system can be divided into the innate and adaptive immune response, with the latter conferring specific immunity.

2.2.1 The innate immune

The innate immune system plays a key role in disease resistance of fish during pathogen infection (Kilpi *et al*., 2013). It is mainly composed of physical barriers, and a variety of cellular and humoral components (Secombes and Wang, 2012). The major cellular components include monocytes, macrophages, neutrophils and cytotoxic cells (Cammarata *et al*., 2012). Phagocytosis is one of the most important cellular processes in innate immunity, and the bacteria are killed after ingestion by production of reactive oxygen species (ROS) or by a range of proteases and hydrolytic enzymes

(Seierstad *et al*., 2009). The main cells involved in phagocytosis in fish are neutrophils and macrophages (Jensch-Junior *et al*., 2006), although B cells are also known to be phagocytic in fish (Li *et al*., 2006).

Pattern recognition receptors (PRRs) are recognition of microbial pathogens and extremely important for the start of innate immune responses. The PRRs is able to detect a conserved molecular structure of pathogen as called pathogen-associated molecular patterns (PAMPs), it stimulates subsequent host immunity via a variety of signaling pathways, helping to eliminate the infection (Janeway and Medzhitov, 2002). The receptors responsible for recognition of PAMPs are found in a variety of regions within the cell including Toll-like receptors (TLRs), Rig-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs).

Additionally, the complement system appears to be one of the major immunological responses in innate immune system of fish. The classical, alternative and possibly also the lectin pathways have been described in several fish groups (Nonaka and Smith, 2000; Zardakis *et al*., 2001). With a recent literature, fish contain multiple functionally active complement components. In spite of differences in the catalytic residues of the protein, fish complement components maintain a differential affinity for a number of substrates, and they are likely the result of several polymorphic genes (Sahu and Lambris, 2001).

2.2.2 The adaptive immunity

The adaptive immunity plays a significant role in protection of host against infection by generating memory cells (cell-mediated immune response) and specific soluble antibodies (humoral immune response) (Yamasaki *et al*., 2013). The responses are affected by lymphocytes, that exist as T or B cells, and possess antigen receptors to recognize foreign molecules. In the case of T cells, the receptors require presentation of peptides by major histocompatibility complex molecules, whereas surface immunoglobulin (Ig) on B cells can recognize soluble proteins (Thompson, 2017). These responses allow the efficient elimination of fish pathogens in a specific way, whereby a second encounter gives both faster and larger responses that are qualitatively different to the primary responses. The recognition of pathogen specific molecules in this way allows vaccination to be used to prime the fish, rather than a

natural first encounter with an infectious agent (Yamasaki *et al*., 2014; Utke *et al*., 2008).

T cells can identify the presence of intracellular pathogens, since infected cells can exhibit pathogen peptides and products on the cell surface. Major histocompatibility complex (MHC) molecules deliver these peptides, which in turn initiate the generation of adaptive immunity against infections. MHC can generally be divided into 2 groups: MHC class I for initiating $CD8^+$ cytotoxic T cell mediated cellular immunity and MHC class II for activating $CD4^+$ helper T cell-mediated humoral immunity.

Immunoglobulins (Igs), which are specially produced by B cells, are essential components of adaptive immunity. Two forms of Igs have been identified: B cell receptor (BCR), a molecule that is membrane-bound and functions as an antigen receptor on the surface of B cells and the well-known antibody that plasma cells release. It has a key role in modulating humoral immunological reactions. In mammals, five classes isotypes of Ig have been described and are named IgM, IgD, IgG, IgA, and IgE, each performs unique effector roles (Abbas *et al*., 2010; Janeway *et al*., 2001). In fish, Igs are discovered and named IgM, IgD and IgT/Z (for teleost/zebrafish). The first Ig class found in fish was IgM. It can be expressed as an antibody on the surface of B cells. IgM multimerizes into a tetrameric form in either serum or mucus and plays a role in both innate and adaptive immunity, as well as complement activation, which lyses and opsonizes pathogens (Boshra *et al*., 2004). While IgD was first discovered in human serum in 1965 (Rowe and Fahey, 1965). Its function is to bind the specific receptor to trigger the production of opsonizing, pro-inflammatory, antibacterial, and B cell activating proteins (Chen *et al*., 2009). IgT/Z is believed to be the most significant Ig of mucosal surfaces in bony fishes, if not a specialized mucosal Ig isotype, according to several datasets (Mashoof *et al*., 2014).

2.3 The cytokine network in fish

Cytokines are produced at the site of entry of a pathogen, and drive inflammatory signals regulating the capacity of resident and newly arrived phagocytes to destroy the invading pathogen. Additionally, they control antigen-presenting cells (APCs) go to lymph nodes to activate the adaptive immune response. In mammals,

Naive $CD4^+$ T cells undergo rapid proliferation and differentiation into at least four distinct T-helper (Th) cell subsets such as Th1, Th2, Th17, and T-regulatory (Treg) cells, when they detect a foreign antigen-derived peptide given in the presence of MHC class II on APCs. The kind and quantity of cytokines released by activated $CD4^+$ T cells and other cell types in response to the invasion of a foreign organism or other danger signals determine to a significant extent whether subsequent immune responses will be advantageous or disadvantageous to the host. The pro-inflammatory cytokines have a crucial role in starting the inflammatory response and regulating host innate immunity. They are released by a variety of immune cells, including T-cells and macrophages. Interleukin (IL)-1β, IL-6, IL-8, IL-11, tumor necrosis factor (TNF)-α are main pro-inflammatory cytokines that are well-known for playing a significant role in regulating inflammation (Takeuchi and Akira, 2010).

2.3.1 Interleukin 6 (IL-6) family

IL-6 is a highly pleotropic cytokine and has a significant role in both innate and adaptive immunity. IL-6 along with IL-1β and TNF-α are important mediators of inflammatory responses. The classification of LIF, CNTF, OSM, IL-11, and CT-1 genes share their similarities in structure and function of IL-6 subfamily as well as their sharing of the same receptor component.

2.3.2 Interleukin 11 (IL-11)

IL-11 is a member of the IL-6 family. It was first discovered in a non-human primate, later discovered in humans, mice and teleost fish (Morris *et al*., 1996). This gene was originally described as a haemotopoietic cytokine. Additionally, this gene has been associated to bone metabolism, immune modulation, and protection of the gastrointestinal mucosa (Keith, 2003). The first report of the isolation of IL-11, outside mammals, was from rainbow trout and green spotted pufferfish (Wang *et al*., 2005). Recently, IL-11 gene has been reported in common carp (*Cyprinus carpio*), Japanese flounder (*Paralichthys olivaceus*), and zebrafish (*Danio rerio*). With recent advances of genome technology, at least two distinct types of IL-11 (putatively named as IL-11a and IL-11b) have been reported from fish that included fugu (*Tetraodon nigroviridis*) (Huising *et al*., 2005). Despite low identities shared among the fish and

mammalian IL-11 genes, critical residues important for the binding to the receptor complex are conserved (Huising *et al*., 2005; Wang *et al*., 2005). The genomic structure of fish IL-11a and IL-11b followed 5 exons and 4 introns structure similar to its mammalian counterparts. The expression of fish IL-11 was mainly in gut and gills that may have the protective effects, as seen in mammals where this gene is found to give protection of the gut mucosa. The presence of 2 distinct IL-11 genes in fish will pave-way for future studies to determine their respective functions.

2.4 The organs of immune in fish

2.4.1 Kidney

Fish hematopoiesis and immune system depend on the kidney, also known as the head kidney. Additionally, it primarily contains lympho-myeloid cells (Press and Evensen, 1999). The entire kidney is involved in the early immune response and the generation of immune cells. Blood flow to the kidney decelerates as fish mature, exposing them to more antigens. Melanoacrophage centers, which are clusters of reticular cells, macrophages, lymphocytes, and plasma cells that may be involved in antigen trapping and possibly contribute to immunologic memory (Galindo-Villegas and Hosokowa, 2004). Two Y-shaped arms that extend beneath the gills create the head kidney, also known as the anterior kidney (pronephros), which is the active immunological component. Additionally, the kidney has anatomy is unique, it is a well innervated organ, and it is also an essential endocrine organ that releases corticosteroids and other hormones similar to the adrenal glands in mammals. As a consequence, the kidney serves as an important organ with important regulatory roles, the center for immune-endocrine interactions, and even for neuroimmuno-endocrine connections (Press and Evensen, 1999; Tort *et al*., 2003).

2.4.2 Thymus

The thymus is a paired bilateral organ found in the dorsolateral area of the gill chamber, beneath the pharyngeal epitelium. But it appears that seasonal shifts and hormonal cycles affect the thymus' growth (Galindo-Villegas and Hosokowa, 2004). The thymus appears as a primary lymphoid organ where the pool of virgin

lymphocytes in the circulation and other lymphoid organs. Additionally, lymphocytes cells that have been identified from fish migrate through the thymus before the kidney and spleen (Tatner and Findlay, 1991). Thymus is responsible for the production of T-lymphocytes, as in other vertebrates (Alvarez-Pellitero, 2008). The data generally show a connection between the development of the cell-mediated immune response, appearance of lymphocytes in peripheral lymphoid organs, and the histological maturation of the fish thymus (Zapata, 1996).

2.4.3 Spleen

The spleen is the major peripheral and a secondary lymphoid organ in fish and is primarily made up of haemopoietic and lymphoid cells. It is thought to play a role in immune response and blood cell development (Galindo-Villegas and Hosokowa, 2004). The majority of fish spleens are not completely divided into red and white pulp, as in mammals, but red and white pulp is still identifiable. It is composed of multiple immature and mature plasma cells, macrophages, and lymphocytes of various sizes, all supported by a network of fibroblastic reticular cells. Fish spleen contains lymphocyte and macrophages, which are located in unique capillary walls known as ellipsoids. Additionally, ellipsoids seem to have a specialized role in immune complex and plasma filtration. It is known that spleen is a malanomacrophage centers, where the majority of macrophages are located. Antigens may be stored for a long time in these sites as immune complexes. Although the lymphoid tissue in the fish spleen is underdeveloped, more lymphoid tissue does form after antigenic stimulation (Galindo-Villegas and Hosokowa, 2004). Additionally, the spleen of fish has been associated to the elimination of immune complexes and bloodborne antigens in splenic ellipsoids, as well as the antigen presentation and the initiation of the adaptive immune response (Alvarez-Pellitero, 2008).

2.4.4 Liver

The liver is one of immune related tissues because it produces acute phase proteins and components of the complement cascade in mammals, both of which are crucial for the animals. It has also been established that the liver of fish serves a similar function (Fletcher, 1981). In vertebrates, the liver is a large organ situated in

the anterior section of the peritoneal cavity. It contains natural killer cells, lymphocytes, and cleaning phagocytic cells that play a role in the innate immune response (Racanelli and Rehermann, 2006). The liver has also been discovered to be a major immunological organ in fish, exposed to circulating antigens and endotoxins. According to a recent study, the fish liver produces a variety of substances that promote the innate immune system and immune homeostasis. This is involved in detoxification and the metabolism of carbohydrate, lipid, and protein into physiologically useful substances in vertebrates (Panserat *et al*., 2009).

2.4.5 The mucosal tissues

In fish, the gut, skin, and gills are mucosa-associated lymphoid tissues that surround the intestine, enhancing the structure's physical and chemical defenses (Doñate Jimeno, 2009). Although there is evidence that skin, gills, and intestine contain populations of leucocytes and that innate and adaptive immunity respond in case of attack by microbes, fish lack structured mucosa-associated lymphoid tissues similar Peyer patches of mammals (Ellis, 2001). Recent study has revealed the immune response in fish mucosal membranes, including the gill generation of nitric oxide and the skin creation of antimicrobial peptides and proteins (Tort *et al*., 2003; Galindo-Villegas and Hosokowa, 2004). Moreover, liver serves as a crucial physical barrier for fish, these tissues also play a role in the host-parasite interaction and secrete proteins or antimicrobials (Tort *et al*., 2003; Galindo-Villegas and Hosokowa, 2004; Alvarez-Pellitero, 2008). The previous studies on Ig presence in mucus suggest that mucus Ig is not a result of antibody transduction from the serum (Shoemarker *et al*., 2001). Mucus, which is secreted by mucous or goblet cellsand acts as a barrier of immune defense. The mucus on skin contains a variety of humoral factors with antimicrobial properties (Galindo-Villegas and Hosokowa, 2004).

2.5 Disease and control strategies

In Thailand, Nile tilapia is classified as the highest produced freshwater fish species. Recently, the production of Nile tilapia has been increasing, with Thailand becoming one of the top five producers worldwide. However, infection with a wide variety of pathogens (viral, bacterial, fungal and parasitic) has accompanied the rapid

expansion in fish production resulting in major financial losses in recent years during outbreaks. Intensive aquaculture of Nile tilapia as well as other tilapia species is often exposed to stressful conditions which have a negative impact on their growth and immunity. Recently, the tilapia aquaculture in Thailand has been hampered by *Streptococcus* spp., which is are the major causative agent. Among different genus, *S. agalactiae* is the main etiological cause of streptococcosis in wild and farmed tilapia fish (Kannika *et al*., 2017). In addition, tilapia lake virus (TiLV) has recently been reported in tilapia culture in Thailand (Tattiyapong *et al*., 2017). For many decades, antibiotics have been used as a bacterial disease prevention tool in aquaculture but with considerable risks to the aquatic species, the environment and to humans. Therefore, alternative ways to avoid disease or induce disease resistance have been explored and are now an important component of farm management practices. To get a better understanding of the infectious disease process is necessary to consider aspects of the host in response to pathogens (Kube *et al*., 2012).

Nowadays, number of fish pathogens, both bacterial and viral, can cause disease in Nile tilapia. Basic methods to control infectious diseases on a farm generally consist of drug therapy and vaccination. However, improving disease resistance by genetic selection is also an alternative because of its prospects for prolonged protection. Genetic improvement has already been shown to improve growth and disease resistance in some fish species (Zhang *et al*., 2014). In addition, the use of probiotics, prebiotics and immunostimulants have also received increasing consideration for use in aquaculture (Huu *et al*., 2016). Importantly, understanding of the immune mechanism can be applied to aquaculture research to improve our understanding of factors that are critical for survival.

2.6 Fish vaccination and administration

The application of fish vaccination is to prevent disease and enhance the specific immune response against pathogens. The main advantage of vaccine 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 (Huang *et al*., 2014). Vaccines have a significant positive impact on reduced usage of antibiotics in fish farming, resulting in a rapid decline of antibiotic drugs (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. To date, vaccines are available for more than 17 species of fish, protecting them against more than 22 different bacterial diseases and 6 viral diseases (Brudeseth *et al*., 2013).

In general, administration of fish vaccines is via a number of different routes, including immersion, injection, or orally (Chettri *et al*., 2013). Injection vaccination is the most commonly used method for administration of commercial fish species. It provides a long duration of protection and allows the incorporation of adjuvants (Mutoloki *et al*., 2008). Injection vaccines can be delivered by intramuscular injection (I.M.) or intraperitoneal injection (I.P.), although the latter is most commonly used to prevent any damage to the fillet. However, the disadvantage of injection vaccination is that it is time consuming and requires skilled personnel. Immersion vaccination is also an effective method for mass vaccination and is widely used for vaccination of small fish for some diseases (Esteve-Gassent *et al*., 2004). This procedure has reduced stress for the fish and it is less labour intensive (Villumsen and Raida, 2013). Some oral vaccines have also been developed and are designed to be administered with the feed. This gives a very easy method of application, improved safety, and substantial reduction on the stress imposed to the fish. However, its effectiveness is limited, likely as a consequence of vaccine degradation in the fish digestive system (Vervarcke *et al*., 2004). Thus, the only commercial use of oral vaccines is limited to booster vaccines. The choice of delivery method is therefore based on a number of factors such as the fish size, duration of protection required, type of pathogen and likelihood of encounter and cost (Deshmukh *et al*., 2012). Several vaccines have been shown to have high protection efficacy for bacterial and viral diseases (Chettri *et al*., 2015). Determination of the effectiveness of vaccines is usually calculated as the relative percent survival (RPS) (Amend, 1981).

2.7 Inactivated whole cell vaccines

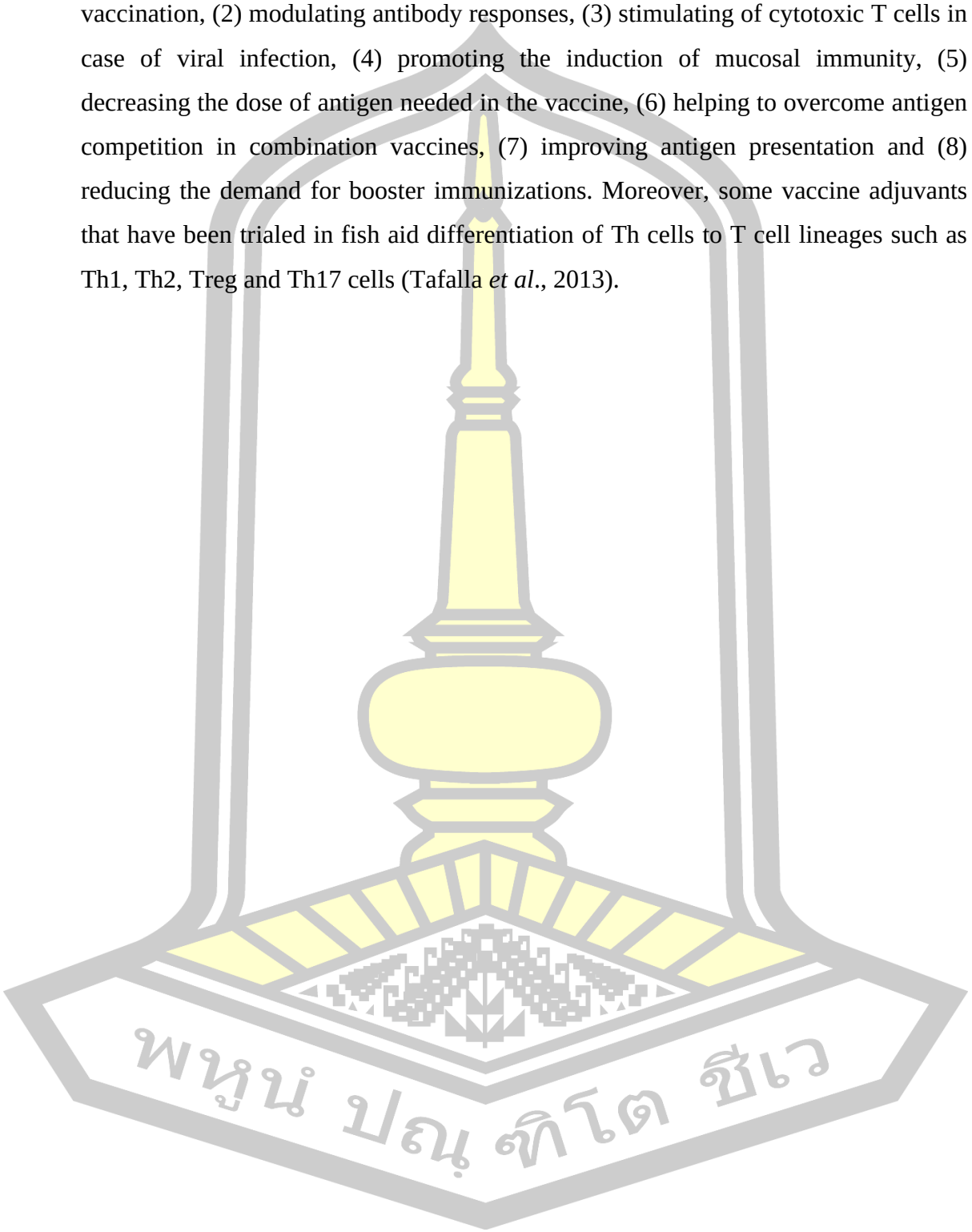
Vaccine development has become an essential tool for preventing diseases in farmed fish. Different types of vaccines exist, broadly categorized as inactivated vaccines, live attenuated vaccines, DNA vaccines and subunit vaccines (Gudding and Muiswinkel, 2013). Inactivated vaccines have been a widely applied and used successful against bacterial diseases in fish for many years (Brudeseth *et al*., 2013). Formalin-killed vaccines (FKC) are most commonly used and have been commercially developed for controlling diseases in aquaculture. The vaccine against ERM, which is the commercial FKC vaccine and was licensed in the late 1970s (Amend *et al*., 1983). The advantage of inactivated bacterial vaccines is the safety offered by formalin treatment, which is a reliable and cheap way to kill the pathogen. However, the disadvantage of this type of vaccine is that it may require an adjuvant to elicit an adequate (long-term) immune response if injected or require booster vaccination if administered by a mucosal route (Thu Lan *et al*., 2021). Currently, commercial fish vaccines are available in North America, Chile, Japan, Australia and Europe.

2.8 Adjuvants for fish vaccines

In fish vaccination, adjuvants have been recommended to combine with vaccine due to its properties can enhance vaccine efficacy (Tafalla *et al*., 2013). Adjuvants can also help reduce the number of doses needed or the quantity of antigen required per dose. Since 1990 oil-adjuvanted vaccines have been applied for aquaculture (Ribeiro and Schijns, 2010), and have been deemed a very real successful in the control of many bacterial diseases.

Basically, adjuvants can be classed according to their chemical nature or physical properties. The activation of specific T and B lymphocytes, which form the adaptive immune system, is a key requirement of vaccine adjuvants (Schijns, 2001). However, it is now recognized that an effective immune response against pathogens involves initial recognition of microbes by the detection of PAMPs, through PRRs such as TLRs, NLRs, and RLRs which are predominantly found on cells of the innate immune system (Awate *et al*., 2013). Consequently, molecules that target these receptors are also useful adjuvants. Hence, the main functions of vaccine adjuvants

are; (1) increasing the immunogenicity of antigens and enhancing the protection after vaccination, (2) modulating antibody responses, (3) stimulating of cytotoxic T cells in case of viral infection, (4) promoting the induction of mucosal immunity, (5) decreasing the dose of antigen needed in the vaccine, (6) helping to overcome antigen competition in combination vaccines, (7) improving antigen presentation and (8) reducing the demand for booster immunizations. Moreover, some vaccine adjuvants that have been trialed in fish aid differentiation of Th cells to T cell lineages such as Th1, Th2, Treg and Th17 cells (Tafalla *et al*., 2013).



Chapter 3
Methodology

3.1 The experimental fish

Healthy Nile tilapia weighing 50 g were purchased from a commercial fish farm at Roi-Et province, Thailand and maintained in a 1000-liters fiberglass tank in the aquarium of the division of fisheries, Mahasarakham University. Fish were fed twice a day on a commercial pellet diet containing 30% crude protein, acclimatized and certified to be pathogen-free for at least 2 weeks before use. Throughout the experiment, the water quality parameters including temperature, dissolved oxygen, ammonia nitrogen and pH were 26±1 ˚C, 6.5±0.3 mg/L, < 0.05 mg/L and 7.7±0.1, respectively.

3.2 Ethical statement

This research was conducted in strict accordance with the recommendations for the use of animals regulated by the Institute of Animals for Scientific Purposes Development (IAD) of Thailand. The experimental protocols were approved by the ethics committee at Mahasarakham University (IACUC-MSU-30/2021).

3.3 Cloning and characterization of IL-11 in Nile tilapia

Taking advantage of the recent release of Nile tilapia whole genome shotgun contigs (WGS), candidate WGSs for IL-11 were identified using the known rainbow trout IL-11. The basic local alignment search tool search was carried out with the NCBI (http://blast.ncbi.nlm. nih.gov/Blast.cgi) and resulted in the identification of IL-11 of Nile tilapia. Primers were designed for the Nile tilapia IL-11a and IL11b to obtain the full-length sequences of IL-11a and IL-11b genes. Amplification template of cDNA was performed using PCR. Amplification conditions were: 1 minute (min) at 94 °C; then 40 cycles at 63 °C for 45 seconds (s), and 72 °C for 45 s. The cloning, sequencing and protein sequence analysis was described previously (Wang *et al*., 2018).

Table 1 Sequences of forward (Fw) and reverse (Rv) primers used for PCR cloning and qRT-PCR analysis.

Gene	Accession no.	Primer	Primer Nucleotide sequence 5'- 3'	Annea-ling T (°C)
B-actin	MM003443127	Fw	ACAGGATGCAGAAGGAGATCACAG	60
		Rv	GTACTCCTGCTTGCTGATCCACAT	
IL-1β	FF280564	Fw	AAGATGAATTGTGGAGCTGTGTT	60
		Rv	AAAAGCATCGACAGTATGTGAAAT	
TNF-α	NM001279533	Fw	AGGGTGATCTGCGGGAATACT	60
		Rv	GCCCAGGTAAATGGCGTTGT	
IFNγ	NM_001287402.1	Fw	GAAACTTCTGCAGGGATTGG	60
		Rv	CTCTGGATCTTGATTTCGGG	
MHC-Iα	MM026157132	Fw	TTCTCACCAACAATGACGGG	60
		Rv	AGGGATGATCAGGGAGAAGG	
MHC-IIβ	JN967618	Fw	GAGGAACAAGCTCGCCATCG	60
		Rv	AGTCGTGCTCTGACCTCGAG	
IgM	JJ676389	Fw	GGATGACGAGGAAGCAGACT	59
		Rv	CATCATCCCTTTGCCACTGG	
IL-8	NM001279704	Fw	GCACTGCCGCTGCATTAAG	58
		Rv	GCAGTGGGAGTTGGGAAGAA	
IL-6	XM_019350387.2	Fw	ACAGAGGAGGCGGAGATG	60
		Rv	GCAGTGCTTCGGGATAGA	
IL-11a[1]	XM_013274585.3	Fw	GTGACAATCAGCGGCATTCATTG	60
		Rv	CAAATGCATGCTCCTTCAGCAGTT	
IL-11b[1]	XM_003450684.5	Fw	TGCCTATCTCTTGGATCCGAACCA	60
		Rv	CAAATGCATGCTCCTTCAGCAGTT	
IL-11a[2]	XM_013274585.3	Fw	TACTTGAACTTGAAAGGACAAGA	63
		Rv	CACATTCTGAAGTTTTTATTTCA	
IL-11b[2]	XM_003450684.5	Fw	ATTGGACTTACCACACTCGCA	63
		Rw	CACGGTCATGAAAAAGGGCTTA	
CD4	MM031744220	Fw	TTCAGTGGCACTTTGCTCCTAA	60
		Rw	TGGGCGATGATTTCCAACA	
TCRβ	HM162889	Fw	GGACCTTCAGAACATGAGTGCAGA	60
		Rw	TCTTCACGCGCAGCTTCATCTGTT	
CD8α	MM031747820	Fw	ATGGACCAAAAATGGCTTCTG	60

		Rw	GCTGAAAGATCCAATGAATTC	

Table 1 Sequences of forward (Fw) and reverse (Rv) primers used for PCR cloning and qRT-PCR analysis (Cont.).

Gene	Accession no.	Primer	Primer Nucleotide sequence 5'- 3'	Annea-ling T (°C)
COX-2	XM_003445052	Fw	TGCTGAAAGAGGTCCACCCATACT	60
		Rw	CGCTCAGATGCTGCACGTAGTC	

Abbreviations: IL-1β: Interleukin 1 beta; TNF-α: Tumor necrosis factor alpha; MHC-Iα: Major histocompatibility complex class I alpha; MHC-IIβ: Major histocompatibility complex class II beta; IgM: Immunoglobulin M; IL-8: Interleukin 8; IL-11a: Interleukin 11a; IL-11b: Interleukin 11b; CD4: Cluster of differentiation 4; TCRβ: T-cell receptor beta; CD8α: Cluster of differentiation 8 alpha; COX-2: cyclooxygenase 2.

Note: 1: primer for qRT-PCR; 2: primer for cloning.

3.4 Bioinformatics analysis

The nucleotide sequences of IL-11 gene of Nile tilapia were searched with the NCBI website (http://blast.ncbi.nlm.Nih.gov /Blast.cgi) and 2 paralogues as called IL-11a and IL-11b were found. ExPASy-Translate (http://web.expasy.org/translate/) was used to translate the nucleotide sequence, and the molecular weight and theoretical isoelectric point (p*I*) of the protein were determined using the ExPASy-Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Amino acid sequence identity and similarity of vertebrate IL-11 were performed using the EMBL-EBI service (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Multiple sequence alignment was evaluated by CLUSTALW and BOXSHAD (http://www.ch.embnet.org/software-/BOX_-form.html).

The synteny and gene organization analysis were predicted by using the Genomicusv100.01program(https://www.genomicus.biologie.ens.fr/genomicus100.01 /cgibin/search.pl). Phylogenetic tree was analysed using the neighbor-joining method with the bootstrap set to 10,000 times in the Molecular Evolutionary Genetics Analysis software version 10 (MEGA10).

3.5 Tissue distribution of Nile tilapia IL-11a and IL-11b genes in Nile tilapia

Gene expression analysis of IL-11a and IL-11b genes in several tissues was investigated by quantitative real-time PCR (qRT-PCR). The primer sequences for gene expression were designed so that at least one primer crossed an intron to prevent amplification of genomic DNA. qRT-PCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The RNA preparation and cDNA synthesis were described previously (Wangkahart *et al*., 2016). Briefly, three healthy Nile tilapia (~100 g) were killed and eleven tissues (gills, skin, muscle, liver, spleen, gonad, head kidney, trunk kidney, intestine, heart and tail fins) were collected and homogenized in TRI reagent (Sigma, UK). Each sample was placed in a new 2 mL microtube containing a magnetic bead, and then 1.5 mL TRI reagent (Sigma, UK) was added and homogenized with a TissueLyser (Qiagen, Germany). After that, the TRI reagent solution containing the disturbed tissue was centrifuged at 12,000 rpm for 5 min. The supernatant was collected into a new 2 mL tube. TRI reagent (Sigma, UK) was added to 1 mL, and the mixture was homogenized once more. Three hundred microliter of chloroform was then added to the sample and mixed by homogenizer. Subsequently, centrifugation step 12,000 rpm for 15 min and was carefully by pipetting the remaining 550 µL of the sample into a clean 1.5 mL tube. Seven hundred microliter of cooled 2-propanol was added into the sample, mixed and centrifuged at 12,000 rpm for 10 min at 4°C. A pellet forms on the side and bottom of the tube. The RNA pellet was washed twice with 1,000 µL cooled 70% ethanol (Sigma-Aldrich) and mixed by vortexing. The solution was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed, and the pellet was recovered and air-dried at room temperature for about 10 to 15 min.

The synthesis of DNA from an RNA template, was applied to reverse transcription, produce complementary DNA (cDNA). After the pellet was air-dried, 32 µL of Oligo dT (d(T)28VN) was added, mixed and then spin-down tube. Afterward, incubated at 72 °C for 2 min. In a new 0.6 mL PCR microtube, 29.4 µL of sample was added, followed by 10.6 µL of cDNA master mix included were: 8 µL of 5x buffer, 1.6 µL of 25 mM dNTP and 1 µL of reverse transcriptase. 500 µL TE

buffer was added into the tube. RT-PCR conditions were: 1 hour (h) at 42 °C; 30 min at 45 °C; 30 min at 50 °C, and 90 °C for 5 min.

The qRT-PCR condition followed by a 10 min denaturation at 95 °C, then 49 cycles of 95 °C for 30 seconds (s), 66 °C for 30 s and 72 °C for 30 s. The qRT-PCR reactions were run as a standard 18 µL reaction included 4 µL template cDNA, 2 µL dH_2O, 10 µL primer (IL-11a and IL-11b), and 10 µL of immolase cocktail solution. The expression level of each gene in different tissues was normalized to the expression of β-actin and expressed as arbitrary units. These calculations were performed according to the double-delta CT ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001) in Microsoft Excel.

3.6 Gene expression of IL-11a and IL-11b genes in Nile tilapia during bacterial infection

Sixteen Nile tilapia were injected with *S. agalactiae* intraperitoneally (I.P.) at the concentration of 1×10^7 CFU in 0.5 mL PBS into fish and four fish of a control group were injected with PBS only (0.5 mL/fish). Basically, the infected fish start to show signs of disease at day 3 under the same challenge dose, which causes 100% mortality, thus five control and infected fish were sampled at 6, 24, 48 and 72 h post infection, with spleen, liver, gills and intestine tissues being collected. The fish tissue collected were anaesthetized by immersing 0.5% of 2-phenoxyethanol overdose for 5 min and checking that they were completely insensitive. Total RNA extraction, cDNA synthesis and qRT-PCR analysis were conducted as described in section 3.5.

3.7 Peripheral blood leucocyte (PBL) preparation by hypotonic lysis

The PBL of Nile tilapia was modified from method previously (Hu *et al*., 2018). Briefly, 3 healthy Nile tilapia was collected blood 1 mL and before collected blood fish were anaesthetized by immersing 0.5% of 2-phenoxyethanol for 5 min and checking that they were completely insensitive. The blood was derived from the caudal vein and using a K3EDTA blood collection tube (MediPlus, China) for anti-coagulation. 9 mL of water for molecular biology (ITW Reagents, USA) and 1 mL of 10X PBS were cooled on ice before using. Blood was transferred to 15 mL Falcon tube, added 9 mL ice-cooled water and mixed inverted for disrupted of red blood cell.

1 mL of 10X PBS was added to return the solution isotonicity and put on ice for 5-10 min immediately. Then passing through a 70 µm EASYstrainer (Greiner Bio One, UK) was using separated PBL from cell debris, centrifugation at 12,000 rpm for 5 minute and washed once with Leibovitz medium L-15 (HyClone, USA). PBL was resuspended into L-15 medium and counted the cell number using Trypan blue exclusion.

3.8 Immune stimulation of PBL

PBL freshly were seeded into 12-well cell culture plates 2 mL (2×10^6 cell/mL) and stimulated with polyinosinic: polycytidylic acid (Poly I:C 100 ng/mL, Sigma, UK) and lipopolysaccharide (LPS, 1 ng/mL, Sigma, UK) at 20°C for 4, 8 and 24 h and stimulated with LPS and Poly I:C different concentration (1 µg/mL, 10 µg/mL and 100 µg/mL) at 4 h. Gene expression analysis of PBLs in several stimulation was investigated by qRT-PCR. The RNA preparation was modified from previously (Wangkahart *et al*., 2016). Briefly, at 4, 8 and 24 h after PBL stimulated were collected and homogenized in TRI reagent (Sigma, UK). Each sample was placed in a new 2 mL microtube, and then 1.5 mL TRI reagent (Sigma, UK) was added and homogenized with a TissueLyser (Qiagen, Germany). After that, the TRI reagent solution containing the disturbed PBL cell was centrifuged at 12,000 rpm for 5 min. Three hundred microliter of chloroform was then added to the sample and mixed by homogenizer for 1 min. Subsequently, centrifugation step 12,000 rpm for 15 min and was carefully by pipetting the remaining 750 µL of the sample into a clean 1.5 mL tube. Seven hundred microliter of cooled 2-propanol containing glycoblue was added into the sample, mixed and centrifuged at 12,000 rpm for 10 min at 4°C. A blue pellet forms on the side and bottom of the tube. The RNA pellet was washed twice with 1,000 µL cooled 70% ethanol (Sigma-Aldrich) and mixed by vortexing. The solution was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed, and the pellet was recovered and air-dried at room temperature for about 10 to 15 min. The synthesis of DNA from an RNA template, was applied to reverse transcription, produce cDNA and qRT-PCR condition followed previously method in section 3.5.

3.9 Preparation of competent cells

The *Escherichia coli* BL21 Star (DE3) and *E. coli* DH5α were used for the competent cell. Both *E. coli* BL21 Star (DE3) and *E. coli* DH5α were streaked on a Luria-Bertani (LB) agar plate for colony isolation and incubated at 37°C overnight. *E. coli* BL21 Star (DE3) and *E. coli* DH5α were cultivated as a single colony in 3 mL of LB medium by shaking at 2,000 rpm, 37°C, overnight. The cell suspension was diluted in LB media 1:50 and cultivated at 37°C with 2,000 rpm of shaking until the bacteria reached late log phase (OD_{600} = 0.5). The culture spent 10 min in an ice bath and 100 mM $CaCl_2$ and 80% glycerol were cooled on ice before using. The cell suspension was centrifuged at 2,400 rpm, 4°C for 10 min and removed of supernatant. Added 1.6 mL of 100 mM $CaCl_2$ cooled to cell pellet for resuspended by swirled gently on ice. The resuspended cell after incubated 30 min was centrifuged at 2,400 rpm, 4°C for 10 min and removed the supernatant. Added 1.6 mL of 100 mM $CaCl_2$ cooled to cell pellet for resuspended cell again by swirled gently on ice and incubated on ice for 20 min. The resuspended after incubated was centrifuged at 2,400 rpm, 4°C for 10 min and removed supernatant. 80% glycerol cooled was added 1 mL to the cell pellet and mixed, pellet cell was aliquoted and stored at −80 °C.

3.10 Production of Nile tilapia recombinant IL-11 (rIL-11)

Two transcripts of both IL-11a and IL-11b are produced by alternative use of the first exon. IL-11a is more likely and has a signal peptide predicted and IL-11b has an upstream ATG that may affect translational efficiency and skip the first ATG of the main ORF. Therefore, the similar structure of both molecules was edited and used for recombinant protein production. Production of a recombinant Nile tilapia IL-11 (rIL-11) genes was performed. Briefly, open reading frames of IL-11a and IL-11b were amplified by PCR using specific primers containing restriction enzyme sites and the high-fidelity enzyme. The amplified product was cloned to a pXN vector (Novagen) and a his-tag (ASSAHHHHHHHHHH) was added at the C-terminus for protein purification (kindly help by Dr. Tiehui Wang, the University of Aberdeen). Principally, the rIL-11 will be 200 amino acids, with a calculated molecular weight of 23.23 kDa and a theoretical p*I* of 9.35. T4 DNA ligase was used to ligate the PCR product and pXN vector. The ligation product was transformed into *E. coli* DH5α

competent cells (Invitrogen) and was isolated from an ampicillin resistant transformant. The positive cloned was plasmid DNA extraction and verified by DNA sequencing.

The *E. coli* heat shock transformation was used to transform the pXN-IL-11 plasmid into *E. coli* BL21 Star competent cells (Invitrogen). Briefly, the 50 µL competent cells were spent 20-30 minute on ice bath and combined 1-5 µL of DNA were extracted from *E. coli* DH5α competent cells, then mixed by vortexing. Competent cells and a DNA mixture tube were incubated on ice for 20-30 minute. After that, the competent cells and DNA mixture was incubated into 42°C for 30-60 second and returned the tube to ice for 2 min. Five hundred microliter of LB medium was added and incubated at 37°C for 1 h.

Following transformation of the pXN-IL-11 plasmid into *E. coli* BL21 Star (DE3) competent cells (Invitrogen) for the production rIL-11 protein. The transformed cell was incubated in LB medium with 100 µg/mL ampicillin at 37°C overnight until the bacteria reached late log phase (OD600 = 0.5). Added IPTG to the final concentration of 0.8 mM and incubated again at 30°C for 18 h. After that, the cultured was centrifuged at 2,400 rpm, 4°C for 10 min and removed of supernatant. The cell pellet was resuspended in dissolving buffer, the dissolving buffer for lysis (LB0) was contained 7 M GuHCl, 500 mM NaCl, 50 mM Tris-HCl, 5 mM Imidazole and 5 mM TCEP. The cell pellet 1 gram was dissolved in 15 mL of LB0 and incubated at 37 °C with 50 rpm of shaking for 3 h. The cell suspension was centrifuged at 4,000 rpm for 30-90 minute and collected of supernatant. The supernatant 10 mL was sonicated at 100% power for 1 minute. Added Triton X-100 to 1.5% (0.75 mL to 10 mL lysate) with 50 rpm of shaking for 1 h. After that, the supernatant was centrifuged at 4,000 rpm for 60 min and supernatant collected for purification.

3.11 Purification of Nile tilapia recombinant IL-11 (rIL-11)

The purification under denaturing conditions, refolding, re-purification under native conditions, SDS-PAGE analysis of proteins and quantification of protein concentration of Nile tilapia rIL-11 was performed as described previously (Wangkahart *et al*., 2016). Briefly, the wash buffer (WB1) was used under denaturing

conditions contained 6 M GuHCl, 500 mM NaCl, 50 mM Tris-HCl, 10 mM Imidazole, 1.5% Triton X-100 and 5 mM TCEP, that effectively remove membrane proteins e.g. Lipopolysaccharide (LPS). The refolding buffer (RF7.4-Refolding) was contained 1X PBS, 10% glycerol, 500 mM Arginine Monohydrochloride, 50 mM Glutamic acid and 1 mM TCEP. Then, the elution buffer (RF7.4-Elution) was contained 1X PBS, 10% glycerol, 500 mM Arginine Monohydrochloride, 10 mM Glutamic acid, 1 mM TCEP and 300 mM Imidazole. The DNEB buffer was contained 6 M GuHCl, 500 mM NaCl, 50 mM Tris-HCl, 500 mM Imidazole and 5 mM TCEP.

Supernatant was poured in the column and added 10 mL WB1 to balance the column and prevent it from drying out during the run. Added 10-20 mL lysate LB0 for lysis and was washed twice with 12.5 mL WB1. Added 12.5 mL of RF7.4-Refolding buffer for refolding and added 6 mL of RF7.4-Elution buffer. After that, 5 mL of DNEB buffer was added for eluting proteins. After sterilization with a 0.2 μm filter, the rIL-11s was aliquoted and stored at −80 °C ready for used as adjuvant.

3.12 SDS-PAGE analysis of Nile tilapia recombinant IL-11 (rIL-11)

After purification the rIL-11 was analyzed by SDS-PAGE. Briefly, 12% separating gel (GenScript, UK) was used and loaded into the wells. The gel was run at 60 volt for 30 min.

3.13 Immunostimulant effect of rIL-11 in PBL

PBLs isolated from blood of Nile tilapia by hypotonic lysis were described in section 3.7. PBLs freshly were seeded into 12-well cell culture plates 2 mL (2×10^6 cell/mL) and stimulated with rIL-11 (5, 50 and 500 ng/mL) at 4 h. Total RNA extraction, cDNA synthesis and gene expression analysis of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 by RT-qPCR analysis were conducted as described in section 3.5.

3.14 Formalin-killed cell (FKC) vaccine preparation and fish vaccination

A FKC vaccine was prepared at the Laboratory of Fish Immunology and Nutrigenomics, Applied Animal and Aquatic Sciences Research Unit, Faculty of Technology, Mahasarakham University. Briefly, *S. agalactiae* was grown in BHI

broth and culturing at 37°C for 24 h. Bacterial cells were then collected by centrifugation at 3,000 rpm. The bacterial suspension was treated with 100 µL of formalin (1% of bacterial suspension volume) and incubated at 37 °C for 24 h, with a shaking speed of 50 rpm. The prepared solution was then centrifuged at 5,000 rpm for 20 min, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 10 mL of sterile 1X PBS.

The rIL-11 of Nile tilapia prepared were adjusted to 10 µg/mL and was used as an adjuvant candidate. Before vaccination, fish were anaesthetized with 0.5% 2-phenoxyethanol and then intraperitoneally vaccinated with each vaccine formulation. Group 1: mock vaccination as the control group, injection of 0.1 mL PBS; Group 2: injection of 0.1 mL FKC (1×10^9 cells/mL); Group 3, injection of 0.1 mL FKC (1×10^9 cells/mL) formulated with rIL-11. Experimental schedule is summarized in figure 1 vaccinated fish was maintained in 500 litter fiberglass tanks.

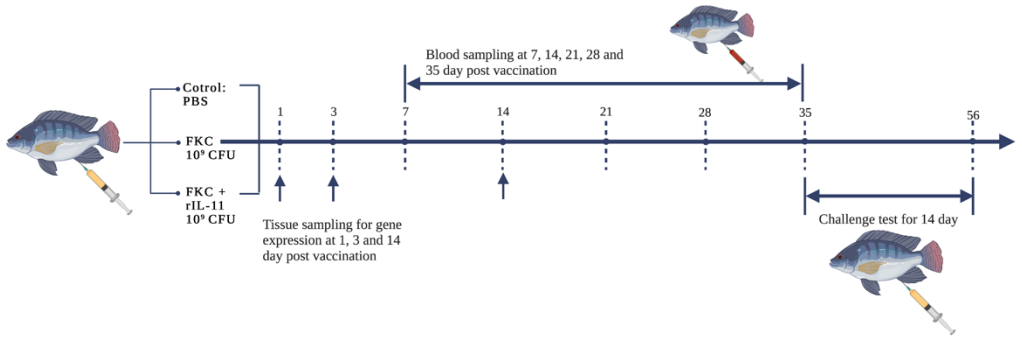


Figure 1 Schematic representation of the trial in this study.

3.15 Gene expression analysis after vaccination

Four fish from the vaccinated and control group were killed at 1, 3 and 14 days post vaccination. Spleen and liver were collected and homogenised separately in TRI reagent (Sigma, UK). Total RNA extraction and cDNA synthesis were performed as described in section 3.5. The fish were anaesthetized by immersing 0.5% of 2-phenoxyethanol overdose for 5 min and checking that they were completely insensitive and tissue were collected. qRT-PCR was used to study the expression of immunology gene included IL-1β, IL-8, TNFα, IL-11a, IL-11b, IL-6, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ.

3.16 IgM antibody titer measurement by enzyme-linked immunosorbent (ELISA) assay

Blood was collected at 7, 14, 21, 28 and 35 days post vaccination (dpv) from 8 fish in each group. Blood was incubated overnight at 25°C. The blood was centrifuged at 5,000 rpm for 10 min and then the serums were collected. The ELISA were used to determine the IgM antibody titres level against *S. agalactiae*. FKC-specific IgM antibody titres in serum samples were determined by ELISA using mouse anti-tilapia IgM provided by Vertebrate Antibody Limited (Aberdeen, UK). Briefly, the concentration of *S. agalactiae* was determined by wet pack cell mass weight, then the sample was re-suspended in PBS and briefly sonicated. The antigen was diluted to 20 µg/mL in coating buffer (carbonate bicarbonate buffer) (Sigma) and 50 µL was added to each well of a 96 well ELISA plate. The plate was incubated at 37°C for 2 h and washed twice using washing buffer (1% w/v skimmed milk in PBS). Serum was serially diluted (2-fold) in washing buffer and 50 µL was added to each well and incubated overnight at 4 °C. After washed in washing buffer 50 µL of anti-tilapia IgM, 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. Afterwards the plates were washed 3 times 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. Plate 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 min. The reaction was stopped by adding 50 µL/well of 0.5 M sulfuric acid (H_2SO_4), and the optical density (OD) was read using an ELISA plate reader at 450 nm. All serum sample was examined in duplicate.

3.17 Innate immune response analysis

Serum lysozyme activity (LZM) in fish serum was measured by turbidimetric assay (Saurabh and Sahoo, 2008). Twenty-five microliter of serum was added into 96-well plate. Then, 100 µL of 0.05 M *Micrococcus lysodeikticus* in PBS, pH 8.4 was added. The absorbance was measured at 450 nm after 30 and 180 seconds. LZM

activity was calculated using the by following formula. LZM activity (Unit/mL) = ((OD30s-OD180s)/2.5) x1,000 in Microsoft Excel.

Myeloperoxidase activity (MPO) was measured as described by Sahoo (Sahoo *et al.*, 2005). Briefly, 20 µL of serum was diluted with 80 µL of 1X PBS in 96-well plate. Then, 35 µL TMB (Sigma, USA) and 35 µL of hydrogen peroxide (H_2O_2) was added. The color change reaction was stopped after 1 min by adding 35 µL of 2% v/v H_2SO_4. The OD was measured at 450 nm using microplate reader (VersaMax™, Molecular devices). MPO activity was calculated by following method. MPO activity = Absorbance OD at 450 nm in Microsoft Excel.

Glutathione reductase activity (GRD) was modified by previously method (Meng *et al.*, 2021). Briefly, Stock solution containing of Tris-EDTA (TE) buffer and 18 mM glutathione (GSSG) in DI water and the solution 50 µL and 50 µL of serum was added in 96-well plate. After incubated at 25°C for 2 min, 50 µL of 3 mM NADPH was added. The OD was measured at 450 nm using microplate reader (VersaMax™, Molecular devices). GRD activity was calculated by following method. GRD activity (U/mL) = (Absorbance OD at 450 nm/60) x 1,000 in Microsoft Excel.

Glutathione peroxidase activity (GPx) was modified by previously method (Meng *et al.*, 2021). Briefly, 20 µL of serum was added in 96-well plate, and then 20 µL of first substrate (50 mM PBS + 0.1% Triton X). Added 20 µL of second substrate (24 µmol glutathione +12 U GRD + 4.8 µmol µ-nicotinamide adenine dinucleotide (NAD+). 35 µL of 1% v/v H_2SO_4 was added. The absorbance measured at 405 nm using microplate reader (VersaMax™, Molecular devices) after incubated at 25 °C for 3 min. GPx activity (U/mL) = Absorbance OD at 405 nm/0.00622 in Microsoft Excel.

3.18 Challenge study

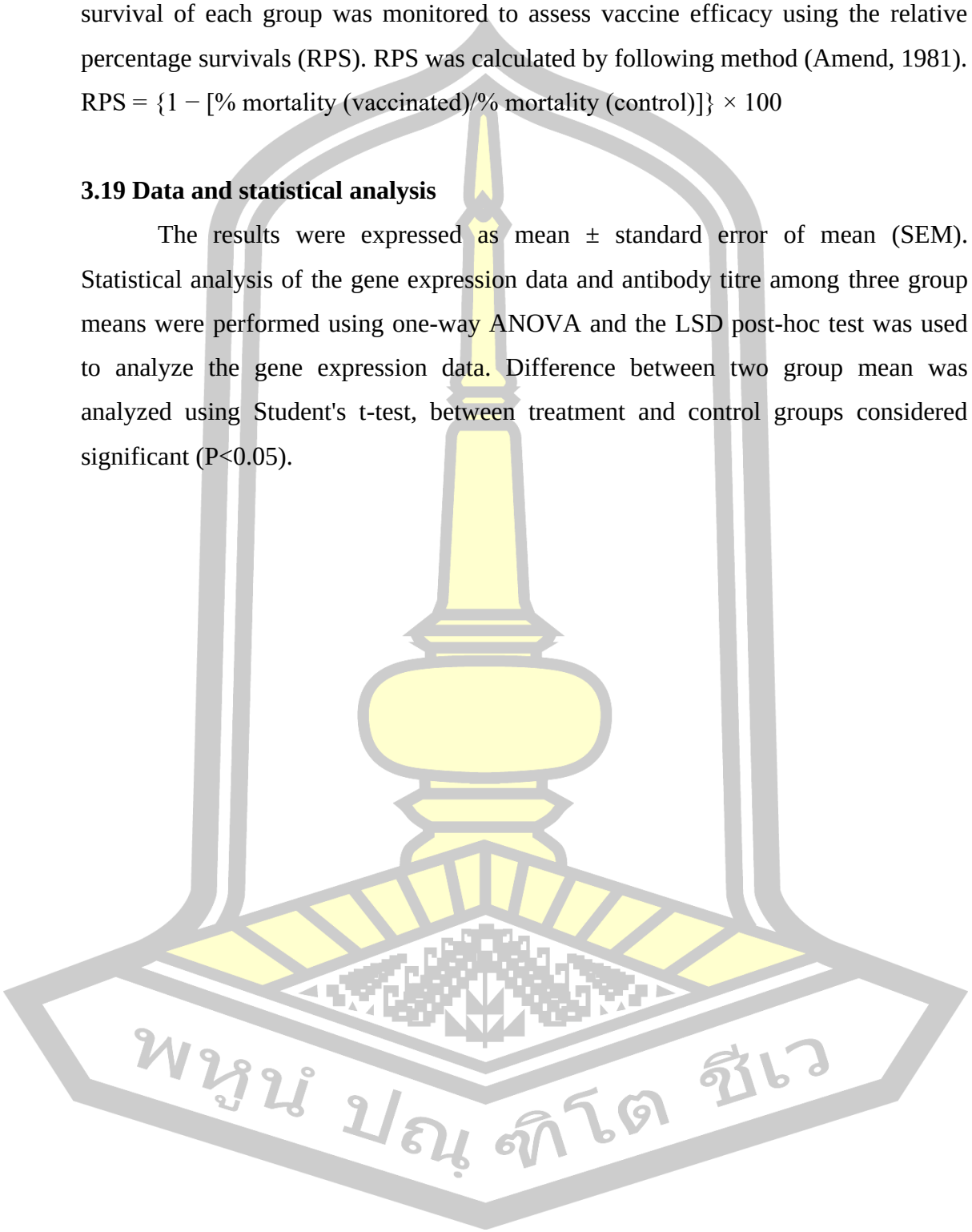
S. agalactiae serotype III was used for the challenge test. In order to determine the LD_{50} concentration of the bacteria used, fish was administered with 0.1 mL of the bacterial suspension by I.P. injection.

After 35 d.p.v., fish from the 3 groups were administered I.P. injections containing 0.2 mL of a 1×10^8 CFU/mL bacterial suspension. The challenged fish was maintained in 500 L fiberglass tanks. Clinical signs of disease and cumulative

mortalities were monitored twice a day for 21 days after injection. The percentage survival of each group was monitored to assess vaccine efficacy using the relative percentage survivals (RPS). RPS was calculated by following method (Amend, 1981).
RPS = {1 − [% mortality (vaccinated)/% mortality (control)]} × 100

3.19 Data and statistical analysis

The results were expressed as mean ± standard error of mean (SEM). Statistical analysis of the gene expression data and antibody titre among three group means were performed using one-way ANOVA and the LSD post-hoc test was used to analyze the gene expression data. Difference between two group mean was analyzed using Student's t-test, between treatment and control groups considered significant ($P<0.05$).



Chapter 4

Results

4.1 Molecular structure of Nile tilapia IL-11 genes

The nucleotide sequences and deduced amino acid sequences of Nile tilapia IL-11a and IL-11b as showed in Figure 2. The cDNA sequence of IL-11a and IL-11b consisted of 5,794 and 10,137 bp encoding 238 and 200 amino acids, respectively. Both molecules contained the potential N-glycosylation site (NQT) at the positions of 80-82 and 42-44, respectively. The 2 cysteines in IL-11a at the positions of 37 and 220, while IL-11b had 1 cysteines at the position of 182. In addition, both of IL-11a and IL-11b had 7 methionine at the positions 1, 60, 86, 113, 165, 169 and 180 for IL-11a and at the position 1, 42, 48, 75, 127, 131 and 142 for IL-11b. The instability motifs (ATTTA) of IL-11a and IL-11b were present in the 3'-UTR and 5'-UTR, respectively.

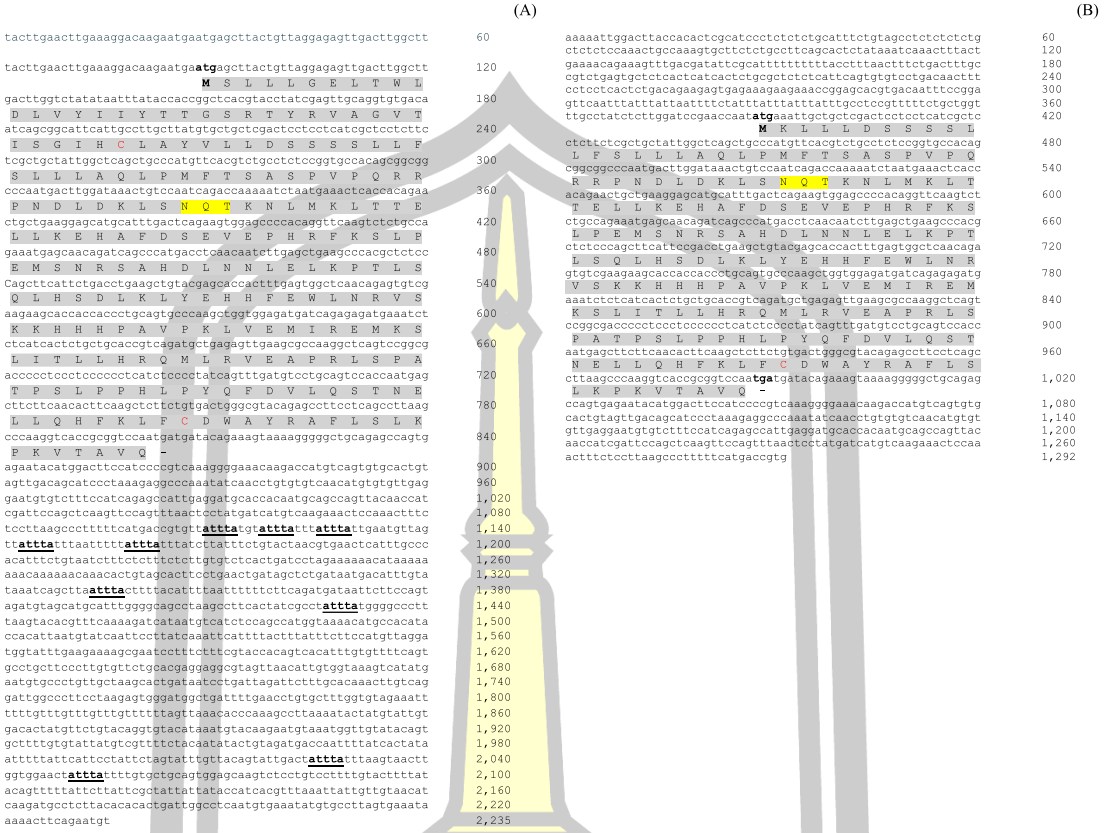


Figure 2 Full-length nucleotide sequences and deduced amino acid sequences of IL-11a (A) and IL-11b (B) in Nile tilapia.

The start (ATG) and stop (TGA) codons were marked with gray and bold, respectively.

4.2 Bioinformatics analysis for IL-11 genes discovery in Nile tilapia

The amino acid length and theoretical p*I*/Mw of IL-11 gene between the Nile tilapia and selected fish and vertebrate species as showed in Table 2. The potential peptide of Nile tilapia IL-11a and IL-11b were 238 and 200 amino acids, respectively. A theoretical isoelectric point (p*I*) of Nile tilapia IL-11a and IL-11b were 9.02 and 8.73 and molecular weight (Mw) were 27.4 and 22.9 kDa, respectively. The isoelectric point (p*I*) of protein was identified as the pH where a protein molecule has no charge. Therefore, the negative charge of proteins at a pH over their p*I* and positive charge at a pH under their p*I*. The protein p*I* ranges from 4.0 to 12.0, which ranges from extremely acid to strongly base values. Moreover, p*I* values have been

applied for protein isolation, separation, purification, crystallization, etc. to identify distinct proteins. In addition, 3D structure of IL-11a and IL-11b were presented in Figure 3.

Table 2 The species name, accession number, the amino acid length and theoretical pI/Mw of IL-11 gene between Nile tilapia and other species.

Species	**Accession no.**	**Length (aa)**	**Theoretical p*I*/Mw (kDa)**
Mammal IL-11			
Gallus gallus	XP_046791319.1	350	10.91 / 21.47
Rattus narvegicus	NP_598203.4	199	10.64 / 21.54
Mus musculus	NP_032376.1	199	10.90 / 21.52
Homo sapiens	NP_000632.1	199	10.64 / 21.43
Sus scrofa	XP_020950667.1	199	10.87 / 21.33
Equus caballus	XP_005596605.1	246	11.10 / 21.39
Felis catus	XP_003997447.1	199	10.87 / 21.30
Fish IL-11a			
O. niloticus	Un-submitted	238	9.02 / 27.40
O. aureus	XP_031600140.1	201	9.35 / 23.20
Nothobranchius furzeri	XP_015830724.1	215	9.52 / 15.39
Mastacembelus armatus	XP_026173364.1	200	9.44 / 23.23
Gasterosteus aculeatus	XP_040021782.1	201	9.52 / 23.19
Takifugu rubripes	XP_003966297.1	201	9.12 / 23.27
Tetraodon nigroviridis	CAI61344.1	201	8.97 / 23.17
Salarias fasciatus	XP_029959064.1	201	9.38 / 23.20
Amphiprion ocellaris	XP_023150780.2	201	9.38 / 23.12
Sphaeramia orbicularis	XP_030013797.1	201	9.24 / 23.34
Oncorhynchus mykiss	XP_036824640.1	241	9.30 / 27.43
Salmo trutta	XP_029603119.1	208	9.99 / 23.77
S. salar	XP_045555938.1	248	9.42 / 27.98

Table 2 The species name, accession number, the amino acid length and theoretical pI/Mw of IL-11 gene between Nile tilapia and other species (Cont.).

Species	**Accession no.**	**Length (aa)**	**Theoretical p*I*/Mw (kDa)**
Fish IL-11b			
O. niloticus		200	8.73 / 22.92
O. aureus	XP_039461968.1	201	8.73 / 22.99
Hippoglossus hippoglossus	XP_034456886.1	201	9.35 / 22.66
Gymnodraco acuticeps	XP_034055634.1	198	6.79 / 22.24
T. rubripes	CAI61343.1	202	6.63 / 23.23
T. nigroviridis	CAI61345.1	211	5.38 / 20.02
Paralichthys olivaceus	BAF80790.1	201	6.82 / 22.90
Xiphias gladius	XP_039977943.1	202	7.20 / 22.58
M. armatus	XP_026156860.1	201	7.82 / 22.88
Echeneis naucrates	XP_029370652.1	228	9.64 / 25.80
Danio rerio	CAI61347.1	194	8.46 / 22.29
O. mykiss	XP_021445673.1	204	9.77 / 23.31
S. salar	XP_045555939.1	205	9.77 / 23.26
S. trutta	XP_029603128.1	204	9.83 / 23.32
Fish IL-11 fish			
Anabarilius grahami	ROI36420.1	151	9.72 / 17.22
Ctenopharyngodon idella	AUN43464.1	199	9.81 / 22.66
Labeo rohita	RXN28148.1	224	9.62 / 25.74
Carassius auratus	AHJ60479.1	198	10.00 / 22.92
Chanos chanos	XP_030641447.1	199	9.67 / 22.76
Pygocentrus nattereri	XP_017561517.1	201	9.52 / 23.0
Bagarius yarrelli	TSR75269.1	226	9.71 / 25.64
Ictalurus punctatus	XP_017325999.1	196	9.76 / 22.53
Clupea harengus	XP_042565075.1	144	9.46 / 16.62

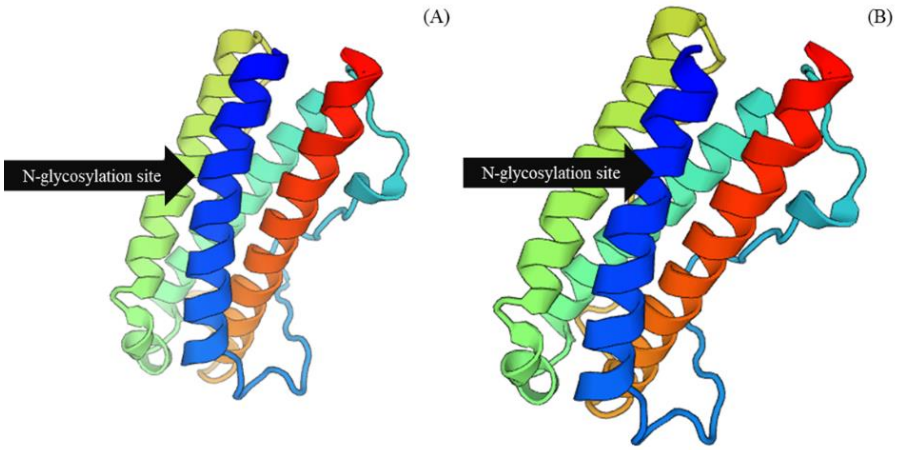


Figure 3 3D protein structures model of IL-11a (A) and IL-11b (B) were predicted by Swiss-model

4.3 Amino acid identity and similarity of the IL-11 gene

The identity and similarity of IL-11 and known IL-11 genes are presented in Table 3. White boxes indicate amino acid similarity and gray boxes indicate amino acid identity. IL-11a of Nile tilapia shared 34.6-81.5% similarity and 20.9-73.9% identity with other fish and mammalian species. IL-11a shared high similarity with *Trachinotus carolinus* (81.5%), *Micropogonias undulatus* (80.3%), *Takifugu rubripes* (79.9%) and *Tetraodon fluviatilis* (79.4%), respectively. The amino acid identity of IL-11a shared with *T. carolinus* (73.9%), *T. rubripes* (73.6%), *M. undulatus* (73.5%) and *T. fluviatilis* (69.7%), respectively. When compared to fish and mammalian species, IL-11b Nile tilapia shared 40.7-98.0% similarity and 23.2-89.5% identity. IL-11b shared high similarities with *T. carolinus* (98.0%), *M. undulatus* (96.5%), *T. rubripes* (96.0%) and *T. fluviatilis* (95.5%), respectively, and shared high identities with *T. carolinus* (89.5%), *T. rubripes* (89.1%), *M. undulatus* (89.0%) and *T. fluviatilis* (84.5%), respectively.

Table 3 Identity and similarity of Nile tilapia IL-11 and known IL-11 genes.
White boxes indicate similarity and gray boxes indicate identity.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Homo sapiens* IL-11		87.4	86.9	27.7	27.4	28.3	25.9	25.6	22.9	25.0	25.2	**20.9**	21.7	24.2	20.6	**24.5**
2	*Mus musculus* IL-11	91.0		97.0	26.4	27.1	26.7	26.4	24.9	22.0	25.6	24.5	**21.4**	22.2	22.4	24.7	**24.4**
3	*Rattus narvegicus* IL-11	89.9	98.0		26.4	27.1	26.7	25.3	24.9	22.0	25.7	24.5	**21.4**	21.7	22.5	23.4	**24.4**
4	*Ctenopharyngodon Idella* IL-11	42.1	41.3	42.3		75.8	78.3	47.8	46.8	55.5	45.5	45.3	**39.7**	26.2	23.9	23.5	**47.3**
5	*Carassius auratus* IL-11	42.1	41.9	42.9	78.8		94.9	55.7	56.2	65.5	55.4	55.2	**46.4**	29.8	26.9	24.5	**56.7**
6	*Cyprinus carpio* IL-11	43.4	42.4	42.9	81.8	96.0		57.7	57.2	67.7	56.9	56.7	**48.1**	31.4	27.1	25.7	**58.7**
7	*Micropogonias undulatus* IL-11	43.5	45.3	44.2	63.2	72.1	73.6		92.5	48.0	88.6	84.0	**73.5**	30.1	28.4	25.8	**89.0**
8	*Trachinotus carolinus* IL-11	44.2	44.1	45.1	63.2	74.1	74.6	98.0		47.1	88.6	86.0	**73.9**	30.7	28.2	23.6	**89.5**
9	*Danio rerio* IL-11a	38.6	37.7	38.1	63.6	74.5	76.4	62.9	63.8		47.1	46.6	**39.0**	25.2	27.8	22.5	**47.1**
10	*Takifugu rubripes* IL-11a	42.1	43.3	43.5	61.4	71.8	72.3	94.0	94.5	61.5		88.6	**73.6**	28.4	28.2	23.4	**89.1**
11	*Tetraodon fluviatilis* IL-11a	40.8	40.7	41.7	62.7	73.6	74.1	93.5	94.0	62.9	94.0		**69.7**	30.1	28.7	24.8	**84.5**
12	*Oreochromis niloticus* IL-11a	**34.6**	**37.5**	**38.7**	**52.7**	**60.3**	**61.1**	**80.3**	**81.5**	**52.5**	**79.9**	**79.4**		25.0	21.9	18.5	**82.8**
13	*Danio rerio* IL-11b	26.7	35.7	36.1	41.9	51.2	53.9	50.0	51.2	44.7	49.3	49.5	41.8		25.1	25.1	**31.1**
14	*Takifugu rubripes* IL-11b	41.1	39.9	40.8	39.0	45.3	48.1	46.5	45.1	45.4	44.1	42.6	35.1	48.3		58.4	**25.8**
15	*Tetraodon fluviatilis* IL-11b	36.7	40.8	39.9	37.3	39.9	42.2	44.0	41.0	40.1	36.9	38.9	33.7	46.3	69.8		**23.2**
16	*Oreochromis niloticus* IL-11b	**40.7**	**42.3**	**43.2**	**62.7**	**72.6**	**73.6**	**96.5**	**98.0**	**62.4**	**96.0**	**95.5**	**83.2**	**50.5**	**41.8**	**40.8**	

4.4 Amino acid sequence analysis of IL-11 genes

Recently, the IL-11 gene has been discovered in several fish genomes such as *T. rubripes, O. niloticus, Mastacembelus armatus* and *O. aureus* and mammalian species such as *Homo sapiens, Mus musculus, Rattus narvegicus and Gallus gallus.* Figure 4 showed multiple alignments of IL-11 were produced using ClustalW (1.2.4) from the predicted peptide sequence to further understand the conservation of IL-11. The alignment showed four α-helices in the boxed. Only Nile tilapia and other fish IL-11 had one conserved cysteine residue (C1). There are sixteen consensuses in the black box or under the star symbol (*) that means the residues in that column are identical in all sequences in the alignment. Twenty-six of conserved under the colon symbol (:) means that conserved substitutions have been observed, and there was nine semi-conserved under period symbol (.) that means amino acid was replaced by one having similar characteristics.

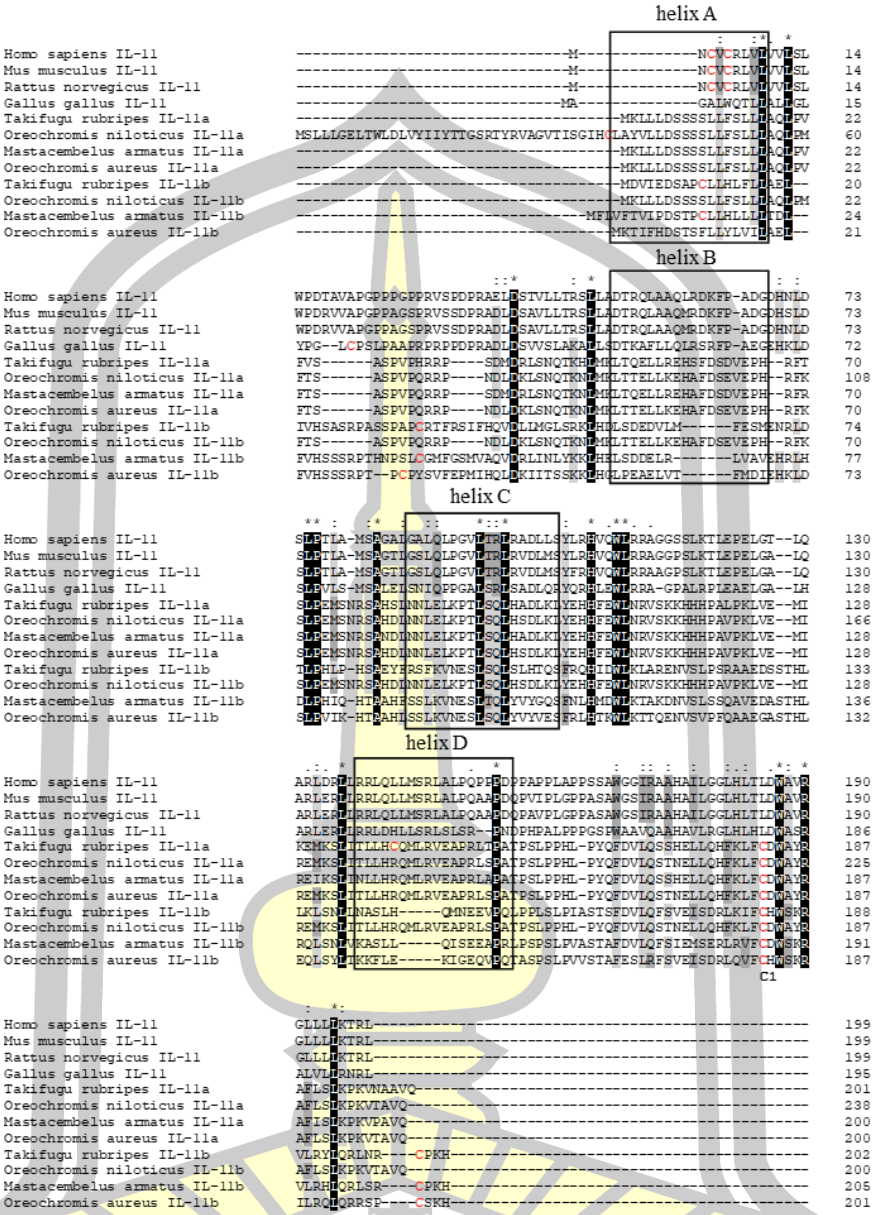


Figure 4 Amino acid sequence alignment of IL-11 from Nile tilapia and other fish and mammalian species.

The multiple alignment was produced using ClustalW (1.2.4).

4.5 Synteny analysis by using Genomicus Database

Synteny and gene organization of IL-11 between the Nile tilapia and other fish and mammalian species are presented in Figure 5. Human, mouse, chicken, horse, fugu, zig-zag eel, and blue tilapia reference genomes were used for the synteny analysis of IL-11a and IL-11b for information to support their relationship. IL-11a of fugu (Chr. 7), Nile tilapia (Chr. LG11), zig-zag eel (Chr. OOHQ01000011.1) and blue tilapia (Chr. VASH01006871.1) shared a conserve syntenic relation as the gene m6pr, zgc:92606, trpv6, ephb6, slc2a3b, cox6b2, rasip1, nat14, aicda, sbk3, epn1, foxj2, isoc2, atg12, si: ch211-149k23.9, and rcvrn2 and IL-11b of fugu (Chr. 12), Nile tilapia (Chr. LG22), zig-zag eel (Chr. OOHQ01000103.1) and blue tilapia (Chr. VASH01000140.1) the gene si:ch211-171h4.3, necap1, zgc:194578, si:ch211-232m10.6, rcvrn3, tmem238a, ccdc106b, shisa7b, and dbpa shared a conserve syntenic relationship. It was observed that the IL-11a of fish syntenic genes were conserved more than IL-11b, and there were not conserved between IL-11a of fish and IL-11 of mammalian.

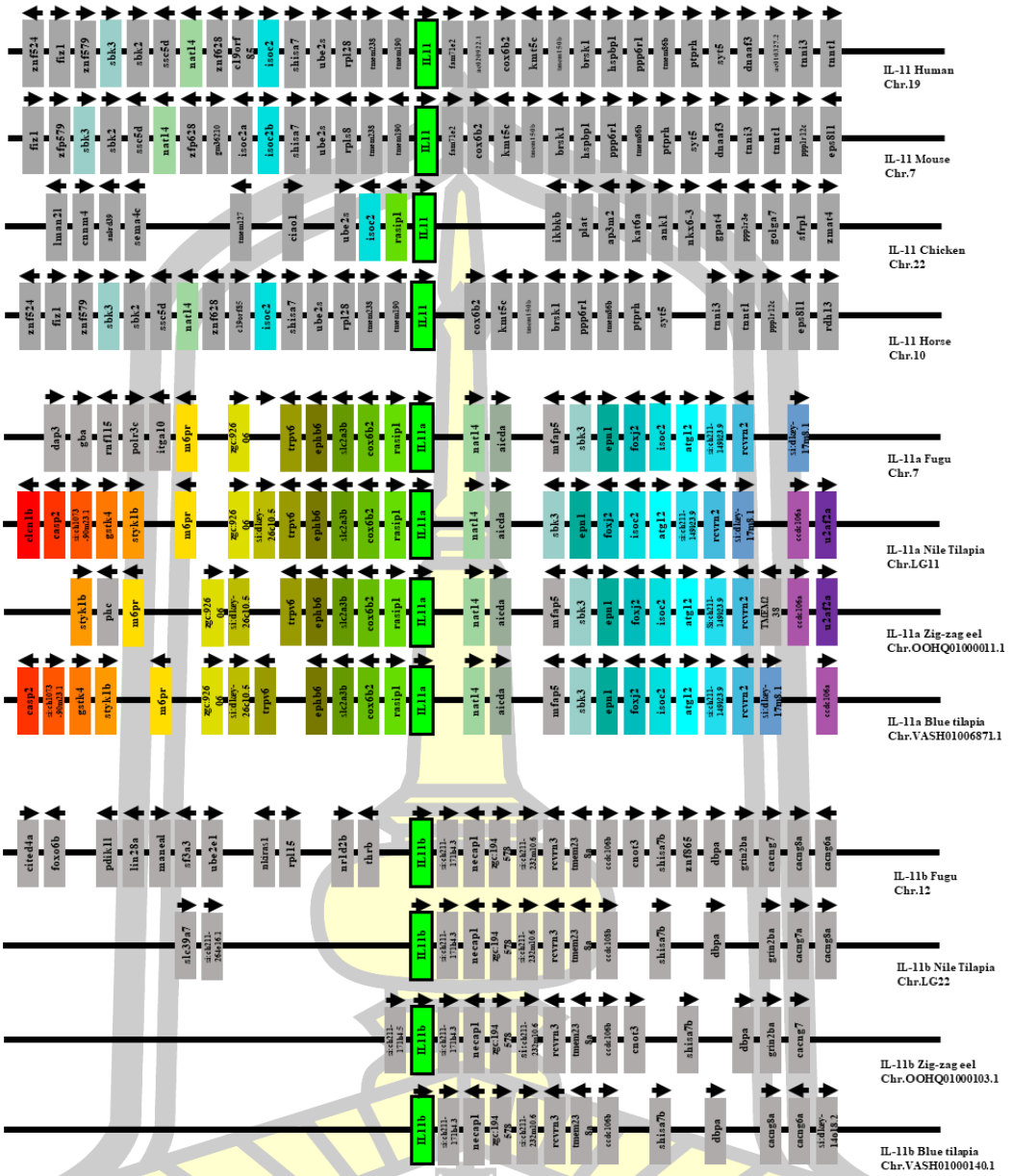


Figure 5 Gene synteny of IL-11 between Nile tilapia and other species were predicted using the Genomicus v100.01 program.
Arrows show the direction of genes.

4.6 Genome organization analysis by using Genomicus database

The exon and intron size of IL-11a and IL-11b genes from fish and mammalian species are presented in Figure 6. The IL-11a and IL-11b genes of fish and IL-11 gene of mammals had 5 exons and 4 introns, except for IL-11b in zig-zag eels, which had 4 exons and 3 introns. Exon 3 of IL-11 mammalian and IL-11a of fish

were relatively similar. Except for the zig-zag eel's IL-11b, all fish IL-11a and IL-11b had a conserved exon in exon 3 (96 bp). The IL-11a of fish had a conserved exon in the exon 3 (96 bp) and exon 4 (162 bp), while IL-11b had a conserved exon in the exon 3 (96 bp) and exon 4 (168 bp). The IL-11a and IL-11b of Nile tilapia were generally relatively similar to those of fish species.

Figure 6 Comparison of the gene intron/exon sizes between Nile tilapia IL-11 and known IL-11 genes.

The number of base pairs in the introns and exons of the IL-11 genes is shown where numbers indicate the regions containing the coding sequence. White boxes indicate coding regions and gray boxes indicate untranslated regions of exons for each gene.

4.7 Evolution relationship of IL-11 genes by phylogenetic tree analysis

The phylogenetic tree of the IL-11 based on multiple alignments of IL-11a and IL-11b as showed in Figure 7. There were 2 major distinct clades, fish and other mammalian IL-11. The IL-11 of teleost fish had 2 clades included IL-11 and IL-11b, while IL-11a were within IL-11 teleost clade. The teleost IL-11a clade contains both IL-11a and IL-11b of *O. niloticus* in addition to the IL-11a paralogues of *Gasterosteus aculeatus, Salarias fasciatus, Amphiprion ocellaris, O. aureus, M. armatus, Sphaeramia orbicularis, T. rubripes and T. nigroviridis*. In addition, both IL-11a and IL-11b *O. niloticus* were within the IL-11a of teleost branch, as same the IL-11a and IL-11b of *Salmo trutta*. Nile tilapia IL-11a and IL-11b, there were closed by *O. aureus* IL-11a.

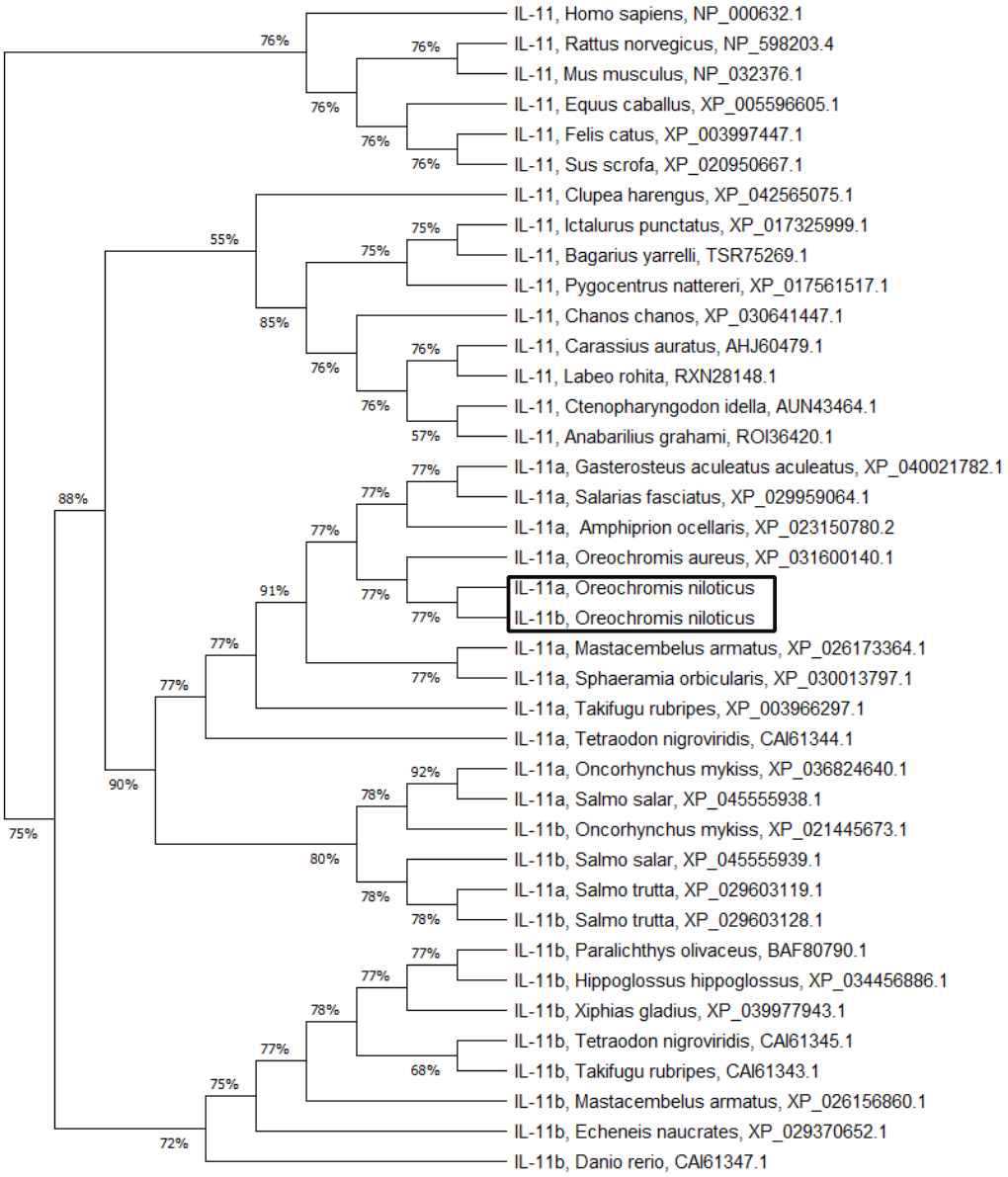


Figure 7 Phylogenetic tree of IL-11.

The phylogenetic tree was constructed using the neighbor-joining method with the bootstrap value of 10,000 times in the Molecular Evolutionary Genetics Analysis software version 10 (MEGA10).

4.8 Tissues distribution analysis of IL-11 genes in Nile tilapia

The expression of IL-11a and IL-11b was analyzed in 11 tissues of healthy Nile tilapia to gain further about their potential functions, and level of β-actin was

used to normalize the relative expression level. As shown in Figure 8, tissue distribution was expressed in all the tissues examined. The expression of IL-11a was found to be higher in the spleen, liver, intestine and trunk kidney, and low expression in the gonad, skin, muscle, head kidney and gills, and low expression in the tail and heart. While IL-11b was higher expression in the spleen and tail, and less expression in heart, liver, gonad, skin and intestine, and low expression in trunk kidney, muscle, head kidney and gills. The results showed that the most predominant expression levels of IL-11a were detected in spleen, liver, intestine and trunk kidney and IL-11b were detected in spleen and tail, respectively.

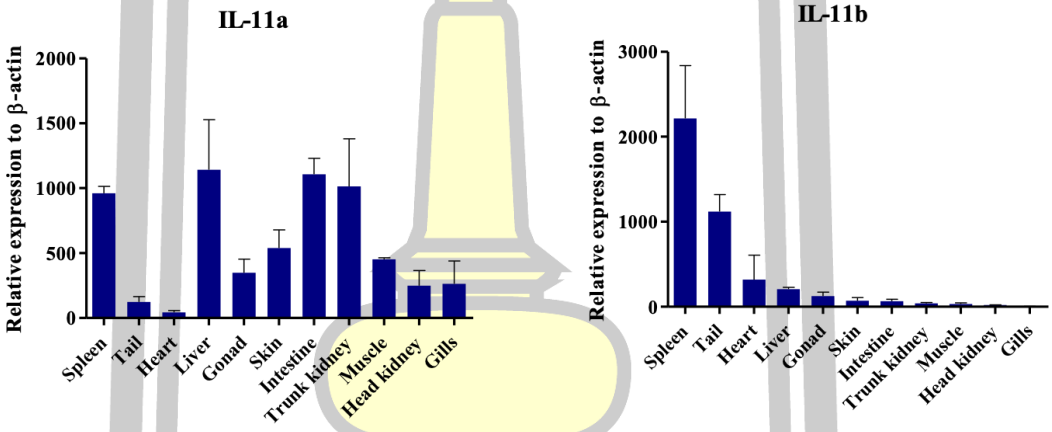


Figure 8 Tissue distribution of IL-11a and IL-11b in 11 tissues of Nile tilapia.
The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of three fish (N=4).

4.9 IL-11 expression analysis in the immune tissues of Nile tilapia after *S. agalactiae* infection

To better clarify the responses and the immunological functions of Nile tilapia IL-11a and IL-11b to *S. agalactia* challenge, its expression in the gills, intestine, spleen and liver of Nile tilapia as different time point from post infection is presented in Figure 9. In the gills, the expression of IL-11b was significantly higher than IL-11a and it was increased from 6 to 72 h and reached the peak at 6 h ($P<0.05$). In the intestine, the expression of IL-11b was significantly higher than of IL-11a and reached the peak at 6 h ($P<0.05$). In the spleen, the expression of IL-11a and IL-11b

were significantly higher expression at 6 h (P<0.05). In the liver, the expression of IL-11a and IL-11b were significantly higher expression from 6 to 24 h and reached the peak at 6 h (P<0.05). The expression of IL-11a gene was not significantly different in the gills and intestine, but in the spleen and liver was up-regulated and reached the peak at 6 h (P<0.05). It was interesting that in all tissues analyzed, IL-11a expression was lower than IL-11b at the peak expression and can be assumed that IL-11b was responsible for the resistance of pathogens when infected.

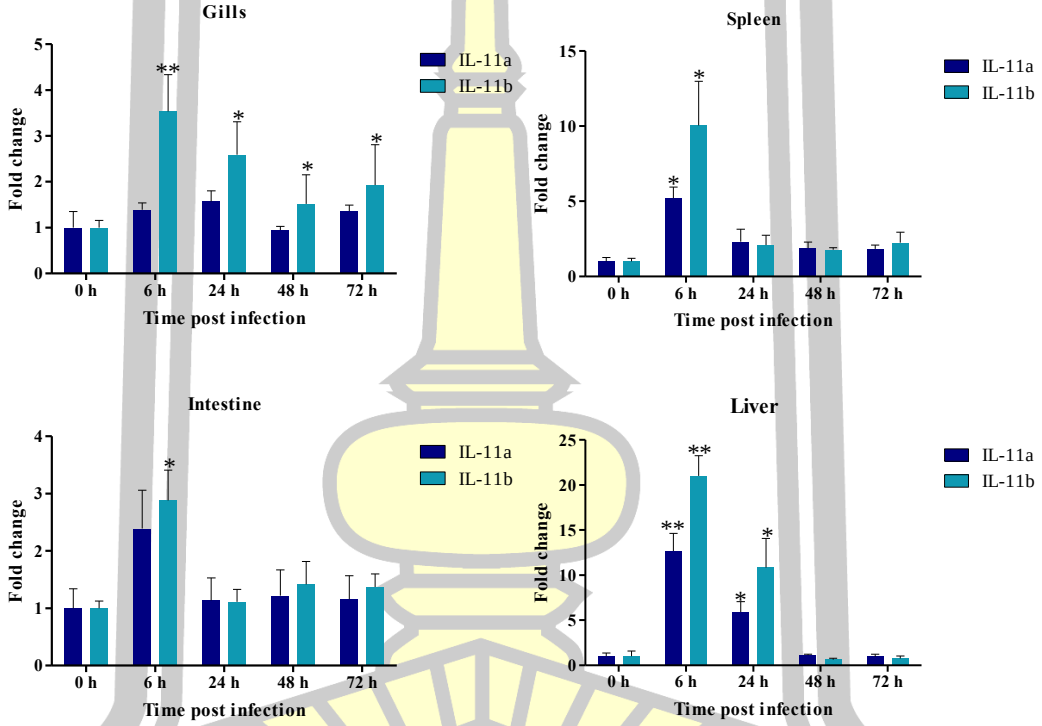


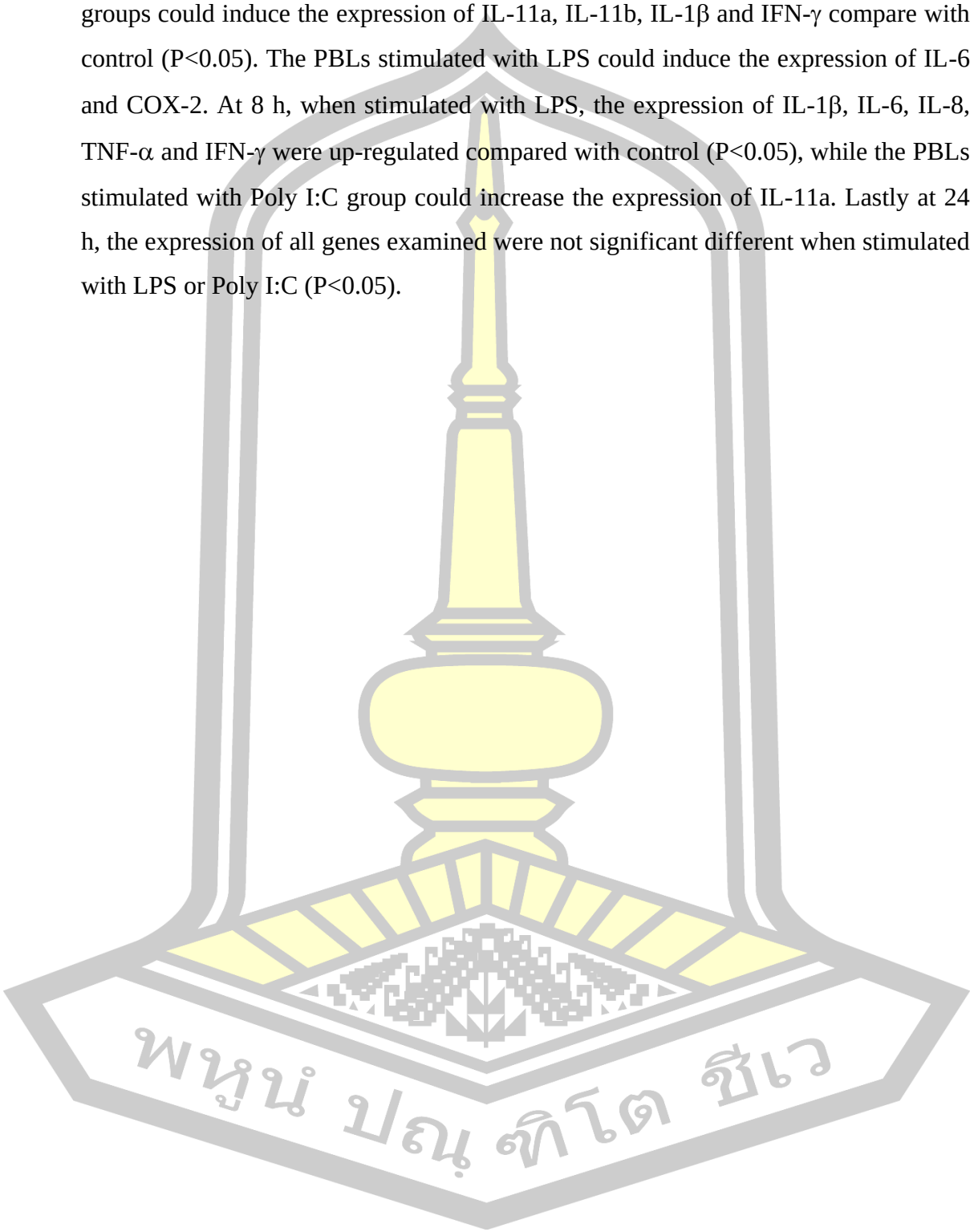
Figure 9 Gene expression of IL-11a and IL-11b in gills, intestine, spleen and liver of Nile tilapia during bacterial infection.

The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of 4 fish.

4.10 Gene expression of immune-related genes in PBL stimulanted by LPS and Poly I:C at different time point

Expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 in PBLs stimulated with LPS (1 μg/mL) and Poly I:C (100 μg/mL) at different time

point is presented in Figure 10. At 4 h, the PBLs stimulated with LPS and Poly I:C groups could induce the expression of IL-11a, IL-11b, IL-1β and IFN-γ compare with control ($P<0.05$). The PBLs stimulated with LPS could induce the expression of IL-6 and COX-2. At 8 h, when stimulated with LPS, the expression of IL-1β, IL-6, IL-8, TNF-α and IFN-γ were up-regulated compared with control ($P<0.05$), while the PBLs stimulated with Poly I:C group could increase the expression of IL-11a. Lastly at 24 h, the expression of all genes examined were not significant different when stimulated with LPS or Poly I:C ($P<0.05$).



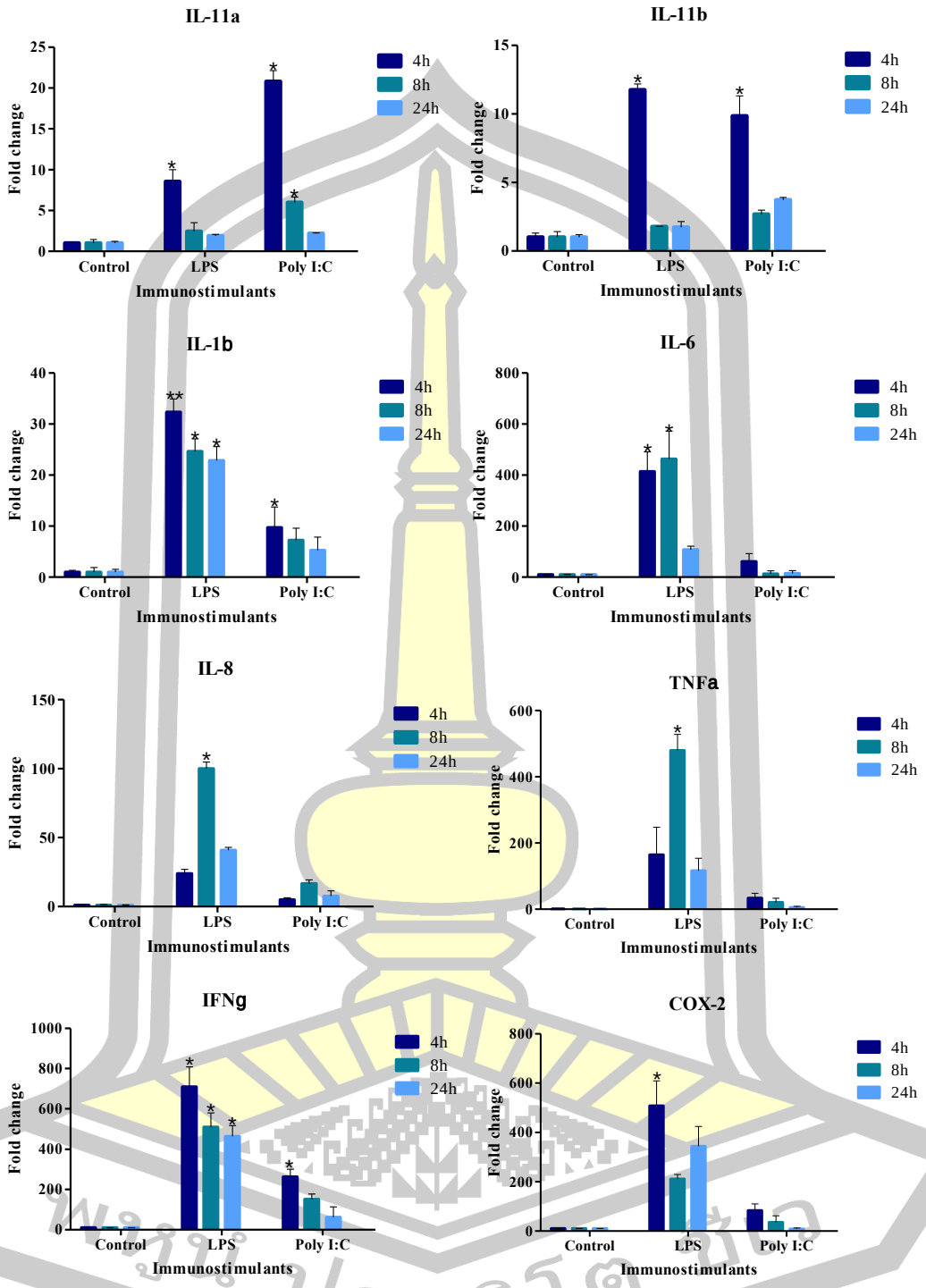


Figure 10 Gene expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 stimulated by LPS (1 ng/mL) and Poly I:C (100 ng/mL) at 4, 8 and 24 h in the PBLs.

The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of 3 fish.

4.11 Gene expression of immune-related genes in PBL stimulant by LPS at different concentration

The expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 in PBLs stimulated with LPS at different concentration (1, 10, 100 ng/mL) is presented in Figure 11. At the concentrations of 1 ng/mL LPS, the expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α and COX-2 were up-regulated, while the expression in gene IFN-γ was up-regulated at 1 and 10 ng/mL LPS compared with control group ($P<0.05$). As a result, the expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α and COX-2 in PBLs stimulated by LPS showed higher at 1 ng/mL, suggesting that 1 ng/mL was optimum concentration to induce PBLs more than 10 and 100 ng/mL.

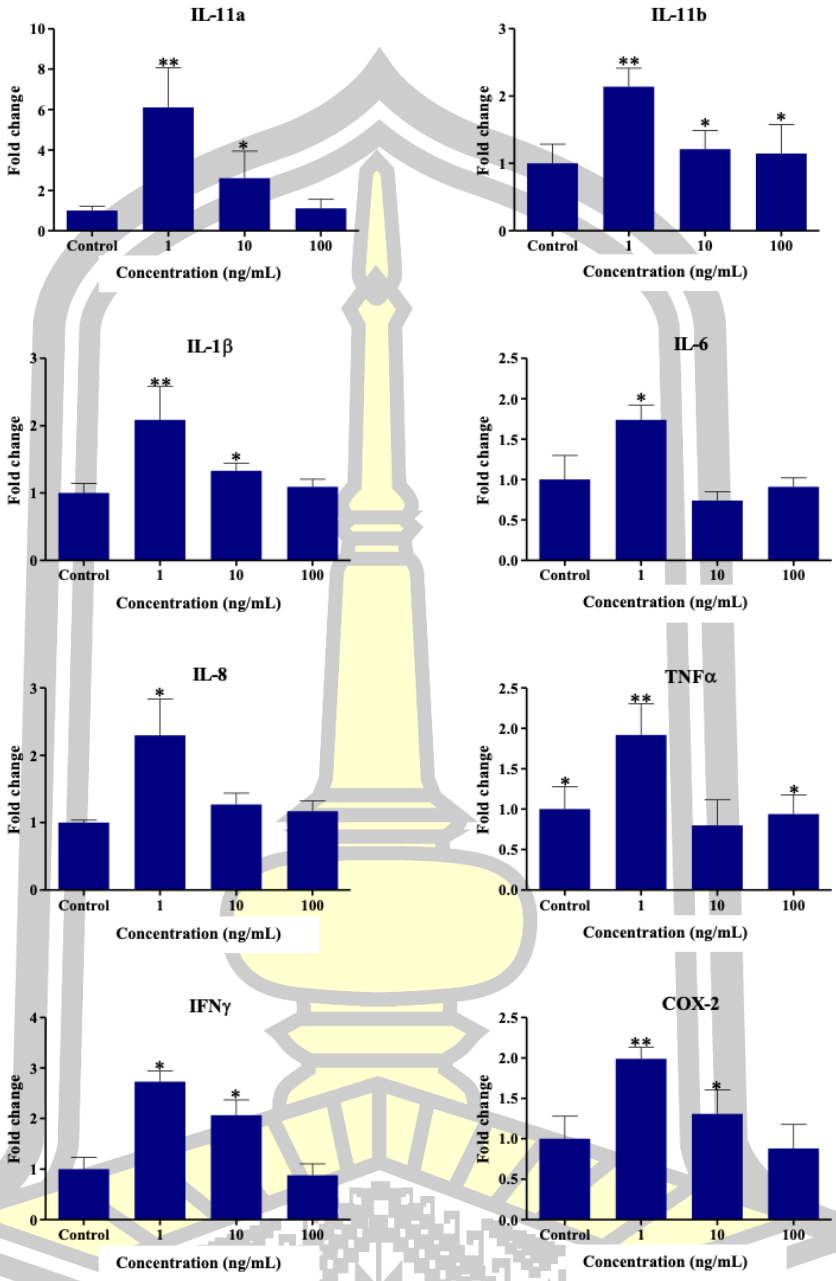


Figure 11 Gene expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 in the PBLs stimulated by LPS with different concentrations.
The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of 3 fish.

4.12 Gene expression of immune-related genes in PBL stimulant by Poly I:C at different concentration

The expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 in PBLs stimulated by Poly I:C at different concentration (1, 10, 100 ng/mL) is presented in Figure 12. At the concentrations of 100 μg/mL Poly I:C, the expression of IL-11a, IL-11b, IL-1β and TNF-α were up-regulated, and the expression in gene COX-2 was up-regulated at 10 and 100 ng/mL Poly I:C, while the expression of gene IL-6, IL-8 and IFN-γ were not significant up-regulated in all concentration compared with control ($P<0.05$). As a result, expression of IL-11a, IL-11b, IL-1β and TNF-α in PBLs stimulant by Poly I:C showed higher at 100 ng/mL, suggesting that 100 ng/mL was optimum concentration to induce PBLs more than 1 and 10 ng/mL.

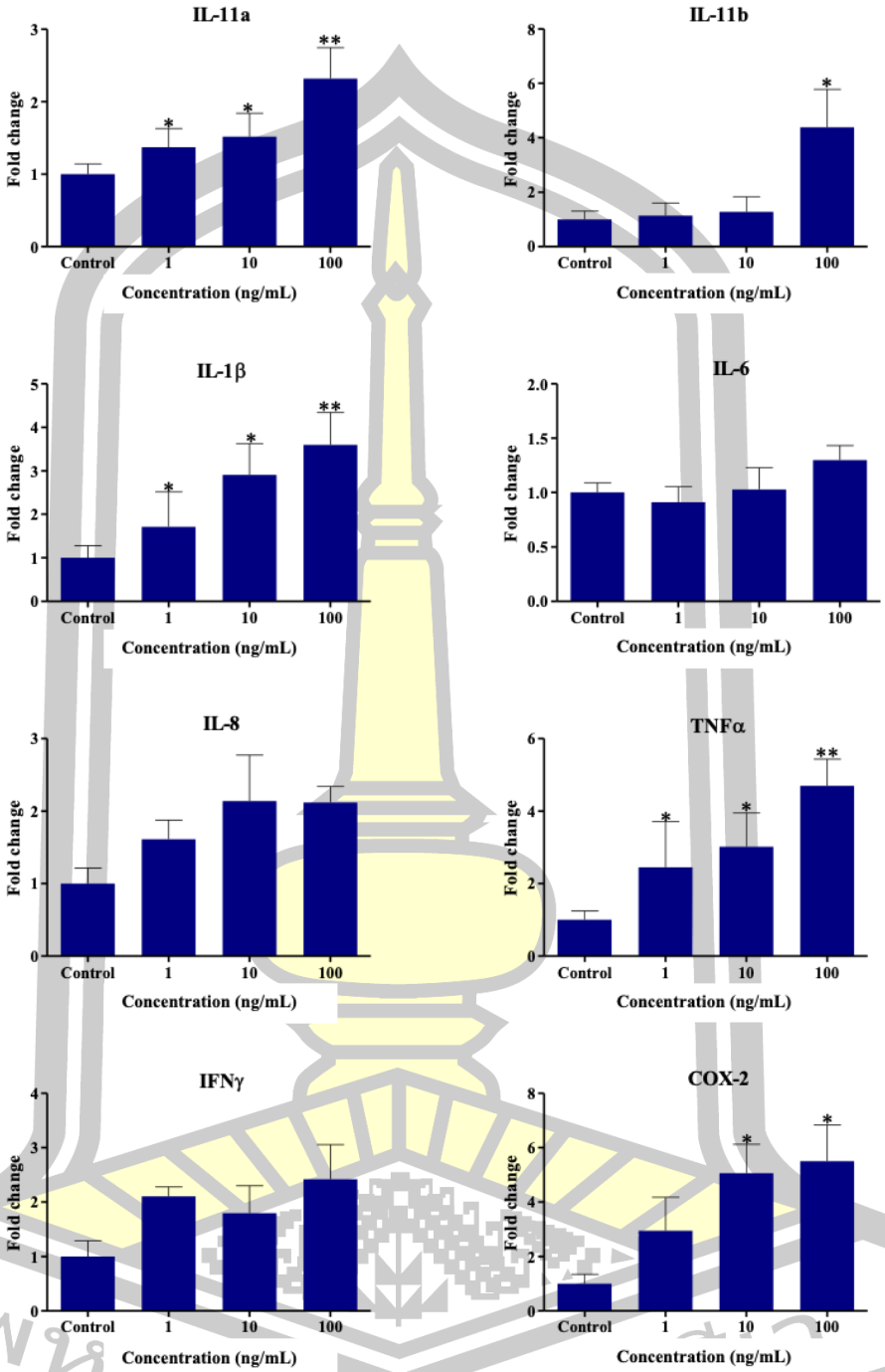


Figure 12 Gene expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 in the PBLs stimulated by Poly I:C with different concentrations.

The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of 3 fish.

4.13 Protein expression and purification

A protein with the predicted molecular weight of 22.005 kDa was induced by IPTG stimulation of transformed BL21 cells and extensive washing in 1% Triton X-100 buffer under denaturing conditions to remove LPS as showed in Figure 13. The rIL-11 gene was efficiently expressed after IPTG induction (lane 2). The expressed rIL-11 was purified and used for adjuvant preparation (lane 3).

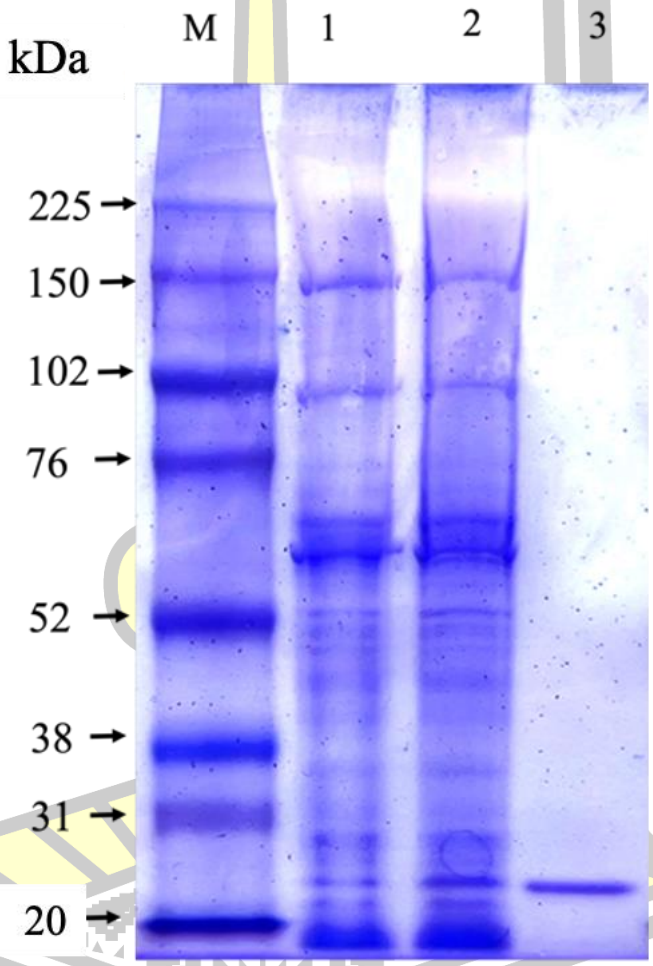


Figure 13 SDS-PAGE.
Lane M, protein molecular marker; lane 1, pXN-IL-11 without IPTG induction; lane 2, pXN-IL-11 with IPTG induction; lane 3, purified rIL-11 protein.

4.14 Gene expression of immune-related genes in PBL stimulant by rIL-11 at different concentration

The expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 in PBLs stimulated with rIL-11 at concentration 5, 50, 500 ng/mL is presented in Figure 14. At the concentrations of 50 ng/mL rIL-11, the expression of IL-11a, IL-11b, IL-1β, IL-6, TNF-α, IFN-γ and COX-2 were significantly up-regulated ($P<0.05$), while the expression of gene IL-8 was not significant up-regulated in all concentration compared with control ($P<0.05$). The results showed that the most predominant expression levels were up-regulated and can be detected in PBLs stimulant by rIL-11 at 50 ng/mL, that was the optimum concentration to induce PBLs more than 5 and 50 µg/mL. In addition, results suggested that PBLs stimulant by rIL-11 induced the expression of IL-11a, IL-11b, IL-1β, IL-6, TNF-α, IFN-γ and COX-2.

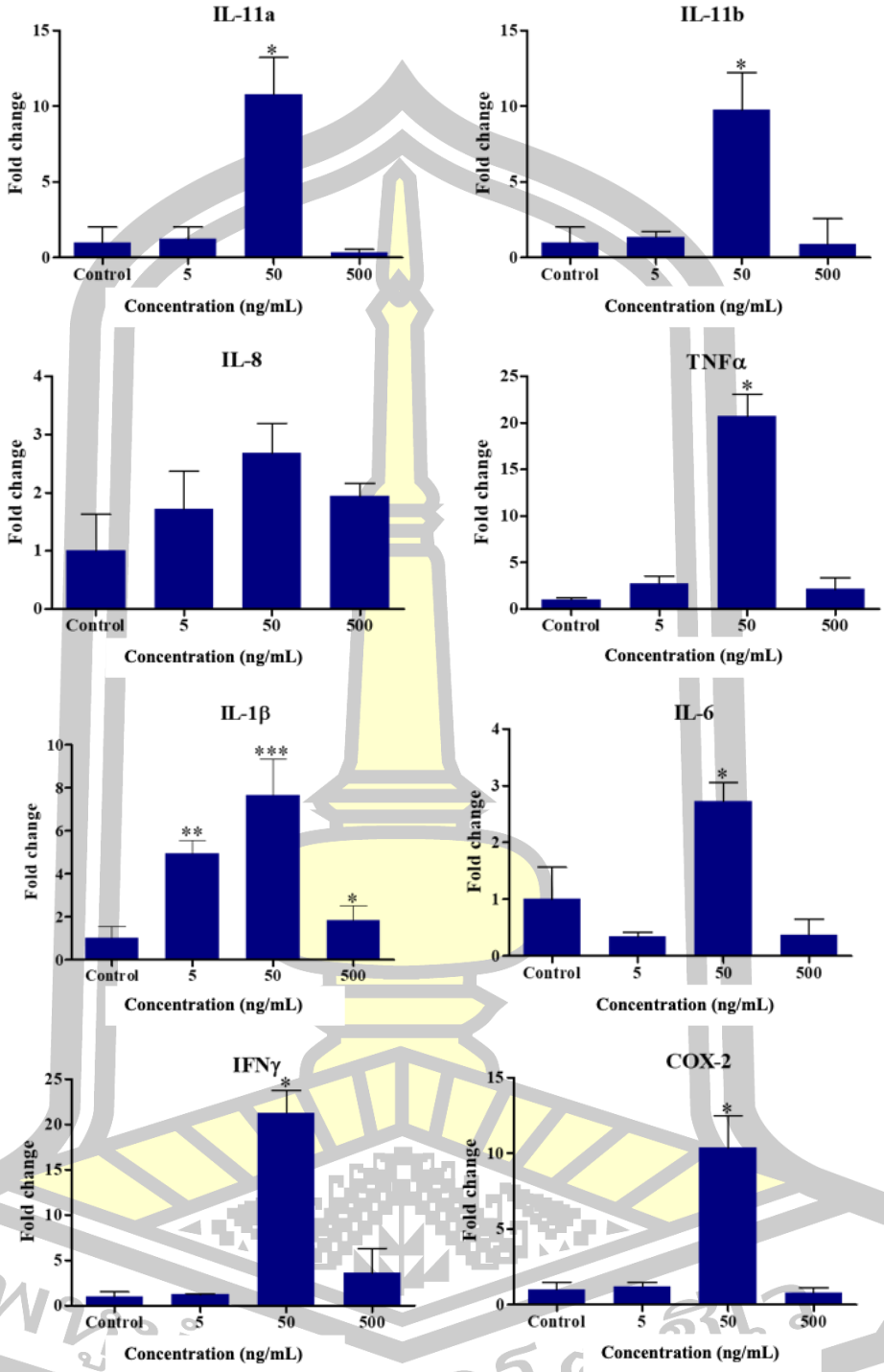


Figure 14 Gene expression of IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNF-α, IFN-γ and COX-2 in the PBLs stimulated by rIL-11 with different concentrations.

The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of 3 fish.

4.15 Adjuvant effect of rIL-11 in the streptococcosis fomalin-killed vaccine on gene expression analysis in spleen after vaccination

The relative expression levels of IL-1β, IL-8, TNF-α, IL-11a, IL-11b, IL-6, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ in spleen were assessed using RT-qPCR is presented in Figure 15. At day 1, the expression of IL-8, TNFα, IL-11a, IL-11b and IL-6 in the FKC + rIL-11 group was significantly up-regulated compared with the PBS group ($P<0.05$). The expression of IL-8 and IL-11a in FKC group was significantly up-regulated compared with the PBS group ($P<0.05$). On the other hand, expression of IL-1β, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ were not significant in all group ($P<0.05$). At day 3, the expression of IL-1β, IL-8, TNF-α and IL-6 in FKC + rIL-11 and FKC group was significantly up-regulated when compared with the PBS group ($P<0.05$). The expression of IL-11a, IL-11b, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ were not express significant in all group ($P<0.05$). While at day 14, all of genes examined were not significant differences in all group ($P<0.05$). As a result, expression of IL-1β, IL-8, TNF-α and IL-6 in FKC + rIL-11 group showed higher tendency at 1 and 3 days post vaccination and returned to the control level at 14 days post vaccination, suggesting that these expression profiles may suggest multiple cells or signaling pathways are activated by rIL-11 in different tissues.

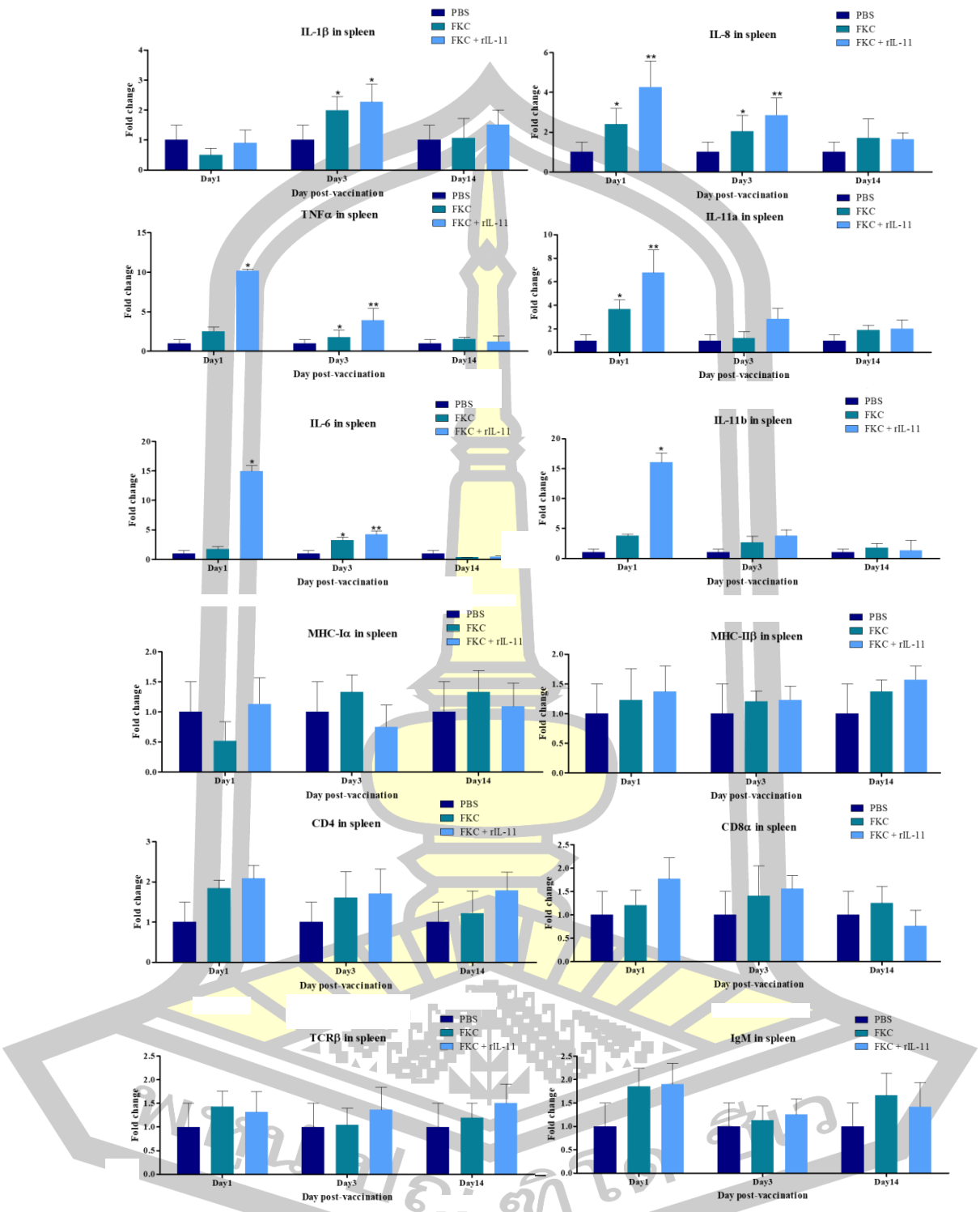


Figure 15 Gene expression in spleen of Nile tilapia post vaccination.

The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of 4 fish.

4.16 Adjuvant effect of rIL-11 in the streptococcosis fomalin-killed vaccine on gene expression analysis in liver after vaccination

Gene expression of IL-1β, IL-8, TNF-α, IL-11a, IL-11b, IL-6, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ in liver collected from vaccinated fish were analyzed and is presented in Figure 16. At day 1, the FKC + rIL-11 and FKC group could be induce the expression of IL-8 and TNF-α compared with the PBS group ($P<0.05$). On the other hand, expression of IL-1β, IL-11a, IL-11b, IL-6, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ were not significant difference in all groups ($P<0.05$). At day 3, the FKC + rIL-11 and FKC group could be induce the expression of IL-8 and TNF-α compared with the PBS group ($P<0.05$). The expression of IL-1β, IL-11a, IL-11b, IL-6, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ were not significantly different in any groups ($P<0.05$). While at day 14, the FKC + rIL-11 group could be induced the expression of IL-8, IL-11a and IL-11b compared with the PBS group ($P<0.05$). The expression of IL-1β, TNFα, IL-6, MHC-Iα, MHC-IIβ, IgM, CD4, CD8α and TCRβ were not significantly different in any groups ($P<0.05$). As a result, expression of TNF-α in FKC + rIL-11 group showed higher tendency at 1 and 3 days post vaccination, and returned to the control level at 14 days post vaccination and IL-8 was expression in all time point. However, expression of IL-11a and IL-11b showed higher at 14 day post vaccination, suggesting that rIL-11 control the expression of humoral and cell-mediated immune response in liver tissue.

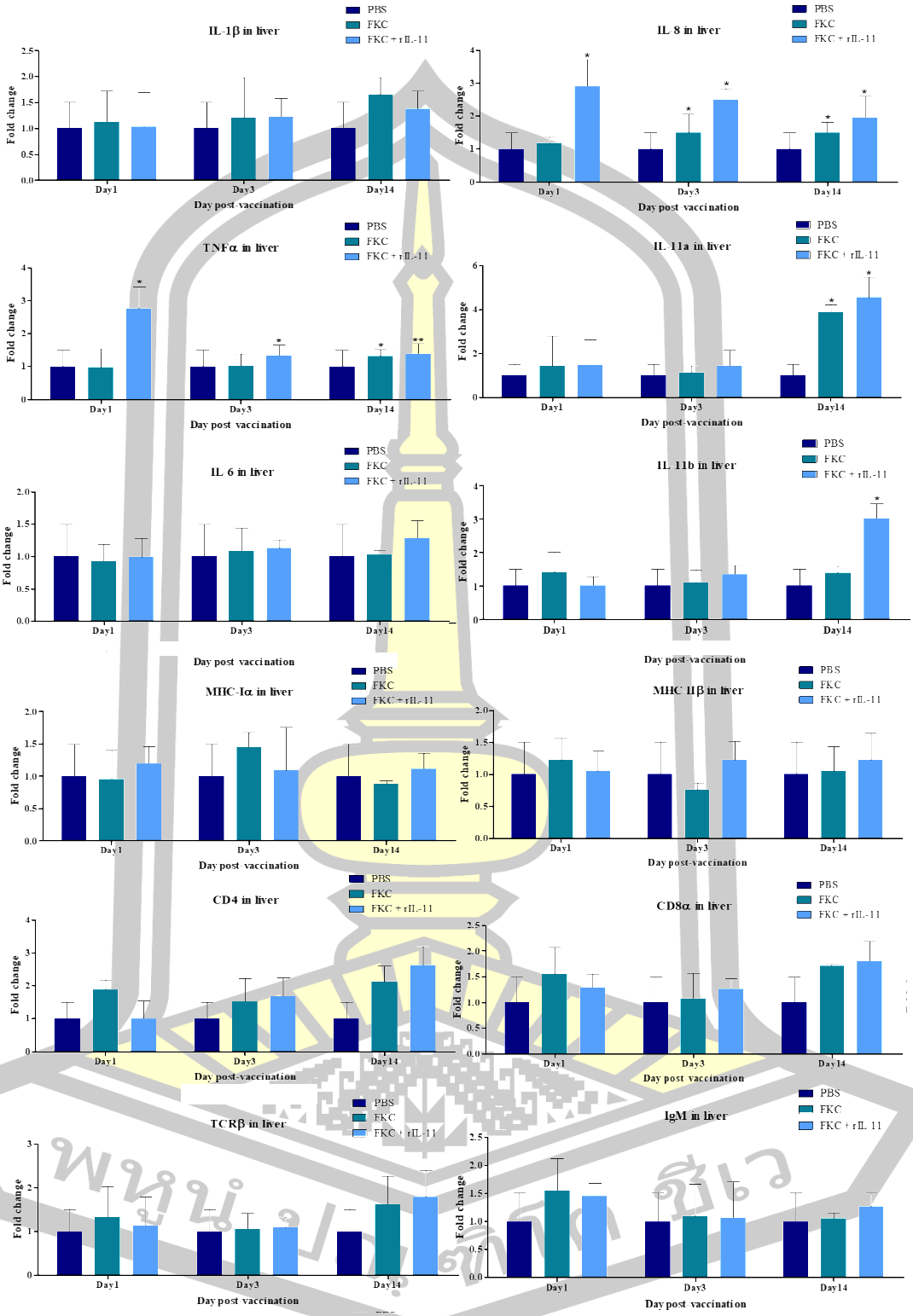


Figure 16 Gene expression in liver of Nile tilapia post vaccination.

The level of β-actin was used to normalize the relative expression level. The results are the mean + SEM of 4 fish.

4.17 Antibody titer measurement by enzyme-linked immunosorbent assay (ELISA)

To examine the IgM antibody production in vaccinated fish, antibody titer was measured by using Nile tilapia serum at 0, 1, 2, 3, 4 and 5 week post vaccination (w.p.v.) and is presented in Figure 17. The result revealed that at each time points, serum from the FKC + rIL-11 group had the highest antibody levels, and significantly higher compared to the control group ($P<0.05$). In fish that injected the adjuvanted and vaccines, the antibody IgM levels increased from 1 to 2 w.p.v. and peak at 2 w.p.v., then decreased until 5 w.p.v. ($P<0.05$). While, the FKC group the IgM antibody level was significantly lower than that of FKC + rIL-11 group at 1-5 w.p.v although the IgM antibody titer decreased at 1-3 w.p.v. ($P<0.05$). These responses suggested that, in the humoral response production can be induced by rIL-11 when combined with FKC vaccine.

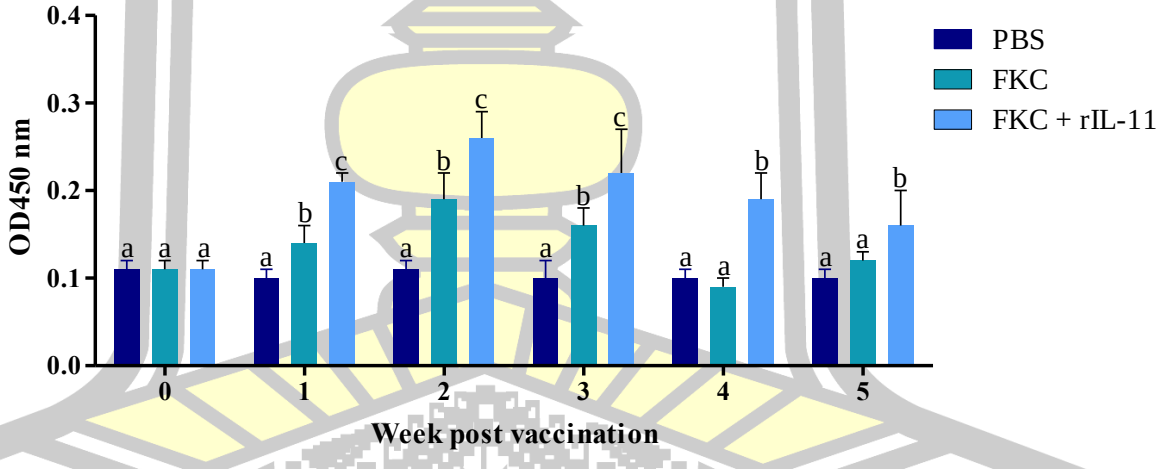


Figure 17 Specific antibody (IgM) levels in serum of Nile tilapia vaccinated with inactivated vaccine.

Serum was collected at different time points post vaccination from fish vaccinated with PBS (control), FKC and FKC + rIL-11, respectively. Data are presented as mean + SEM (N=6). Specific IgM antibody level were determined by ELISA measuring at OD450 nm. Different letters above each bar showed the significant different ($P<0.05$).

4.18 Innate immune response analysis

Serum LZM activity of Nile tilapia vaccinated with FKC, FKC + rIL-11 compared with PBS as a control group is presented in Table 4. The LZM activity of the FKC + rIL-11 group was significantly higher than that of the PBS and FKC groups at 2 and 4 w.p.v. ($P<0.05$). At 1, 3 and 5 w.p.v. the LZM activity was not different. The highest LZM activity was found in the FKC + rIL-11 group at 4 w.p.v. ($P<0.05$). These results suggested that the LZM activity can be induced by rIL-11.

Serum MPO activity of Nile tilapia vaccinated with FKC, FKC + rIL-11 compared with PBS as a control group is presented in Table 4. The result showed the MPO activity of FKC + rIL-11 group was significantly higher than that of the PBS and FKC groups at all the time points post-vaccination excepted week 3 ($P<0.05$). The highest MPO activity was found in the FKC + rIL-11 group at 4 w.p.v. ($P<0.05$). These results suggested that the MPO activity can be induced by rIL-11.

Serum GRD activity of Nile tilapia vaccinated with FKC, FKC + rIL-11 compared with PBS as a control group is presented in Table 4. The group vaccination with rIL-11 showed significantly higher levels of GRD at all the time point post-vaccinated experimental when compared to those treated with PBS and FKC only. The highest GRD activity was found in the FKC + rIL-11 group at 5 w.p.v. ($P<0.05$). These results suggested that the GRD activity can be induced by rIL-11.

Serum GPx activity of Nile tilapia vaccinated with FKC, FKC + rIL-11 compared with PBS as a control group is presented in Table 4. Serum was collected at different time points post-vaccination, the highest increase in GPx activity was observed in FKC + rIL-11 group and the lowest increase was observed in PBS control group. The result showed the GPx activity of FKC + rIL-11 group was significantly higher than that of the PBS and FKC groups at 2 and 4 w.p.v. ($P<0.05$). At week 1, 3 and 5, the GPx activities were not significantly different.

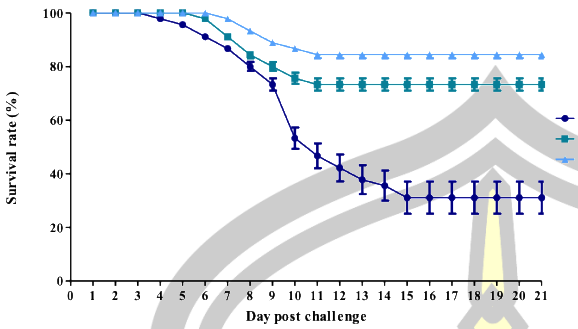
Table 4 Serum LZM activity, MPO activity, GRD activity and GPx activity of Nile tilapia vaccinated with inactivated vaccine.

Serum was collected at different time points post vaccination from fish vaccinated with PBS (control), FKC and FKC + rIL-11 (N=8).

Parameters	**Control**	**FKC**	**FKC + rIL-11**	**Pooled SEM**	**P-value**
LZM(U/mL)					
Week 1	1.68	1.84	1.76	0.37	0.956
Week 2	2.08^{ab}	1.76^{a}	2.72^{b}	0.20	0.049
Week 3	2.32	3.20	3.28	0.31	0.182
Week 4	2.72^{a}	3.84^{ab}	5.30^{b}	0.42	0.008
Week 5	4.00	4.32	4.56	0.40	0.734
MPO (OD at 450 nm)					
Week 1	1.52^{a}	1.50^{a}	2.31^{b}	0.21	0.044
Week 2	1.23^{a}	1.30^{a}	2.09^{b}	0.14	0.005
Week 3	0.97	0.95	1.64	0.24	0.157
Week 4	1.35^{a}	1.36^{a}	1.87^{b}	0.09	0.008
Week 5	2.19^{a}	2.37^{ab}	2.65^{b}	0.08	0.012
GPx (U/mL)					
Week 1	17.36	17.49	19.16	0.71	0.305
Week 2	16.94^{a}	15.18^{a}	21.54^{b}	0.71	0.000
Week 3	25.34	24.44	28.07	1.13	0.145
Week 4	15.24^{a}	17.46^{ab}	21.00^{b}	1.47	0.050
Week 5	25.88	30.32	23.83	2.36	0.187
GRD(U/mL)					
Week 1	3.32^{a}	3.60^{a}	4.74^{b}	0.21	0.003
Week 2	3.52^{b}	2.81^{a}	5.38^{c}	0.14	0.000
Week 3	5.66^{ab}	5.03^{a}	7.18^{b}	0.24	0.026
Week 4	4.35^{a}	5.62^{ab}	6.75^{b}	0.09	0.012
Week 5	5.67^{a}	5.56^{a}	12.77^{b}	0.08	0.000

4.19 Survival rate of vaccinated fish in challenge study

The survival rate statistics may show as effectively the non-specific immune system defends against bacteria. In all groups, the dead fish was not seen during the vaccination period (35 days after vaccination). As showed as Figure 18, after challenge by *S. agalactiae* injection, the dead fish were first observed 3 days post injection. RPS was calculated to determine the effectiveness of the FKC + rIL-11 vaccine. The RPS of FKC and FKC + rIL-11 vaccinated fish were 61.28 and 77.41%, respectively.



	No. mortality	% mortality	RPS
Control	31	68.89	-
FKC	12	26.67	61.28
FKC + rIL-11	7	15.56	77.41

Figure 18 Survival rate of vaccinated Nile tilapia challenged with *S. agalactiae* for long immunity duration.

After vaccination, fish in vaccinated group and control group were I.P. challenged with 1.0×10^8 CFU/fish of *S. agalactiae*. Fish mortality was recorded for 21 days until there were no dead fish. The experiment was conducted in triplicate. Error bars represented standard deviations.

Chapter 5
Discussions and Conclusions

5.1 Discussions

IL-11, IL-6 family members plays an important role in the host immunological response to infection and the cytokine network. It promotes the proliferation and differentiation of both primary and secondary hematopoietic progenitors, either by itself or in conjunction with a number of other growth factors (Du and Williams, 1994; Du and Williams, 1997). IL-11 is able to stimulate generation of variety acute phase plasma protein in hepatocytes as well as promotes B cell immunoglobulin synthesis. Through reducing macrophage and production of ROS, IL-11 has an impact on the inflammatory response (Waxman *et al*., 1998). It also defends and repairs mucosal of respiratory and gastrointestinal in response to injury (Redlich *et al*., 1996). In fish, IL-11 plays a key role as the coordinator of immune response and antibacterial and antiviral mechanisms (Mulero *et al*., 2005; Huising *et al*., 2005). Recently, the IL-11 molecule has been discovered and characteriezed in fish (Huising *et al*., 2005). In this study, we characterized and sequence analysis of both IL-11a and IL-11b of Nile tilapia between known other species IL-11 gene.

Characterization of IL-11a and IL-11b gene in Nile tilapia, with ORF of 717 and 603 bp and the amino acid sequence consisted of 238 and 200, respectively. The potential N-glycosylation site (NQT) were located at positions 80-82 and 42-44 in IL-11a and IL-11b, respectively, while it was located at positions 42-45 in IL-11 of common carp (Huising *et al*., 2005). 3D structure was predicted by Swissprot databases and encoded amino acid sequence IL-11a and IL-11b Nile tilapia by BLAST. Additionally, the Nile tilapia IL-11a and IL-11b predicted structure was in support of a topology with four α-helices that is maintained by the interaction of hydrophobic internal surfaces and hydrophilic solvent exposed surfaces.

The presence of an IL-11 gene is confirmed across all teleost fish species, and the similarity between IL-11 peptides of different fish species reduces with increasing phylogenetic distance, according to a systematic study of the genome databases of other teleost fish species (Huising *et al*., 2005). Interestingly, our genome alignment

investigations of fugu, zig-zag eel, and blue tilapia fish found that the synteny and gene organization of fish IL-11b shares only identity some gene with the mammalian IL-11 sequence and also share limited gene identity with IL-11a fish. Furthermore, the fish IL-11a and IL-11b and mammalian IL-11 are encoded by genes having 5 exons and 4 introns, with the exception of IL-11b in zig-zag eels, which had 4 exons and 3 introns. Moreover, there were no significant variances in exon sizes across the different IL-11 genes, although exon 3 of the IL-11 mammalian species and IL-11a of fish were had relatively similar. In addition, IL-6 family cytokines are encoded by less than 5 exons are CNTF, LIF, OSM, M17 and by more than 5 exons are IL-12p35 (Fujiki et al., 2003; Bi *et al.*, 2018). The identity of IL-11a and IL-11b Nile tilapia with other species shared between 20.9-73.9% and 23.2-89.5%, while the similarity shared with other species as 34.6-81.5% and 40.7-98.0%, respectively. IL-11a and IL-11b shared the highest identities and similarities with *Trachinotus carolinus* IL-11.

In phylogenetic analyses and amino acid sequence alignment of IL-11 teleost fish with mammalian IL-11, by using high bootstrap value supported. The alignment between IL-11a and IL-11b teleost fish and IL-11 mammalian contained 4-helix sequences (Huising *et al*., 2005). The phylogenetic tree revealed that IL-11a and IL-11b of fish were clustered in the clades of their teleost, but separated from the mammalian IL-11. Also, IL-11a and IL-11b of Nile tilapia were clustered within IL-11a of teleost fish which was similar to *Oreochromis aureus*.

Distribution of IL-11a in several tissues revealed that the spleen, liver, intestine and trunk kidney were the mainly sites of gene expression, while the spleen and tail were higher expression levels of IL-11b. However, it was widely expressed in the heart of crucian carp (Podok *et al*., 2014), and mainly expressed in the intestine and gills of rainbow trout (Wang *et al*., 2005). Basically, intestine is key location tissue where fish is exposed to foreign disturbances such as bacterial, viral, and parasitic infection as well as environmental pressures. Hematogenesis, as well as the production of antibodies and granulocytes, depend on the spleen and liver (Long *et al*., 2021). Additionally, it was found that stomach, colon, and lungs were the main tissue of IL-11 expression in mice (Davidson *et al*., 1997). It hypothesizes that IL-11 serves as an anti-inflammatory cytokine in the gills, lung, and intestine, suggesting a

conserved role for this substance (Fung *et al*., 2022), although the gills, head kidney and trunk kidney of healthy fish express IL-11 at low levels.

The expression of IL-11a and IL-11b were significantly up-regulated at 6h in the gills, intestine, liver and spleen post bacterial infection. Previous studies showed that IL-11 expression was significantly up-regulated in liver gills at 12, 24 and 48 h and in spleen at 6, 12 and 24 h after *S. agalactiae* infection in golden pompano (Wu *et al*., 2019b), and up-regulated in kidney at 6 h, then decreased at 12, 24, 48 and 72 h of crucian carp after *A. hydrophila* infection (Podok *et al.,* 2014). According to these results, IL-11 might play function in the host defense against bacterial infection through these immune organs (Wang *et al*., 2005). Recent studies have suggested that the gills contain a high level of interleukin expression and is a primary site where foreign insults are first encountered (Øvergård *et al*., 2011; Wang *et al*., 2013b).

Peripheral blood leucocytes (PBLs) are mature lymphocytes, including neutrophils, monocytes, eosinophils, basophils, and lymphocytes (Carrick and Begg, 2008). The results of the present study showed that after stimulation by LPS and Poly I:C, the expression of IL-11a, IL-11b, IL-1β and IFNγ were significantly up-regulated at 4 h in PBLs. However, IL-11 in head kidney macrophages stimulated with LPS was significantly up-regulated within 2 h in common carp (Huising *et al*., 2005), and all increased expression of 3, 7 and 24 h in RTS-11 cell line stimulanted with LPS in rainbow trout (Wang *et al*., 2005). The mRNA expression in macrophages cells of large yellow croaker and found that the expression of IL-1β and IL-6 were significantly up-regulated within 3 to 6 h post Poly I:C stimulation (Li *et al*., 2020). Among the pro-inflammatory cytokines, IL-1β is the first cytokine involving in inflammation and control of other cytokines and chemokines (Guo *et al*., 2018). IL-6 is inflammatory cytokines induced by IL-1β, demonstrating its functional role as pro- and anti-inflammatory properties. IL-8 is a cytokine promoting the leukocyte proliferation and migration in lymphoid organs and protects host against bacterial and viral infection (Guo *et al*., 2018). IFN-γ is regulated of gene transcription and has a variety of immunoregulatory roles in innate and adaptive immunity (Øvergård *et al*., 2011).

In the present study, PBLs stimulated by LPS with different concentration, showed IL-11a, IL-11b, IL-1β, IL-6, IL-8, TNFα and COX-2 were up-regulated at 1

µg/mL. LPS is the main components of the outer surface membrane found in Gram-negative bacteria, and potent inducer of innate immune response (Steimle *et al*., 2016). The receptor responsible for recognizing LPS in mammals is Toll-like receptor (TLR) 4 when the receptor interacting with microbial ligands, it stimulates the signaling pathway and induced proinflammatory cytokines. In teleost fish, the NOD1 receptor can respond to LPS and then encourage the expression of inflammatory cytokines by activating the NF-$_K$B signal pathway (Bi *et al*., 2018). Poly I:C is an immunostimulant structurally comparable to the double-stranded RNA found in several viruses, which interact with TLR3 (Li *et al*., 2015). Our result found that IL-11a, IL-11b, IL-1β and TNF-α were significantly up-regulated at 100 µg/mL post Poly I:C stimulation. Moreover, PBLs stimulated by rIL-11, showed IL-11a, IL-11b, IL-1β, IL-6, TNF-α, IFN-γ and COX-2 were higher expression at 50 µg/mL.

The expression of key immune-related genes was studied at 1, 3 and 14 day by RT-qPCR in spleen and liver post vaccination. The spleen is an important systemic lymphoid tissue, contains a variety of immune cell types, including B cells and T cells, which are essential for triggering the immunological response (Kobayashi *et al*., 2016). Acute phase proteins (such as SAA), antimicrobial peptides, complement factors, and proinflammatory cytokines are all produced mainly in the liver (Wangkahart *et al*., 2016). In teleost, TNF-α has been shown to have important biological effects, such as increasing the expression of pro-inflammatory cytokines, stimulating the growth of leukocytes, and attracting granulopoiesis to inflammatory sites. IgM is the first antibody molecule made during an immune response, and it is essential for fish humoral immunity (Wu *et al*., 2019a). The T cell recepter molecule, which is present on the surface of T cells, is in charge of identifying antigen fragments as peptides attached to MHC molecules (Yazawa *et al*., 2008). Our result showed that at 1 d.p.v. the expression of IL-8, TNF-α, IL-11a, Il-11b and IL-6 in spleen and IL-8 and TNF-α in liver in all vaccination groups were significantly higher than the PBS group. At 3 d.p.v. the expression of IL-1β, IL-8, TNFα and IL-6 in spleen and IL-8 and IL-11a in liver in all vaccination groups were significantly higher than the PBS group. The expression of IL-8, IL-11a and IL-11b was significantly higher in liver, while in spleen were not significant expression at 14 d.p.v. compared

with control group. The expression of IL-11a was significantly higher at 1 d.p.v. in spleen and in liver at 14 d.p.v. of all vaccination group. However, the expression of IL-11b was significantly higher in spleen and liver of the FKC + rIL-11 group at 1 and 14 d.p.v., respectively when compared with control group. Thus, this is evidences that the vaccine and adjuvant were able to increase the immune response and may stimulate the adaptive immunity afterward as seen in other fish species post vaccination (Guo *et al.*, 2017; Wangkaghart *et al*., 2021).

Antigen-specific IgM antibody levels is frequently used to assess the effectiveness of vaccines in fish and other animals (Adams, 2019). The result of this study showed that the level of specific IgM antibody of fish vaccinated with FKC + rIL-11 was significantly higher than compared with PBS and FKC groups at 1-5 w.p.v.. In earier studies, the antibody IgM level vaccinated flounder was increased and reached their peak levels at 3 w.p.v. (Huang *et al*., 2019), and in Nile tilapia receiving the adjuvanted vaccines remained at high levels from 1 w.p.v. to the end of the experiment (Wangkaghart *et al*., 2021). IgM is a recognized key player in the humoral immune system and is thought to be the primary serum antibody in teleost fish (Park *et al*., 2019). Research on Nile tilapia and other fish species demonstrates a continuous correlation between the production of antibodies and the protective effects of the vaccine (Wang *et al*., 2019a). Therefore, the present results support the monitoring of serum IgM levels as a correlate of *S. agalactiae* defense in Nile tilapia.

The innate immune response plays a crucial function in the first protection from pathogen infection. Several innate immune parameters in this studied included LZM, MPO, GPx and GRD showed significantly higher in the FKC + rIL-11 group than that of the PBS and FKC groups ($P<0.05$). LZM is found in all organism and functions as a significant indicator of fish innate immunity (Wang *et al.*, 2013a). MPO is a neutrophil antioxidant enzyme that produces hypohalous acids to destroy microorganisms during an infection (Noia *et al.*, 2021). GPx is a preventative intracellular antioxidant enzyme that helps to protect molecules from oxidative damage (Rocha-Santos *et al.*, 2018). GRD is an essential molecule for protecting cells from oxidative damage and preserving their reducing environment (Deponte, 2013). In the previously studied in Nile tilapia showed that the level of LZM showed

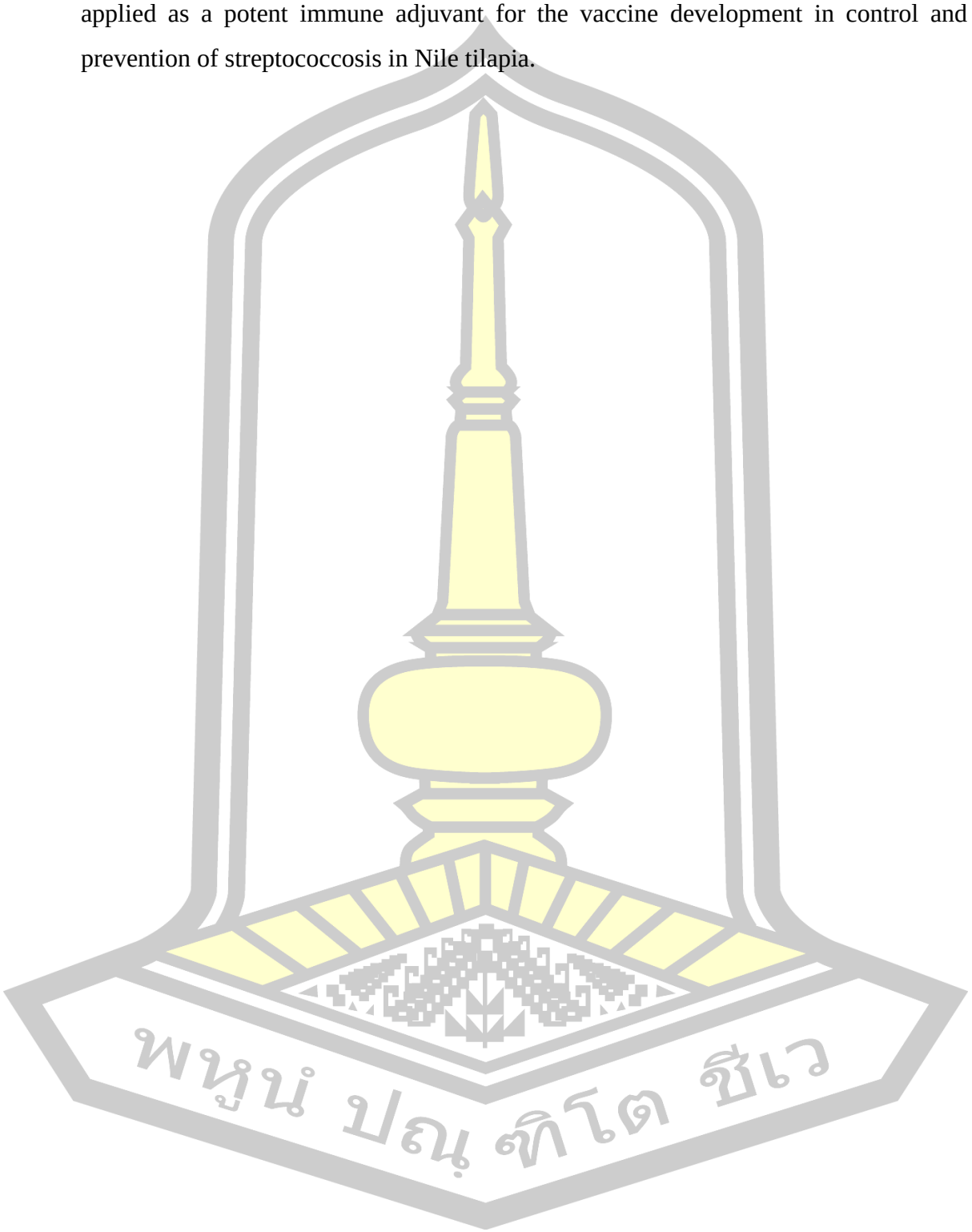
significantly increased in serum from vaccine + rIL-8 group compared with PBS + Freund's adjuvant and PBS groups (Ma *et al*., 2020). A previous study in Nile tilapia vaccinated with bacterial ghost vaccine mixed with 2 commercial adjuvants, Montanide 763B and Montanide GEL02 found to increased significantly when compared with control group. Also, the MPO activity in vaccinated + GEL02 group was increased compared with control group (Wangkaghart *et al*., 2021). The result suggested that the innate immune response of vaccinated Nile tilapia was activated and offered some disease resistance and defense against *S. agalactiae* infection. Due to the bacterial membrane remains intact of membrane protein and immune-stimulatory components, dendritic cells and macrophages that are associated to the host can distinguish between different antigens and then consume them to stimulate the immune response (Wang and Lu, 2009).

The survival rate in our study showed, vaccinated group were significantly higher than when compare with control group. The RPS of FKC and FKC + rIL-11 vaccinated fish were 61.28 and 77.41%, respectively. As a previous study, In Nile tilapia were immunized with FKC of *S. agalactiae* showed RPS values of 92.3% after challenged (Wang *et al*., 2019b) and vaccinated fish showed the highest RPS value after challenge with *S. agalactiae* (Ma *et al*., 2020). In general, over 60% RPS is necessary demonstrate sufficient vaccination effects (Amend, 1981).

5.2 Conclusions

In this study, molecular cloning and characterization of IL-11a and IL-11b in Nile tilapia were investigated. It found that IL-11a and IL-11b were expressed widely in tissues of Nile tilapia, especially in the major immune organs, and that displayed significant and varied alterations in mRNA levels in response to *S. agalactiae* infections. Furthermore, both IL-11a and IL-11b were expressed in PBLs cell when stimulated by LPS and Poly I:C. In general, LPS and and poly I:C represents as bacterial infection and viral infection, respectively. The expression of immune-relative genes was dependent on the type of pathogen, tissue type, and infection stage. These results provide the study of IL-11a and IL-11b against bacteria, thus promoting the current understanding of the function of these cytokines in the immune system against pathogen infection. Additionally, the FKC vaccine and rIL-11 increased level

of protective immunity after *S. agalactiae* challenged, indicating that rIL-11 could be applied as a potent immune adjuvant for the vaccine development in control and prevention of streptococcosis in Nile tilapia.



REFERENCES

Reference

Abbas, A. K., Lichtman, A. H., and Pillai, S. (2010). *Cellular and Molecular Immunology*. Elsevier Health Sciences.

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish and Shellfish Immunology*, 90, 210-214.

Alvarez-Pellitero, P. (2008). Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary Immunology and Immunopathology*, 126, 171–198.

Amend, D. F. (1981). Potency testing of fish vaccines. *Developments in Biological Standardization*, 49, 447-454.

Amend, D. F., Johnson, K. J., Croy, T. R., and Mccarthy, D. H. (1983). Some factors affecting the potency of *Yersinia ruckeri* bacterins. *Journal of Fish Diseases*, 6, 337-344.

Austin, B. (2012). Developments in vaccination against fish bacterial disease. *Infectious Disease in Aquaculture*, 218-243.

Awate, S., Babiuk, L. A., and Mutwiri, G. (2013). Mechanisms of action of adjuvants. *Frontiers in Immunology*, 4, 114.

Bi, D., Wang, Y., Gao, Y., Li, X., Chu, Q., Cui, J., and Xu, T. (2018). Recognition of lipopolysaccharide and activation of NF-κB by cytosolic sensor NOD1 in teleost fish. *Frontiers in Immunology*, 9, 1413.

Boshra, H., Gelman, A. E., and Sunyer, J. O. (2004). Structural and functional characterization of complement C4 and C1s-like molecules in teleost fish: Insights into the evolution of classical and alternative pathways. *The Journal of Immunology*, 173, 349–359.

Brudeseth, B. E., Wiulsrød, R., Fredriksen, B. N., Lindmo, K., Løkling, K. -E., Bordevik, M., Steine, N., Klevan, A., and Gravningen, K. (2013). Status and future perspectives of vaccines for industrialised fin-fish farming. *Fish and Shellfish Immunology*, 35, 1759-1768.

Cammarata, M., Vazzana, M., Accardi, D., and Parrinello, N. (2012). Seabream (*Sparus aurata*) long-term dominant-subordinate interplay affects phagocytosis by peritoneal cavity cells. *Brain, Behavior, and Immunity*, 26(4), 580-587.

Carrick, J. B., and Begg, A. P. (2008). Peripheral blood leukocytes. *Veterinary Clinics of North America: Equine Practice*, 24, 239–259.

Chen, K., Xu, W., Wilson, M., He, B., Miller, N.W., Bengten, E., Edholm, E. S., Santini, P. A., Rath P., and Chiu A. (2009). Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nature Immunology*, 10, 889–898.

Chettri, J. K., Skov, J., Jaafar, R. M., Krossøy, B., Kania, P. W., Dalsgaard, I., and Buchmann, K. (2015). Comparative evaluation of infection methods and environmental factors on challenge success: *Aeromonas salmonicida* infection in vaccinated rainbow trout. *Fish and Shellfish Immunology*, 44, 485-495.

Chettri, J. K., Deshmukh, S., Holten-Andersen, L., Jafaar, R. M., Dalsgaard, I., and Buchmann, K. (2013). Comparative evaluation of administration methods for a vaccine protecting rainbow trout against *Yersinia ruckeri* O1 biotype 2 infections. *Veterinary Immunology and Immunopathology*, 154, 42-47.

Davidson, A. J., Freeman, S. A., Crosier, K. E., Wood, C. R., and Crosier, P. S. (1997). Expression of murine interleukin 11 and its receptor alpha-chain in adult and embryonic tissues. *Stem Cells*, 15, 119–124.

Deponte, M. (2013). Glutathione catalysis and the reaction mechanisms of Glutathione – dependent enzymes. *Biochimica et Biophysica Acta*, 1830, 3217–3266.

Deshmukh, S., Raida, M. K., Dalsgaard, I., Chettri, J. K., Kania, P. W., and Buchmann, K. (2012). Comparative protection of two different commercial vaccines against *Yersinia ruckeri* serotype O1 and biotype 2 in rainbow trout (*Oncorhynchus mykiss*). *Veterinary Immunology and Immunopathology*, 145, 379-385.

Doñate Jimeno, C. (2009). A transcriptomic approach toward understanding PAMP-driven macrophage activation and dietary immunostimulation in fish, PhD Thesis. University of Barcelona.

Du, X. X., and Williams, D. A. (1994). IL-11: a multifunctional growth factor derived from the hematopoietic microenvironment. *Blood*, 83, 2023-2030.

Du, X. X., and Williams, D. A. (1997). Interleukin-11: review of molecular, cell biology, and clinical use. *Blood*, 89, 3897-3908.

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and Comparative Immunology*, 25, 827-839.

Esteve-Gassent, M. D., Fouz, B., Barrera, R., and Amaro, C. (2004). Efficacy of oral reimmunisation after immersion vaccination against *Vibrio vulnificus* in farmed European eels. *Aquaculture*, 231, 9-22.

Evans, J. J., Klesius, P. H., Pasnik, D. J., and Bohnsack, J. F. (2009). Human *Streptococcus agalactiae* isolate in Nile tilapia (*Oreochromis niloticus*). *Emerging infectious diseases*, 15(5), 774.

FAO. (2017). Fishstat Plus Version 2.30. Available Source: http://www.fao.org/fi/-statist/FISOFT/

Fletcher, T. C. (1981). Non-antibody molecules and the defence mechanisms of fish. *Stress and fish.*

Fujiki, K., Nakao, M., and Dixon, B. (2003). Molecular cloning and characterisation of a carp (*Cyprinus carpio*) cytokine-like cDNA that shares sequence similarity with IL-6 subfamily cytokines CNTF, OSM and LIF. *Developmental and Comparative Immunology*, 27, 127–136.

Fung, K. Y., Louis, C., Metcalfa, R. D., Kosasih, C. C., Wicks, I. P., Griffin, M. D. W., and Putoczki, T. L. (2022). Emerging roles for IL-11 in inflammatory diseases. *Cytokine*, 149, 155750.

Galindo-Villegas, J., and Hosokawa H. (2004). Immunostimulants: Towards temporary prevention of diseases in marine fish. In: *Avances en Nutrición Acuícola VII. Memorias del VII Simposium Internacional de Nutrición Acuícola*.

Garcia, J. C., Klesius, P. H., Evans, J. J., and Shoemaker, C. A. (2008). Non-infectivity of cattle *Streptococcus agalactiae* in Nile tilapia, *Oreochromis niloticus* and channel catfish, *Ictalurus punctatus*. *Aquaculture*, 281, 151-154.

Gudding, R., and Muiswinkel, Van W. (2013). A history of fish vaccination: Science-based disease prevention in aquaculture. *Fish and Shellfish Immunology* 35, 1683-1688.

Guo, M., Tang, X., Sheng, X., Xing, J., and Zhan, W. (2017). The immune adjuvant effects of flounder (*Paralichthys olivaceus*) interleukin-6 on *E. tarda* subunit vaccine OmpV. *International Journal of Molecular Sciences*, 18, 1445.

Guo, M., Tang, X., Sheng, X., Xing, J., and Zhan, W. (2018). The effects of IL-1β, IL-8, G-CSF and TNF-α as molecular adjuvant on the immune response to an *E. tarda* subunit vaccine in flounder (*Paralichthys olivaceus*). *Fish and Shellfish Immunology*, 77, 374–384.

Hu, Y., Maisey, K., Subramani, P. A., Liu, F., Flores-Kossack, C., Imarai, M., Secombes, C. J., and Wang, T. (2018). Characterisation of rainbow trout peripheral blood leucocytes prepared by hypotonic lysis of erythrocytes, and analysis of their phagocytic activity, proliferation and response to PAMPs and proinflammatory cytokines. *Developmental and Comparative Immunology*, 88, 104–113.

Huang, H. Y., Chen Y. C., Wang, P. C., Tsai, M. A., Yeh, S. C., Liang, H. J., and Chen, S. C. (2014). Efficacy of a formalin-inactivated vaccine against *Streptococcus iniae* infection in the farmed grouper *Epinephelus coioides* by intraperitoneal immunization. *Vaccine*, 32, 7014-7020.

Huang, P., Cai, J., Yu, D., Tang, J., Lu, Y., Wu, Z., Huang, Y., and Jian, J. (2019). An IL-6 gene in humphead snapper (*Lutjanus sanguineus*): Identification, expression analysis and its adjuvant effects on *Vibrio harveyi* OmpW DNA vaccine. *Fish and Shellfish Immunology*, 95, 546-555.

Huising, M. O., Kruiswijk, C. P., van Schijndel, J. E., Savelkoul, H. F. J., Flik, G., and Verburg- van Kemenade, B. M. L. (2005). Multiple and highly divergent IL-11 genes in teleost fish. *Immunogenetics*, 57, 432–443.

Huu, H. D., Sang, H. M., and Thuy, N. T. T. (2016). Dietary β-glucan improved growth performance, *Vibrio* counts, haematological parameters and stress resistance of pompano fish, *Trachinotus ovatus* Linnaeus, 1758. *Fish and Shellfish Immunology*, 54, 402-410.

Janeway, Jr., C. A., and Medzhitov, R. (2002). Innate immune recognition. *Annual review of immunology*, 20, 197–216.

Janeway Jr, C. A., Travers, P., Walport, M., and Shlomchik, M. J. (2001). The components of the immune system. In *Immunobiology: The Immune System in Health and Disease. 5th edition*. Garland Science.

Jensch-Junior, B. E., Pressinotti, L. N., Borges, J. C. S., and da Silva, J. R. M. C. (2006). Characterization of macrophage phagocytosis of the tropical fish *Prochilodus scrofa* (Steindachner, 1881). *Aquaculture*, 251, 509-515.

Kannika, K., Pisuttharachai, D., Srisapoome, P., Wongtavatchai, J., Kondo, H., Hirono, I., Unajak, S., and Areechon, N. (2017). Molecular serotyping, virulence gene profiling and pathogenicity of *Streptococcus agalactiae* isolated from tilapia farms in Thailand by multiplex PCR. *Journal of Applied Microbiology*, 122, 1497-1507.

Keith Jr, J. C. (2003). Interleukin-11. *The Cytokine Handbook*, 363-381.

Kilpi, M., Nikoskelainen, S., Grannas, S., Nuutila, J., Järvisalo, O., Kause, A., and Lilius, E. (2013). Resistance to bacterial infectious diseases in rainbow trout (*Oncorhynchus mykiss*). *Veterinary Immunology and Immunopathology*, 153(3-4), 267-278.

Kobayashi, I., Katakura, F., and Moritomo, T. (2016). Isolation and characterization of hematopoietic stem cells in teleost fish. *Developmental and Comparative Immunology*, 58, 86–94.

Kube, P. D., Taylor, R. S. and Elliott, N. G. (2012). Genetic variation in parasite resistance of Atlantic salmon to amoebic gill disease over multiple infections. *Aquaculture*, 364, 165-172.

Kumar, G., and Engle, C. R. (2016) Technological advances that led to growth of shrimp, salmon, and tilapia farming. *Reviews in Fisheries Science and Aquaculture*, 24(2), 136-152.

Li, J., Bardera, D. R., Zhang, Y. -A., Boshra, H., Gelman, A. E., LaPatra, S., Tort, L., and Sunyer, J. O. (2006). B lymphocyte from early vertebrates have potent phagocytic and microbicidal abilities. *Nature Immunology*, 7, 1116-1124.

Li, Q., Wu, M., Cui, K., Zhu, S., Mai, K., and Ai, Q. (2020). Characterization of antiviral immune response induced by poly (I:C) in macrophages of farmed large yellow croaker (*Larimichthys crocea*). *Fish and Shellfish Immunology*, 104, 663-672.

Li, Y., Xu, X. L., Zhao, D., Pan, L. N., Huang, C. W., Guo, L. J., Lu, Q., and Wang, J. (2015). TLR3 ligand Poly IC attenuates reactive astrogliosis and improves recovery of rats after focal cerebral ischemia CNS. *Neuroscience and Therapeutics*, 21, 905–913.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402–408.

Long, S., Li, Z., Dong, X., Yan, X., Liu, H., Tan, B., Zhang, S., Pan, S., Li, T., Suo, X., and Yang, Y. (2021). The effect of oxidized fish oil on the spleen index, antioxidant activity, histology and transcriptome in juvenile hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *Epinephelus lanceolatus*). *Frontiers in Marine Science*, 8, 1747.

Ma, Y., Hao, L., Liang, Z., Ma, J., Ke, H., Kang, H., Yang, H., Wu, J., Feng, G., and Liu, Z. (2020). Characterization of novel antigenic vaccine candidates for nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae* infection. *Fish and Shellfish Immunology*, 105, 405-414.

Mashoof, S., Pohlenz, C., Chen, P. L., Deiss, T. C., Gatlin, D., Buentello, A., and Criscitiello, M. F. (2014). Expressed IgH μ and τ transcripts share diversity segment in ranched *Thunnus orientalis*. *Developmental and Comparative Immunology*, 43, 76–86.

Meng, S., Chen, X., Song, C., Fan, L., Qiu, L., Zheng, Y., Chen, J., and Xu, P. (2021). Effect of chronic exposure to pesticide methomyl on antioxidant defense system in testis of tilapia (*Oreochromis niloticus*) and its recovery pattern. *Applied Sciences*, 11(8), 3332.

Morris, J. C., Neben, S., Bennett, F., Finnerty, H., Long, A., Beier, D. R., Kovacic, S., McCoy, J. M., DiBlasio-Smith, E., La Vallie, E. R., Caruso, A., Calvetti, J., Morris, G., Weich, N., Paul, S. R., Crosier, P. S., Turner, K. J., and Wood, C.

R. (1996). Molecular cloning and characterization of murine interleukin-11. *Experimental Hematology*, 24, 1369–1376.

Mulero, V., García, A. A., and Meseguer, J. (2005). What can we learn from fish? An evolutionary perspective of pro-inflammatory cytokines. *Inmunología*, 24, 17-22.

Mutoloki, S., Alexandersen, S., Gravningen, K., and Evensen, Ø. (2008). Time-course study of injection site inflammatory reactions following intraperitoneal injection of Atlantic cod (*Gadus morhua* L.) with oil-adjuvanted vaccines. *Fish and Shellfish Immunology*, 24, 386-393.

Noia, M., Fontenla-Iglesias, F., Valle, A., Blanco-Abad, V., Leiro, J. M., and Lamas, J. (2021). Characterization of the turbot *Scophthalmus maximus (L.)* myeloperoxidase. An insight into the evolution of vertebrate peroxidases. *Developmental and Comparative Immunology*, 118, 103993.

Nonaka, M., and Smith, S. L. (2000). Complement system of bony and cartilaginous fish. *Fish and Shellfish Immunology*, 10, 215-228.

Øvergård, A. -C., Nepstad, I., Nerland, A. H., and Patel, S. (2011). Characterisation and expression analysis of the Atlantic halibut (*Hippoglossus hippoglossus* L.) cytokines: IL-1β, IL-6, IL-11, IL-12β and IFNγ. *Molecular Biology Reports*, 39(3), 2201–2213.

Panserat, S., Hortopan, G. A., Plagnes-Juan, E., Kolditz, C., Lansard, M., Skiba-Cassy, S., Esquerré, D., Geurden, I., Médale, F., Kaushik, S., and Corraze, G. (2009). Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. *Aquaculture*, 294, 123–131.

Park, J., Kwon, W., Kim, W. -S., Jeong, H. -D., and Hong, S. (2019). Cloning and expressional analysis of secretory and membrane-bound IgM in rock bream (*Oplegnathus fasciatus*) under megalocytivirus infection and vaccination. *Fish and Shellfish Immunology*, 87, 275-285.

Podok, P., Xu, L., Xu, D., and Lu, L. (2014). Different expression profiles of interleukin 11 (IL-11), intelectin (ITLN) and purine nucleoside phosphorylase 5a (PNP 5a) in crucian carp (*Carassius auratus gibelio*) in response to

Cyprinid herpesvirus 2 and *Aeromonas hydrophila*. *Fish and Shellfish Immunology*, 38, 65–73.

Press, C. M., and Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology*, 9, 309-318.

Pridgeon, J. W., and Klesius, P. H. (2013). Development of live attenuated *Streptococcus agalactiae* as potential vaccines by selecting for resistance to sparfloxacin. *Vaccine*, 31, 2705-2712.

Racanelli, V., and Rehermann, B. (2006). The liver as an immunological organ. *Hepatology*, 43, 54–62.

Redlich, C. A., Gao, X., Rockwell, S., Kelley, M., and Elias, J. A. (1996). IL-11 enhances survival and decreases TNF production after radiation-induced thoracic injury. *The Journal of Immunology*, 157, 1705-10.

Ribeiro, C. M. S, and Schijns, V. E. J. C. (2010). Immunology of vaccine adjuvants. *Methods in molecular biology*, 626, 1-14.

Rocha-Santos, C., Bastos, F. F., Dantas, R. F., Hauser-Davis, R. A., Rodrigues, L. C., Cunha Bastos, V. L. F., and Cunha Bastos, J. (2018). Glutathione peroxidase and glutathione S-transferase in blood and liver from a hypoxia-tolerant fish under oxygen deprivation. *Ecotoxicology and Environmental Safety*, 163, 604–611.

Rowe, D. S., and Fahey, J. L. (1965). A new class of human immunoglobulins: I. A unique myeloma protein. *Journal of Experimental Med*icine, 121, 171–184.

Sahoo, P. K., Kumari, J., and Mishra, B. K. (2005). Non-specific immune responses in juveniles of Indian major carps. *Applied Ichthyology*, 21, 151-155

Sahu, A., and Lambris, J. D. (2001). Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunological Reviews*, 180, 35-48.

Santos, M. D., Yasuike, M., Kondo, H., Hirono, I., and Aoki, T. (2008). Teleostean IL11b exhibits complementing function to IL11a and expansive involvement in antibacterial and antiviral responses. *Molecular Immunology*, 45, 3494-3501.

Saurabh, S., and Sahoo, P. K. (2008). Lysozyme: an important defence molecule of

fish innate immune system. *Aquaculture Research*. 39, 223–239.

Schijns, V. E. J. C. (2001). Activation and programming of adaptive immune responses by vaccine adjuvants. *Veterinary Sciences Tomorrow*, 3, 1-7.

Secombes, C. J., and Wang, T. (2012). The innate and adaptive immune system of fish. *Infectious disease in aquaculture: prevention and control*, 3-68.

Seierstad, S. L., Haugland, Ø., Larsen, S., Waagbø, R., and Evensen, Ø. (2009). Pro-inflammatory cytokine expression and respiratory burst activity following replacement of fish oil with rapeseed oil in the feed for Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 289, 212-218.

Shoemaker, C. A., Klesius, P. H., and Lim, C. (2001). Immunity and disease resistance in fish. *Nutrition and Fish Health (ed. by Lim C., Webster CD)*, 149-162.

Steimle, A., Autenrieth, I. B., and Frick, J.-S. (2016). Structure and function: Lipid A modifications in commensals and pathogens. *International Journal of Medical Microbiology*, 306, 290–301.

Tafalla, C., Bogwald, J., and Dalmo, R. A. (2013). Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives. *Fish and Shellfish Immunology*, 35, 1740-50.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, 140(6), 805–820.

Tatner, M. F. and Findlay, C. (1991). Lymphocyte migration and localization patterns in rainbow trout, *Onchorhynchus mykiss*, studies using the tracer sample method. *Fish and Shellfish Immunology*, 1, 107-117.

Tattiyapong, P., Dachavichitlead, W., and Surachetpong, W. (2017). Experimental infection of Tilapia Lake Virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.). *Veterinary Microbiology*, 207, 170-177.

Thompson, K. D. (2017). Immunology: improvement of innate and adaptive immunity. In *Fish Diseases* (pp. 1-17). Academic Press.

Thu Lan, N. G., Salin, K. R., Longyant, S., Senapin, S., and Dong, H. T. (2021).

Systemic and mucosal antibody response of freshwater cultured Asian seabass (*Lates calcarifer*) to monovalent and bivalent vaccines against *Streptococcus agalactiae* and *Streptococcus iniae*. *Fish and Shellfish Immunology*, 108, 7-13

Tort, L., Balasch, J. C., and Mackenzi, S. (2003). Fish immune system. A crossroads between innate and adaptive responses. *Inmunología*, 22, 277-286.

Tran, H. B., Chen, S. C., Chaung, H. C., and Cheng, T. C. (2019). Molecular cloning of IL-6, IL-10, IL-11, IFN-γ and modulation of pro- and anti-inflammatory cytokines in cobia (*Rachycentron canadum*) after *Photobacterium damselae* subsp. *piscicida* infection. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 230, 10-18.

Trepicchio, W., Wang, L., Bozza, M., and Dorner, A. (1997). IL-11 regulates macrophage effector function through the inhibition of nuclear factor-kappaB. *Journal of Immunology*, 159, 5661-5670.

Tu, F. P., Chu, W. H., Zhuang, X. Y., and Lu, C. P. (2010). Effect of oral immunization with *Aeromonas hydrophila* ghost on protection against experimental fish infection. *Journal of Applied Microbiology*, 50, 13-17.

Utke, K., Kock, H., Schuetze, H., Bergmann, S. M., Lorenzen, N., Einer-Jensen, K., Köllner, B., Dalmo, R. A., Vesely, T., Ototake, M., and Fischer, U. (2008). Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. *Developmental and Comparative Immunology*, 32(3), 239-252.

Vervarcke, S., Ollevier, F., Kinget, R., and Michoel, A. (2004). Oral vaccination of African catfish with *Vibrio anguillarum* O2: effect on antigen uptake and immune response by absorption enhancers in lag time coated pellets. *Fish and Shellfish Immunology*, 16, 407-414.

Villumsen, K. R., and Raida, M. K. (2013). Long-lasting protection induced by bath vaccination against *Aeromonas salmonicida* subsp. *salmonicida* in rainbow trout. *Fish and Shellfish Immunology*, 35, 1649-1653.

Wang, T., Holland, J., Bols, W. N., and Secombes, C. J. (2005). Cloning and expression of the first nonmammalian interleukin-11 gene in rainbow trout *Oncorhynchus mykiss*. *FEBS Journal*, 272, 1136-1147.

Wang, J., Du, J. J., Jiang, B., He, R. Z., and Li, A. X. (2019a). Effects of short-term fasting on the resistance of Nile tilapia (*Oreochromis niloticus*) to *Streptococcus agalactiae* infection. *Fish and Shellfish Immunology*, 94, 889-895

Wang, Q., Fu, T., Li, X., Luo, Q., Huang, J., Sun, Y., and Wang, X. (2019b). Cross-immunity in Nile tilapia vaccinated with *Streptococcus agalactiae* and *Streptococcus iniae* vaccines. *Fish and Shellfish Immunology*, 97, 382-389.

Wang, R., Feng, J., Li, C., Liu, S., Zhang, Y., and Liu, Z. (2013a). Four lysozymes (one c-type and three g-type) in catfish are drastically but differentially induced after bacterial infection. *Fish and Shellfish Immunology*, 35(1), 136–145.

Wang, T., and Secombes, C. J. (2013). The cytokine networks of adaptive immunity in fish. *Fish and Shellfish Immunology*, 35(6), 1703–1718.

Wang, T., Hu, Y., Wangkahart, E., Liu, F., Wang, A., Zahran, E., and Secombes, C. J. (2018). Interleukin (IL)-2 is a key regulator of T helper 1 and T helper 2 cytokine expression in fish: functional characterization of two divergent IL2 paralogs in salmonids. *Frontiers in immunology*, 9, 1683.

Wang, T., Kono, T., Monte, M. M. Kuse, H. Costa, M. M., Korenaga, H., Maehr, T., Husain, M., Sakai, M., and Secombes, C. J. (2013b). Identification of IL-34 in teleost fish: differential expression of rainbow trout IL-34, MCSF1 and MCSF2, ligands of the MCSF receptor. *Molecular Immunology*, 53, 398–409.

Wang, X., and Lu, C. (2009). Mice orally vaccinated with *Edwardsiella tarda* ghosts are significantly protected against infection. *Vaccine*, 27, 1571–1578.

Wangkaghart, E., Deville, S., Wang, B., Srisapoome, P., Wang, T., and Secombes, C. J. (2021). Immune response and protective efficacy of two new adjuvants, Montanide™ ISA 763B VG and Montanide™ GEL02, administered with a *Streptococcus agalactiae* ghost vaccine in Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology*, 116, 19–29.

Wangkahart, E., Scott, C., Secombes, C. J., and Wang, T. (2016). Re-examination of the rainbow trout (*Oncorhynchus mykiss*) immune response to flagellin: *Yersinia ruckeri* flagellin is a potent activator of acute phase proteins, anti-

microbial peptides and pro-inflammatory cytokines in vitro. *Developmental and Comparative Immunology*, 57, 75-87.

Waxman, A. B., Einarsson, O., Seres, T., Knickelbein, R. G., Warshaw, J. B., Johnston, R., Homer, R. J., and Elias, J. A. (1998). Targeted lung expression of interleukin-11 enhances murine tolerance of 100% oxygen and diminishes hyperoxia-induced DNA fragmentation. *The Journal of Clinical Investigation*, 101, 1970-1982.

Wu, L., Yang, Y., Kong, L., Bian, X., Guo, Z., Fu, S., Liang, F., Li, B., and Ye, J. (2019a). Comparative transcriptome analysis of the transcriptional heterogeneity in different IgM+ cell subsets from peripheral blood of Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology*, 93, 612-622.

Wu, Y., Zhou, Y., Cao, Z., Sun, Y., Chen, Y., Xiang, Y., Wang, L., Zhang, S., and Guo, W. (2019b). Comparative analysis of the expression patterns of IL-1β, IL-11, and IL-34 in golden pompano (*Trachinotus ovatus*) following different pathogens challenge. *Fish and Shellfish Immunology*, 93, 863-870.

Yamasaki, M., Araki, K., Nakanishi, T., Nakayasu, C., and Yamamoto, A. (2014). Role of $CD4^+$ and $CD8^+$ T cells in protective immunity against *Edwardsiella tarda* infection of ginbuna crucian carp, *Carassius auratus langsdorfi*. *Fish and Shellfish Immunology*, 36(1), 299-304.

Yamasaki, M., Araki, K., Nakanishi, T., Nakayasu, C., Yoshiura, Y., Iida, T., and Yamamoto, A. (2013). Adaptive immune response to *Edwardsiella tarda* infection in ginbuna crucian carp, *Carassius auratus langsdorfii*. *Veterinary Immunology and Immunopathology*, 153(1-2), 83-90.

Yazawa, R., Cooper, G. A., Beetz-Sargent, M., Robb, A., McKinnel, L., Davidson, W. S., and Koop, B. F. (2008). Functional adaptive diversity of the Atlantic salmon T-cell receptor gamma locus. *Molecular Immunology*, 45, 2150–2157.

Zapata, A. G. (1996). Cells and tissues of the immune system of fish. *The fish immune system. Organism, pathogen, and environment.*

Zardakis, I. K., Mastellos, D., and Lambris, J. D. (2001). Phylogenetic aspects of the

complement system. *Development and Comparative Immunology*, 25, 745-762.

Zhang, Y., Zhang, T., Li, J., Liu, X., and Li, L. (2014). Design and evaluation of a tandemly arranged outer membrane protein U (OmpU) multi-epitope as a potential vaccine antigen against *Vibrio mimicus* in grass carps (*Ctenopharyngodon idella*). *Veterinary Immunology and Immunopathology*, 160, 61-69.

APPENDICES

10X Phosphate buffered saline (PBS)

NaCl	80.00	g
KCl	2.00	g
KH_2PO	1.20	g
Na_2HPO_4 (anhydrous)	9.10	g
Deionized distilled water to	1,000.00	mL

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

1X PBS

10X PBS	100.00	mL
Deionized distilled water	900.00	mL

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

1X Tris (pH 8.0)

Tris-base	121.00	g
Deionized distilled water	1,000.00	mL

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

1X TE buffer

1 M Tris pH 8.0	1.21	g

0.5 M EDTA	9.31	g
Deionized distilled water to	1,000.00	mL

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

2XYT

Tryptone	16.00	g
Yeast extract	10.00	g
NaCl	5.00	g
Deionized distilled water to	900.00	mL

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

Brain heart 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
Deionized distilled water to	1,000.00	mL

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

Brain heart infusion agar (BHI agar, 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
Deionized distilled water to	1,000.00	mL

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

Reagents for competent cells preparation

1. Luria-Bertani broth (LB broth)

Yeast extract	10.00	g
Tryptone	10.00	g
NaCl	5.00	g
Deionized distilled water to	1,000.00	mL

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

2. Luria-Bertani agar (LB agar)

Yeast extract	10.00	g
Tryptone	10.00	g
NaCl	5.00	g
Agar	15.00	g
Deionized distilled water to	1,000.00	mL

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

3. 100 mM $CaCl_2$

$CaCl_2$	1.47	g
Deionized distilled water to	100.00	mL

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

Reagents for agarose gel electrophoresis

1. Agarose gel preparation

	Agarose	1X TAE or 1X TBE
0.8% agarose	0.80 g	100.00 mL
1.5% agarose	1.50 g	100.00 mL
2.0% agarose	2.00 g	100.00 mL

2. 10X Loading dye

Bromophenol blue	0.04	g

Glycerol 85%	500.00	µL
10X TAE	500.00	µL

3. 100 bp DNA marker

100 bp DNA	2.50	µL
6X Loading dye	2.50	µL
Deionized distilled water t	100.00	µL

4. 50X Tris-acetate buffer (TAE)

Tris-base	242.00	g
CH_3COOH	57.10	mL
0.5 M EDTA pH 8.0	100.00	mL
Deionized distilled water to	1,000.00	mL

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

5. 1X TAE

50X TAE	20.00	mL
Deionized distilled water	980.00	mL

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

6. 10X Tris-boric buffer (TBE)

Tris-base	242.00	g
CH_3COOH	57.10	mL
0.5 M EDTA pH 8.0	100.00	mL
Deionized distilled water to	1,000.00	mL

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

7. 1X TBE

10X TBE	100.00	mL
Deionized distilled water	900.00	mL

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

Reagents for His-tagged protein purification

1. LB0 for lysis

GuHCl	668.70	g
5 M NaCl	100.00	mL
1 M Tris pH 8.0	50.00	mL
2 M Imidazol	2.50	mL
0.7 M TCEP	7.00	mL
Deionized distilled water to	1,000.00	mL

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

2. WB1 + 1.5% Triton X-100

GuHCl	573.20	g
5 M NaCl	100.00	mL
1 M Tris pH 8.0	50.00	mL
2 M Imidazol	5.00	mL
0.7 M TCEP	7.00	mL
20% Triton X-100	75.00	mL
Deionized distilled water to	1,000.00	mL

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

3. DNEB

GuHCl	573.20	g
5 M NaCl	100.00	mL
1 M Tris-HCl pH 8.0	50.00	mL
2 M Imidazol	250.00	mL
0.7 M TCEP	7.00	mL
Deionized distilled water to	1,000.00	mL

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

4. RF7.4-Refolding

10X PBS	100.00	mL
50% Glycerol	200.00	mL
1 M Arg	500.00	mL
1 M Glu	50.00	mL
0.7 M TCEP	1.40	mL
Deionized distilled water to	1,000.00	mL

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

5. RF7.4-Elution

10X PBS	100.00	mL
50% Glycerol	200.00	mL
1 M Arg	500.00	mL
1 M Glu	10.00	mL
0.7 M TCEP	1.40	mL
2 M Imidazol	150.00	mL
Deionized distilled water to	1,000.00	mL

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

6. Storage buffer

10X PBS	100.00	mL
50% Glycerol	200.00	mL
1 M Arg	500.00	mL
1 M Glu	50.00	mL
0.7 M TCEP	1.40	mL
Deionized distilled water to	1,000.00	mL

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

Reagents for real-time PCR preparation

1. Immolase cocktail

Immolase buffer	2.00	mL
50 mM $MgCl_2$	2.36	mL

25 mM dNTP	160.00	µL
SYB green	40.00	µL
0.7 M TCEP	1.40	µL
Immolase enzyme	1	tube
Deionized distilled water to	10.00	mL

2. Mix for real-time

Deionized distilled water	2.00	µL
Primer	2.00	µL
Immolase cocktail	10.00	µL
cDNA template	4.00	µL

Reagents for cDNA synthesis preparation

1. Mix for cDNA

5X buffer	8.00	µL
25 mM dNTP	1.60	µL
Reverse transcriptase	1.00	µL
Sample	29.40	µL

2. 25 mM dNTP

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

Reagents for PCR reaction preparation

2X Taq mastermix	25.00	µL
50 mM $MgCl_2$	2.00	µL
Primer	2.00	µL
DNA template	4.00	µL

Deionized distilled water to	50.00	μL

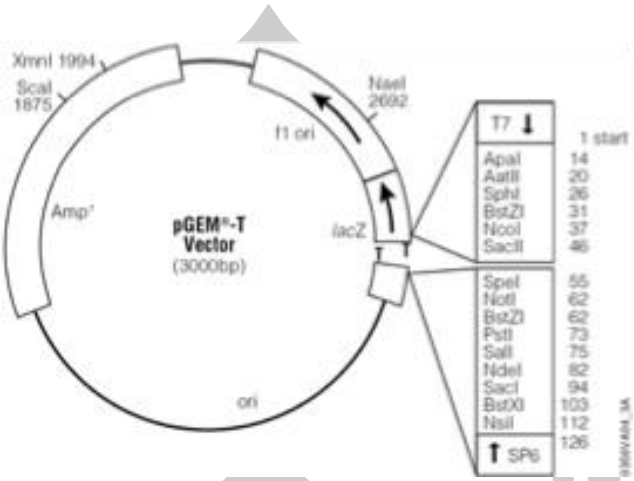


Figure A1 pGEM-T easy vector for cloning.

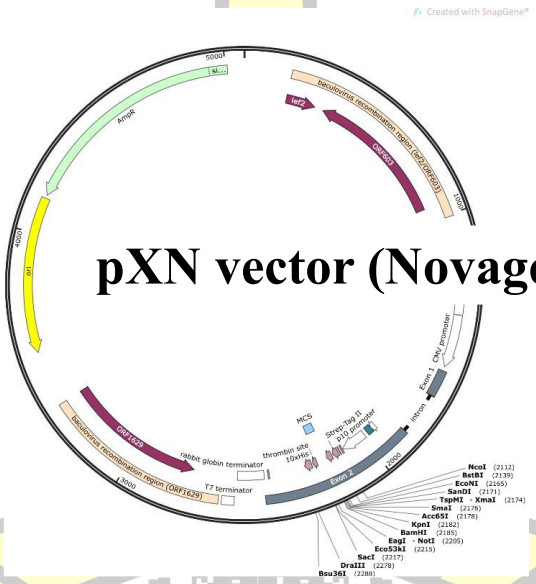


Figure A2 pXN expression vector for expression protein.

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