EFFECTS OF DIETARY SYNBIOTICS ON GROWTH PERFORMANCES AND IMMUNE IMPROVEMENT IN NILE TILAPIA *(Oreochromis niloticus)*

ARPORN PANASE

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT
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ชื่อเรื่อง ผลของการใช้ซินไบโอติกผสมในอาหารต่อการเจริญเติบโตและเสริมภูมิคุ้มกันในปลานิล

ชื่อผู้เขียน นางสาวอาพร ปะนาเส

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อาจารย์ที่ปรึกษาหลัก รองศาสตราจารย์ ดร.มงคล ถิรบุญยานนท์

บทคัดย่อ

งานวิจัยนี้ได้แบ่งการทดลองออกเป็น 2 ส่วน มีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพของการใช้พรีไบโอติก Fructooligosaccharide (FOS) โปรไบโอติก (*Bacillus subtilis*) และซินไบโอติก (*B. subtilis* และ FOS) ผสมในอาหารต่อการเจริญเติบโต การเสริมภูมิคุ้มกัน และการต้านทานต่อการเกิดโรคในปลานิล การทดลองที่ 1 ใช้ปลานิลน้ำหนักเริ่มต้น 24.5 ± 1.6 กรัม แบ่งการทดลองออกเป็น 7 กลุ่ม ได้แก่กลุ่มควบคุมเลี้ยงด้วยอาหารปกติ (G1) กลุ่มทดลองเลี้ยงด้วยอาหารผสมพรีไบโอติก FOS 1 g/kg (G2), 3 g/kg (G3) และ 5 g/kg (G4) และอาหารผสมโปรไบโอติก *B. subtilis* $1x10^9$ CFU/g (G5), $3x10^9$ CFU/g (G6) และ $5x10^9$ CFU/g (G7) เลี้ยงเป็นเวลา 56 วัน จากผลการศึกษาพบว่า อาหารเสริมพรีไบโอติกหรือโปรไบโอติกไม่มีผลต่อการเจริญเติบโตและอัตราการรอดตาย แต่ปลาที่เลี้ยงด้วยอาหารเสริมกลุ่มทดลอง G4, G5, G6, G7 มีค่ากิจกรรมไลโซไซม์มากกว่า (P >0.05) เมื่อเทียบกับกับควบคุมและกลุ่มการทดลองอื่น และปลาที่เลี้ยงด้วยอาหารเสริมทุกกลุ่มการทดลองมีค่า Respiratory burst activity สูงกว่ากลุ่มควบคุม (P>0.05) เมื่อศึกษาการแสดงออก ของยีนด้วยเทคนิค Quantitative Real-time PCR พบว่า ปลาที่เลี้ยงด้วยอาหารผสม FOS g/kg อาหารเสริมผสม *B. subtilis* ทุกความเข้มข้น มีค่าการแสดงออกของยีน *C3, IL-1*β*, TNF-*α*, IFN-*γ และ *hsp-70* จากตับ มากกว่ากลุ่มอื่น (P > 0.05) ปลานิลที่เลี้ยงด้วยอาหารเสริมทุกกลุ่มมีความ ต้านทานต่อการเกิดโรคมากกว่ากลุ่มควบคุมหลังทดสอบการติดเชื้อ *Streptococcus agalactiae* เป็นเวลา 14 วัน

การทดลองที่ 2 เป็นการศึกษาประสิทธิภาพของการซินไบโอติก (*B. subtilis* + FOS) ผสมในอาหารต่อการเจริญเติบโต การเสริมภูมิคุ้มกันและความต้านทานต่อการเกิดโรค แบ่งการทดลองออกเป็น 4 กลุ่ม ได้แก่ กลุ่มควบคุมเลี้ยงด้วยอาหารปกติ (G1) กลุ่มทดลองเลี้ยงด้วยอาหารผสม 1 g/kg FOS + $1x10^9$ CFU/g *B. subtilis* (G2), 3 g/kg FOS + $3x10^9$ CFU/g *B. subtilis* (G3) และ 5 g/kg FOS + $5x10^9$ CFU/g *B. subtilis* (G4) พบว่า ปลานิลที่เลี้ยงด้วยอาหารเสริม มีค่า ADG และอัตราการรอดมากกว่ากลุ่มอื่น (P < 0.05) และกลุ่มปลาที่เลี้ยงด้วยอาหารเสริม 3 g/kg

FOS + *B. subtilis* (G3) และ 5 g/kg FOS + 5x10^{9} CFU/g *B. subtilis* มีค่ากิจกรรมไลโซไซม์ ค่า Respiratory burst activity สูงกว่ากลุ่มควบคุมและกลุ่มการทดลองอื่น ($P < 0.05$) สำหรับการแสดงออกของยีน พบว่า กลุ่มปลานิที่เลี้ยงด้วย 5 g FOS/kg feed + 5x10^{9} CFU/g of *B. subtilis* มีการแสดงออกของยีน complement *C3, IL-1β, IL*-8 และ *Hsp70* ในตับสูงกว่ากลุ่มควบคุมและกลุ่มทดลองอื่น ($P< 0.05$) และการแสดงออกของยีน *TNF-α* และ *IFN-γ* ของปลานิลที่ด้วยอาหารเสริม 3 g FOS/kg feed + 3x10^{9} CFU/g *B. subtilis* มีค่าสูงกว่ากลุ่มควบคุมและกลุ่มทดลองอื่น ($P<0.05$) กลุ่มปลานิลที่เลี้ยงด้วยอาหารเสริมซินไบโอติกมีความต้านทานต่อการติดเชื้อและมีอัตราการรอดสูงกว่ากลุ่มควบคุม ($P<0.05$)

จากผลการทดลองจะเห็นได้ว่าปลานิลที่เลี้ยงด้วยอาหารเลี้ยงเสริม FOS, *B. subtilis* และ FOS + *B.subtilis* สามารถกระตุ้นภูมิคุ้มกันแบบไม่จำเพาะ และมีผลต่อการแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกัน อย่างไรก็ตามในอนาคตอาจมีการศึกษาการใช้พรีไบโอติกชนิดอื่นผสมโปรไบโอติก *B. subtilis* ต่อการเจริญเติบโตและสร้างภูมิคุ้มกันในปลานิล

คำสำคัญ : โปรไบโอติก, พรีไบโอติก, ซินไบโอติก, ปลานิล, ภูมิคุ้มกัน, การแสดงออกของยีน

Title	EFFECTS OF DIETARY SYNBIOTICS ON GROWTH PERFORMANCES AND IMMUNE IMPROVEMENT IN NILE TILAPIA *(Oreochromis niloticus)*
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Degree	Doctor of Philosophy in Biotechnology
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ABSTRACT

This research was divided into two parts to investigate the effects of *Bacillus subtilis*, Fructooligosaccharide (FOS), and synbiotic (*B. subtilis* and FOS combination) on growth performances, immunity improvement, and disease resistance of Nile tilapia (*Oreochromis niloticus*). The first trial, fish (24.5 ± 1.6 g) were fed a basal diet (G1), supplemented with 1 g/kg (G2), 3 g/kg (G3) and 5 g/kg (G4) of FOS as well as diets supplemented with $1x10^9$ CFU/g (G5), $3x10^9$ CFU/g (G6) and $5x10^9$ CFU/g (G7) of *B. subtilis* for 56 days. After the feeding trial, the complement *C3, IL-1*β*, TNF-*α*, IFN-*γ and *hsp70* gene expression in the liver was then analyzed by a quantitative Real-time PCR. Then, fish were infected with *Streptococcus agalactiae*, and the survival rate was recorded. The results showed that FOS and *B. subtilis* had no significant effect ($P>0.05$) on growth performances and survival rate. Lysozyme activity was significantly greater in the G4, G5, G6, and G7 groups. Also, all fish fed FOS and *B. subtilis* showed significantly ($P<0.05$) higher respiratory burst activity than other groups. The expressions of complement *C3, IL-1*β*, TNF-*α*, IFN-*γ and hsp-70 in the liver were significantly higher for fish fed 5 g/kg of FOS as well as for fish that received any concentration level of *B. subtilis* ($P<0.05$) used in the study. However, after the *S. agalactiae* challenge test, the survival rate of fish-fed diets supplemented with FOS and *B. subtilis* was slightly higher than the control group.

The second trial, the effects of synbiotics between *B. subtilis* and FOS combination on growth performances, immunity improvement, and disease

resistance of Nile tilapia. The fish (24.5 ± 1.6 g) were fed a basal diet (G1), diets supplemented with 1 g/kg FOS + $1x10^9$ CFU/g *B. subtilis* (G2), 3 g/kg FOS + $3x10^9$ CFU/g *B. subtilis* (G3) and 5 g/kg FOS + $5x10^9$ CFU/g *B. subtilis* (G4) for 56 days. The results showed that synbiotics had significant effect ($P<0.05$) on growth performances including average daily gain (ADG) and survival rate compared with a control. Lysozyme activity and respiratory burst activity were significantly ($P<0.05$) greater in the G3 and G4 groups. The gene expressions of complement *C3*, *IL-1β*, *IL8* and *Hsp70* in the liver were significantly up-regulated in tilapia fed with 5 g FOS/kg feed + $5x10^9$ CFU/g of *B. subtilis* ($P<0.05$) synbiotics. The *TNF-α* and *IFN-γ* gene expression of tilapia fed with 3 g FOS/kg feed + $3x10^9$CFU/g of *B. subtilis* were significantly higher compared with the control group ($P<0.05$). After the *S. agalactiae* challenge test, the survival rates of fish-fed diets supplemented with synbiotics were higher than for the control group.

The results indicated that the FOS, *B. subtilis* and synbiotic supplementary feeds could stimulate immune responses in tilapia. However, further investigation of other prebiotics or herbs in combination with *B. subtilis* is encouraged at molecular levels and screening for beneficial metabolites that may increasingly improve digestive enzymes, growth performances, and health benefits in tilapia. In addition, on-farm experiments are needed.

Keywords : Probiotic, Prebiotic, Synbiotic, Nile tilapia, Immunity, Gene expression

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Arporn Panase

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Chapter 1
Introduction

1.1. Significances of problem

Nile Tilapia (*Oreochromis niloticus*) is one of the most popular freshwater fish and is farmed over 100 countries worldwide because of its fast growth, suitability for aquaculture, and high marketability (Chen et al., 2019). However, the rapid expansion of Nile tilapia farming has been negatively affected by infectious diseases and climate uncertainties, causing economic losses. The most common pathogenic bacteria influencing the Nile tilapia farming are *Streptococcus agalactiae, Flavobacterium columnare*, and *Aeromonas hydrophila* (Sookchaiyaporn et al., 2020). Chemicals and antibiotics have been applied for these disease prevention and treatment, possibly resulting in the development of resistant bacteria, residue in flesh and destruction of the microbial population in the aquatic environment. Therefore, dietary supplements with prebiotics and probiotics are promising alternatives because it is safe for customers and environment as well as it is able to stimulate beneficial bacteria in gastrointestinal track (Pandiyan et al., 2013).

Prebiotics are non-digestible food ingredients that usefully influence the host by enhancing the growth performances and interacting with bacteria in the gastrointestinal tract and thus improve host health (Gibson and Roberfroid, 1995). The common prebiotics used in aquaculture include mannanoligosaccharide (Rahmani et al., 2020), fructooligosaccharide (Baroiller et al., 1995), inulin, and galactooligosaccharide (Grisdale-Helland et al., 2008). FOS, short and medium chains of β-D-fructans, is one of the most studied prebiotics in fish. It improved immune responses, growth rate, and survivability. Tilapia fed with 20 – 30 g FOS/ kg supplemented diet could enhance immune responses, reduce oxidative stress, and increase survivability after challenging with *A. hydrophila* (Abd El-Gawad et al., 2015). Caspian roach (*Rutilus rutilus*) fry fed 2% and 3% FOS improved digestive enzyme

activities, enhanced growth performances, significantly elevated resistance to a salinity stress challenge (Soleimani et al., 2012). Tambaqui (*Colossoma macropomum)* fed 0.1 and 0.5% FOS presented a better growth performance (de Lima Paz et al., 2019). Dietary supplementation of FOS at a dose of 1% increased growth performance and stimulated immune responses of stellate sturgeon (*Acipenser stellatus*) juvenile (Akrami et al., 2013).

Besides prebiotics associated with aquaculture business, probiotics are living microorganisms such as *Bacillus* sp., *Lactobacillus* sp. and *Saccharomyces* sp. which are applied via the feed or to the rearing water. *Bacillus* spp. are widely used in aquafeeds for feed utilization improvement, growth performance promotion, innate immune response regulation, and disease resistance as well as improvement of water quality for sustainable aquaculture (Kuebutornye et al., 2019). These nonpathogenic bacteria can produce robust spores so they can endure high temperature, dehydration, and resistance to gastric environments (Elshaghabee et al., 2017). The optimal concentration of *B. licheniformis* in diets for juvenile tilapia was greater than or equal to 4.4×10^6 CFU/g feed in order to enhance growth performances, immune responses, and disease resistance (Han et al., 2015). In addition, the supplementation of *B. subtilis* in the red sea bream feed at 1×10^8 and 1×10^{10} CFU/ kg diet could increase the growth, feed utilization, health condition, and immune response (Zaineldin et al., 2018). The *Bacillus* sp. KUQ1 and *Bacillus* sp. KUQ2 dietary supplementation increased lysozyme activity, phagocytic activity and respiratory burst activity in tilapia (Sookchaiyaporn et al., 2020).

Synbiotics, are a combination of prebiotics and probiotics in the form of synergism (Cerezuela et al., 2011) which have been reported positive effects in many fish species. Prebiotics, probiotics and synbiotic can enhance growth performance, stimulate immunity, and increase pathogenic bacteria resistance in fish. However, to the best our knowledge, there is limited information application of *Bacillus* spp. and FOS in freshwater fish. In this study, the effects of dietary supplementation of

commercial *Bacillus subtilis* and FOS on growth performances, expression of immune related gene, non-specific immunity responses, and resistance against *Streptococcus agalactiae* infection in tilapia was carried out.

1.2. Objectives

1. To study dietary supplements with prebiotic, probiotic, and synbiotic effects on growth performances and immune responses of Nile tilapia (*Oreochromis niloticus*).

2. To study the cost of the use of prebiotics, probiotics, and synbiotics in fish farming.

1.3. Benefits

1. Known the appropriate level of prebiotic, probiotic, and synbiotic mixtures in tilapia feed to improve growth performances, strengthen the immune system, and modulate resistance to a bacterial disease was elucidated.

2. The knowledge gained should be very useful for studying and finding a way to effectively prevent bacterial disease in tilapia in the near future.

3. To generate more income for tilapia farmers.

4. Nile tilapia fed with prebiotic, probiotic, and synbiotic supplements have a better immune system and higher survival rate.

Chapter 2
Review of related literature

2.1. Biology and Taxonomy

The taxonomic classification of tilapia is still complicated due to the similarity and overlap of their morphological characteristics, and because many tilapia species freely hybridize in nature. It was later divided, based on breeding behavior and feeding habits, into two subgenera: *Tilapia* (substrate spawners) and *Sarotherodon* (mouthbrooders). Mouth brooders incubate the fertilized eggs and hatched fry in the mouth of the male or female parents or both male and female. Later, the subgenus *Sarotherodon* was raised to a genus and further classified into two genera, *Oreochromis* (mountain cichlids) and *Sarotherodon*, based on whether parental females (*Oreochromis*), males (*Sarotherodon*) or both parental sexes (*Sarotherodon*) perform the mouth brooding behavior (El-Sayed, 2006).

Phylum Vertebrata

Class Actinopterygii

Order Perciformes

Family Cichlidae

Genus *Oreochromis*

Species *Oreochromis niloticus*

2.2. Genus Oreochromis

Nile tilapia *(Oreochromis niloticus)*, Blue tilapia *(O. aureus)* and Mozambique tilapia *(O. mossambicus)* are examples of tilapia in the genus *Oreochromis*. Nile Tilapia has been intentionally introduced across the world as an aquaculture food fish. It is the second most cultured fish because of its high nutritional values and

disease resistance traits (Stankus, 2021). Global annual Tilapia production has been expanded from 379,169 t in 1990 to 6,100,719 t in 2020 (Stankus, 2021). The largest producers of Nile tilapia are China, Indonesia, Egypt, Brazil, and Thailand (Abdel-Latif et al., 2020). It is the main source of high-quality affordable protein in many of the developing countries (Carolan, 2016).

2.3. Growth of Tilapia

Growth of tilapia is dependent on their genetics, stocking rates, food supply, culture system, and water quality. Males grow faster by 10 – 20 % than females. Therefore, sex reversal of females to males is desirable in tilapia farming. The growth of fish will be drastically reduced if fingerling production is not controlled. The growth of Tilapia is directly related to the amount of food available in the pond while the growth of fish raised in cages located in public reservoirs or river depends on the pellet feed.

2.4. Feed and Feeding

Feed is the most expensive component in the intensive aquaculture industry, where it represents over 50% of operating costs. Newly hatched fry depends on their yolk sacs until consumed. Then, they eat the smallest phytoplankton present in the pond. As the fry becomes bigger, they eat larger organisms and supplemental feeds such as rice-bran, fishmeal, and others. Tilapia feeds on a variety of phytoplankton as their primary food items. They are cannibalistic and will feed on their fry if food is not abundant. The tilapia has a short esophagus leading to a small sac-like stomach with an exceptionally long intestine (4x the body long). The *O. niloticus* has firm pharyngeal teeth set on a triangular blade. Its role is to prepare food for digestion, shredding the coarser materials and breaking some of the cell walls before passing it on to the stomach.

The feeding regime (level, amount, frequency) of cultured tilapias is influenced by several factors, including fish species, size and age, diet form (pellets, crumbles, mash, dry, moist, floating, sinking) and culture system.

2.5. Environmental requirements

There is a variety of Tilapia culture systems including extensive, semi-intensive, and intensive systems under different environmental conditions, stocking densities and management strategies. Pond culture is the most popular method of growing tilapia. Tilapia can be cultured with other species to take advantage of many natural foods available in ponds. In addition, integrated systems; for example, Chicken/fish farming or Pig/fish farming, are applied for tilapia culture so the waste or manure generated from land animals serves as feed for the fishes in the pond. Tilapia can be cage cultured at higher densities in lakes, large reservoirs, and rivers where farmers don't have to own the land for constructing ponds. Major environmental factors affecting tilapia under aquaculture conditions include water temperature, dissolved oxygen, pH, ammonia, and nitrites. It is therefore important to understand the major water quality parameters and their interrelationships influencing fish growth, health, and their survival. Good water management is the key to successful fingerling and food fish production. For this reason, the water quality should be monitored regularly.

2.5.1. Temperature

Temperature is one of the most crucial factors involving physiology, metabolism, growth, and reproduction of tilapia. A temperature of about 28 – 30°C appears suitable for growth performance of Nile tilapia. Tilapia cannot tolerate temperatures below 10–12°C for more than a few days, while reproduction ends at 22°C and feeding decreases at 20 C (Hassan et al., 2013). *Oreochromis aureus* shows

better growth tolerance at a lower temperature as compared to other tilapia species. Baroiller et al. (1995) reported that increasing water temperature to 34 – 36 °C significantly raised the proportion of Nile tilapia males (69 –91 %), while low temperature (19 – 23°C) had no effects on sex ratio.

2.5.2. Dissolved oxygen (DO)

Dissolved oxygen (DO) is one of the limiting environmental factors affecting fish feeding, metabolism, stress, growth, and survival. DO fluctuation is disturbed by respiration, phytoplankton concentration, sunlight variation, photosynthesis, and water temperature. Tilapia can tolerate DO levels as low as 0.1 – 0.5 mg/l. They can even survive at zero DO concentration if they are allowed access to surface air. But larger tilapia especially, the ones that are closed to be harvested have gained the high risk for a massive death when they are suffering from depleted DO. The suitable DO for tilapia is higher than 3 ppm.

2.5.3. Ammonia and nitrite

Most of the nitrogenous waste of fish is excreted through gills in the form of ammonia. Excreted ammonia exists in un-ionized NH_3 form, which is toxic to fish, and ionized NH_{4+}, which is non-toxic (Chervinski, 1982). The toxicity of ammonia depends on DO, CO_2 and pH. The toxicity increases with decreasing DO and decreases with systems also affect the toxicity of ammonia to fish. The toxic level of NH_3 -N which causes a negative effect on the growth performance ranges from 0.07 to 0.14 mg/l, it is recommended that the NH_3 -N concentration should be maintained below 0.1 mg/l.

Ammonia is oxidized into nitrite (NO_2) and then into nitrate (NO_3) through nitrifying bacteria grown on suspended organic matter. The bacteria remove the

organic matter from the culture system by using it as food, while the bacteria themselves can be used as natural food for filter-feeding fish such as tilapia and carp. Nitrate is relatively non-toxic to tilapia; however, prolonged exposure to elevated levels of nitrate may decrease immune response and induce mortality (Plumb, 1997). Nitrite is highly toxic to fish, including tilapia since it disturbs the physiological functions of the fish and leads to growth retard.

2.5.4. pH

Tilapia were reported to tolerate a pH range of 5 to 11 for at least 24 h, but they die at pH < 3.5 and >12 (Reite et al., 1974). They overcome the problem of ammonia excretion by excreting about 90% of their nitrogenous wastes as urea. This process is facilitated by seawater-type gill chloride cells (Wilkie and Wood, 1996).

2.6. Tilapia diseases

Tilapia are more resistant to viral, bacterial and parasitic diseases than other commonly cultured fish. “Ich,” caused by the protozoan *Ichthyopthirius multifiliis*, can cause serious losses of fry and juveniles in intensive recirculating systems. External protozoans such as *Trichodina* and *Epistylis* also may reach epidemic densities on stressed fry in intensive culture. Common bacterial pathogens and diseases of tilapia include *Aeromonas hydrophila*, *Streptococcus iniae* and *Streptococcus agalactiae*, columnaris disease (caused by *Flavobacterium columnare*) and Francisellosis. In recent years the bacterial infection *Steptococcus iniae* has caused heavy losses, primarily in recirculating and intensive flow-through systems (Popma and Masser, 1999).

2.6.1. Streptococcosis

Streptococcosis is one of the most devastating diseases causing massive kills of large size fish and is responsible for heavy economic losses. *Streptococcus* spp. are Gram positive, non-acid fast, non-motile, oxidase-positive, catalase-negative cocci. *Streptococcus agalactiae* is the major cause of streptococcosis in farmed tilapia while *S. iniae* also causes mortality but to a lesser extent. Stressful culture conditions, including high water temperature, high salinity, and alkalinity (pH > 8), low dissolved oxygen, high nitrite, and high stocking density, increase the susceptibility of tilapia to streptococcal infection (Chang and Plumb, 1996; Shoemaker et al., 2000).

The major clinical signs of this bacterial disease included lethargy and erratic swimming, petechiae (pin-point) hemorrhage, exophthalmia with corneal opacity (Figure. 1), and body discoloration, ascites, enlarged spleen and kidney.

Figure 1 Exophthalmia in a moribund tilapia

2.7. Preventions and treatments

2.7.1. Good management practice in tilapia hatchery and farm

Farmers should minimize unnecessary handling or transportation of fish to reduce the stress of fish, which may contribute to disease outbreaks. It is also recommended to keep the hatcheries and farms clean all the time to reduce the risks of disease transmission and outbreaks. Furthermore, periodic cleaning and disinfection of all production units and equipment are also suggested as these will decrease the transmission of pathogens. In farm, for example, cleaning of the culture net cages, tanks and ponds should be conducted before the introduction of new fry by physical cleaning and drying, while drying and liming (calcite or dolomite) for the ponds. Equipment such as scoop nets, buckets, small tanks, containers, etc., should be regularly cleaned using the recommended commercial bleach (sodium hypochlorite at 200 – 500 ppm) (Dvorak, 2009).

Partial reduction or completely stop feeding helps to control or reduce the mortality during streptococcosis outbreaks. This is because feeding facilitates proliferation of bacteria in the water. Furthermore, uneaten, or excessive feed leads to deterioration of water quality. Besides, infected fish are low in appetite until they are recovered from the infection or in other words, the sick fish lose their appetite and will not eat. Therefore, feed reduction is one of the factors that can reduce and control the mortality rate of streptococcosis. The use of contaminated trash fish as feed has also been implicated in the outbreaks of *Streptococcus* in Korea (Kim et al., 2007), especially in the marine water fish aquaculture.

2.7.2. Reasonable Fish Stocking Density in the Culture System

Farmers should reduce fish stocking density to be reasonable according to the size of cages, size of fish or type of culture systems. High productivity in Tilapia farming is achieved by balancing stocking density with a good survival rate. When mortality increases, lowering the stocking density helps to lower both stress level

and pathogen load within the population. Extensive research has been carried out on the effects of stocking density on tilapia production in different intensive culture systems (El-Sayed, 2002).

2.7.3. Antibiotics

Antibiotic treatment is generally ineffective as the infected fish have a reduced appetite, and the need for a proper vaccine has become a must (Klesius et al., 2000). However, Darwish andGriffin (2002) found that oxytetracycline was effective in controlling *S. iniae* in blue tilapia (*O. aureus*). Oxytetracycline was incorporated into the feed at 0, 25, 50, 75 and 100 mg/kg body weight. The 75 and 100 mg dose significantly increased the survival of the infected fish from 7% in the infected non-medicated to 85 and 98%, respectively. The negative consequences of using antibiotics, such as emergence of antibiotic resistant bacteria and antibiotic residues in meat, must be carefully evaluated.

2.8. Immune systems in fish

An immune system is a biological process within organisms that detects a wide variety of foreign agents, from viruses to parasitic worms, distinguishes them from the organism's own healthy tissue, eliminates pathogens, and protects against diseases. The immune system of fish is physiologically like that of higher vertebrates. Fish possess both innate and adaptive immune defense systems.

2.8.1. The innate immune system

The innate system is the earliest immune mechanism that defends the host from infection by other organisms in a nonspecific manner. This means that the cells of the innate system recognize and respond to pathogens in a generic way. It also possesses memory as the host evolves its innate immune components based on

evolutionary experience of its ancestors encountering similar pathogens (Kurtz, 2005). Nonspecific or innate immunity is a fundamental defense mechanism in fish.

2.8.2. Innate defense mechanisms

The innate defense mechanisms are divided into three compartments including the epithelial/mucosal barrier, the humoral parameters, and the cellular components.

2.8.3. Physical barrier defense

Skin mucus and gills act as the first barrier to infection. The mucus of fish contains lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides, and immunoglobulin M (IgM), which have an important role in inhibiting the entry of pathogens (Boshra et al., 2006; Saurabh and Sahoo, 2008). In addition, the epidermis is able to react to different attacks (thickening and cellular hyperplasia), and its integrity is essential for osmotic balance and to prevent the entry of foreign agents (Takashima and Hibiya, 1995). On the other hand, defending cells are present, such as lymphocytes, macrophages, and eosinophilic granular cells (Ellis, 1999; Sveinbjornsson et al., 1996).

2.8.4. The humoral innate components

2.8.4.1. Lysozyme

This enzyme is found in fish mucus, serum and eggs (Ellis, 1999) and is able to digest the peptidoglycan layer of bacterial cell walls. Lysozyme is produced by macrophages and neutrophilic granulocytes (Murray and Fletcher, 1976) and is bactericidal even for serious pathogens such as *Aeromonas salmonicida* and *Aeromonas hydrophila* (Ellis, 1999). In fish, lysozyme is synthesized in both the liver and the extra hepatic sites (Bayne and Gerwick, 2001).

2.8.4.2. Complement

The complement system is one of the first internal defenses. The complement system consists of a group of protein and non-protein components that are involved in both innate defense mechanisms and specific adaptive immunity. The complement system can be activated in three ways: (i) the classical pathway, which is triggered by antibody binding to the cell surface (Holland and Lambris, 2002), (ii) the alternative pathway, which is independent of antibodies and is activated directly by foreign microorganisms, and (iii) the lectin pathway, which is activated by the binding of a protein complex consisting of mannose/mannan-binding lectin in bacterial cells (Sakai, 1992). Activation results in the opsonization and/or lysis of foreign cells. Yano (1996) shows that most classes of fish, including jawless fishes, possess a lytic complement system. Isolated C3 and C5 from rainbow trout plasma (Nonaka et al., 1984; Nonaka et al., 1981). Yano (1996) has shown that C1–C9 are present in carp (*Cyprinus carpio*) plasma.

2.8.5. Cytokines

Cytokines, a class of small molecular signaling proteins with a wide range of biological activities, are secreted mainly by immune cells and function by binding to the corresponding receptors to regulate cell growth, differentiation, and effects. Cytokines can have a stimulatory or inhibitory effect on the immune response, which can be further influenced by the presence of other cytokines. Recent genomic studies have confirmed that fish have almost all the same cytokines as mammals. Cytokines play an important role in both innate and adaptive immune responses.

2.8.5.1. Interleukin-1 family

Interleukin (IL) 1 is a pro-inflammatory cytokine that plays an important role in innate immunity. Kono et al. (2002), reported that IL-1 plays an important role in

fish immunity by activating lymphocytes and phagocytic cells and increasing resistance to *Aeromonas hydrophila* infection. Proinflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological pain.

2.8.5.2. Interleukin (*IL-1* β)

IL-1β is released primarily by monocytes and macrophages as well as by nonimmune cells, such as fibroblasts and endothelial cells, during cell injury, infection, invasion, and inflammation. IL-1β is major immunostimulants influencing fish immune response. Nile tilapia fed with supplement diet with *Bacillus subtilis* at 10^7 CFU/g and *Lactococcus lactis* at 10^8 CFU/g diets after an eight-week feeding trial, IL-1β gene expression significantly higher than those of fish fed control group (Won et al., 2020).

2.8.5.3. Interleukin-8 (*IL-8*)

Interleukin-8 (IL-8; also known as CXCL8, NAF-1, MDNCF-1, and NAP-1) is a heparin-binding protein comprised of a three stranded β-sheet and an α-helix, is an inflammatory cytokine. IL-8 is produced by various cells, including macrophages, when inflammation is induced, and it activates inflammatory cells by the synergistic action of NFkB, C/EBP, and AP-1 binding elements in the inflammatory reaction (Zlotnik and Yoshie, 2012). This cytokine plays an important role in the inflammatory response through the activation of neutrophil cells (Kim et al., 2019) and initiates the oxidative burst in neutrophils and induces wound healing by promoting angiogenesis (Li and Yao, 2013). The expression of IL-8 increased after the initiation of the immune system rather than at the early stage of development, and high expression was

observed in the gills and spleen, the organs associated with immunity and metabolism. In addition, IL-8 gene expression after infection by viral hemorrhagic septicemia virus (VHSV) significantly increased in the fin, gill, muscles, and spleen. IL-8 is closely related to inflammation and immune regulation in the immune response of the olive flounder (*Paralichthys olivaceus*) (Kim et al., 2019). Nile tilapia fed Dietary supplementation with β-glucan and/or *Bacillus coagulans* showed synergistic immune responses by increasing IL-8 gene expression (Fath El-Bab et al., 2022).

2.8.5.4. Tumor necrosis factor (*TNF-α*)

TNF-α is the most immunologically important molecule in the TNF family. TNF-α, also known as cachectin, is another inflammatory cytokine that plays a major role in some pain models. In fish, two genes, tnfa and tnfn are duplicated in tandem on the chromosome (Savan et al., 2005). In tilapia, TNF-α has been shown to upregulate granzyme expression in non-specific cytotoxic cells and to protect these cells from activation-induced cell death (Praveen et al., 2006). The activation of TNF-α is indispensable because their activation affects several innate immune responses including phagocytosis (Sakai, 1999). Nile tilapia fed with supplement diet with *Bacillus subtilis* at 10^7 CFU/g and *Lactococcus lactis* at 10^8 CFU/g diets after an eight-week feeding trial, had TNF-α gene expression significantly higher than those of fish fed control group (Won et al., 2020).

2.8.5.8. Interferon family *(IFN-γ)*

Interferons are types of cytokines produced by animal cells in response to the invasion of viruses, heterologous RNA, and certain sugars. Cells infected with viruses are known to exhibit an interference phenomenon that blocks infection by other viruses. In fish, type I IFNs (homologs to the human IFN-α and IFN-β) exhibit

antiviral activity, and type II IFN (IFN-γ) have also been reported to exhibit bactericidal activity against intracellular parasitic bacteria. A type I IFN in fish has been shown to have antiviral activity (Zou and Secombes, 2016). Nile tilapia fed supplement diet with 10^7 CFU/g *Bacillus subtilis* and 10^8 CFU/g *Lactococcus lactis* diets after an eight-week feeding trial, showed interferon-gamma (IFN-γ) gene expression significantly higher than those of fish fed control group (Won et al., 2020).

2.8.5.6. Heat-shock proteins, HSPs

Heat-shock proteins, known as stress proteins and extrinsic chaperones, play important roles in the folding, translocation, and refolding/degradation of proteins (Zhang, C. N. et al., 2014). Heat shock protein 70 (HSP70) is widely distributed group of HSPs found in numerous organisms from bacteria to mammals especially HSP70 in fish**.** HSPs are induced in response to a variety of stress conditions, such as elevated temperature, UV, chemical exposure, and metabolic insults. The expression of Hsp70 was significantly increased in the liver, head kidney, spleen, and gill of Nile tilapia after six-hour *Streptococcus agalactiae* infection (Zhang, C. N. et al., 2014). HSP70 overexpression in fish hepatocytes under stress may aid cell survival by protecting against oxidative and nitrative stress-induced changes (Padmini and Rani, 2008). Nile tilapia fed with supplement diet with *Bacillus subtilis* at 10^7 CFU/g and *Lactococcus lactis* at 10^8 CFU/g diets after an eight-week feeding trial, had heat shock protein HSP70 gene expression significantly higher than those of fish fed control (Won et al., 2020).

2.8.6. The cellular innate components

2.8.6.1. Phagocytic cells

Macrophages and neutrophilic granulocytes in fish are the principal phagocytic cells (Kemenade et al., 1994; Secombes and Fletcher, 1992). These cells recognize evolutionarily conserved epitopes present on microorganisms, using so-called pattern recognition receptors' (PRRs). Different types of PRRs have been described for fish, including Toll-like receptors (Bricknell and Dalmo, 2005). Upon stimulation through PRRs, these cells phagocytize antigenic material and/or exert cytotoxic activity. The killing of intracellular or extracellular pathogens is based upon the release of a number of oxygen radical species and nitric oxide (NO) (Campos-Pérez et al., 2000; Saeij et al., 2002). Phagocytosis of antigenic material by macrophages is not only an activity of the non-specific innate defense system but is also the initial step in the specific adaptive immune response. Macrophages from immune fish are more active in phagocytosis than those from control animals. This is probably due to the opsonization of the antigen by antibodies or to metabolic activation of the macrophages (Griffin, 1983). Sakai (1984) has even suggested that salmonid macrophages have Fc and C3 receptors on their surface facilitating the binding and gut of carp bind purified Ig, which is an indication for Fc receptors on these cells (Koumans-van Diepen et al., 1994). This is another example of cooperation between the innate immune system (phagocytes) and the acquired immune system (Ig molecules).

2.8.7. Adaptive immunity

The adaptive immunity or specific immune response occurs through mechanisms that involve a complex network of specialized cells, proteins, genes, and biochemical messages that provide the mechanisms for the body to respond

specifically to antigens, antibodies and effector cells with high specificity and affinity. The adaptive immune system of teleost fish consists of two major components.

2.8.7.1. Humoral responses

The predominant immunoglobulin in teleosts is a tetramer of the IgM class and contains eight antigenic combining sites (Acton et al., 1971). Some teleosts have a monomer of IgM in their serum, although the factors leading to its expression are still unknown (Wilson and Warr, 1992).

IgD was the second immunoglobulin isotype identified in fish, specifically catfish, due to sequence similarity with IgD in mammals, its location immediately under the IgM gene and its expression in B cells (Wilson et al., 1997). Moreover, the concentration of IgM in the serum of salmonids is extremely low compared to that of other teleosts such as Japanese eel (*Anguilla japonica*) (Uchida et al., 2000), cyprinids (Vilain et al., 1984) and some Perciformes (Scapigliati et al., 1997). However, the amounts of IgM in the serum of brown trout (Salmo trutta) and rainbow trout that are infected or acclimated to high temperature (19 °C) reach values similar to those of the common cod and haddock (*Melanogrammus aeglefinus*). In addition, IgM levels in salmon and Atlantic cod (Gadus morhua) vary with size (Magnadottir et al., 2001; Sanchez et al., 1993) temperature (Sanchez et al., 1993) and water quality season (Magnadottir et al., 2001; Olesen and Vestergard Jorgensen, 1986). Teleost antibodies are found in the skin (Hatten et al., 2001), intestine (Rombout et al., 1986), gill mucus (Lumsden et al., 1993), bile (Jenkins et al., 1994) and systemically in the plasma. The immune response of the skin and gills is important because these organs are in direct contact with the environment. Specific antibodies can be generated in the skin (Cain et al., 2000), intestine (Jones et al., 1999) and gills (Lumsden et al., 1993) without necessarily generating a systemic response.

2.8.7.2. Cell-mediated responses

T cells play a fundamental role in cell-mediated responses of adaptive immunity by either involving the regulation of other leukocytes functions or directly killing infected host cells. T cells are developed in the thymus; therefore, they are also called thymocytes.

2.9. Molecular Techniques

2.9.1 Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is currently regarded as the gold standard for targeted quantification of RNA gene expression, especially because of its high sensitivity, specificity, accuracy, precision, and also because of its practical simplicity and processing speed. However, different critical factors can influence the outcome of RT-qPCR studies, including isolation of RNA, reverse transcription to cDNA, and data analysis. These factors need to be addressed in order to obtain biologically meaningful results (Vanhauwaert et al., 2016). Successful quantitative RT-PCR involves correction for experimental variations in individual RT and PCR efficiencies The simplest detection technique for newly synthesized PCR products in real-time PCR uses SYBR Green I Fluorescence DNA (Freeman and Walker, 2016). qRT-PCR (real-time reverse transcription-PCR) for the detection and quantification of RNA targets and is being utilized increasingly in novel clinical diagnostic assays. Quantitative results obtained by this technology are not only more informative than qualitative data, but simplify assay standardization and quality management Therefore, although it is evident that qRT-PCR assay has become a useful and important technology in the clinical diagnostic laboratory, it must be used appropriately and it is essential to be aware of its limitations if it is to fulfil its potential (Bustin, 2005). The qRT-PCR was used to detect immune-related genes in liver, spleen, and head kidney of grass carps after feeding with probiotic *Pediococcus pentosaceus* strain (SL001) supplemented diets for 30 days. It was found

that immunoglobulin M (IgM) and complement 3 (C3) were significantly increased ($p < 0.05$), whereas the interleukin-8 (IL-8) was down-regulated ($p < 0.05$) (Gong et al., 2019). Quantitative Real-time PCR analysis was applied for immune-related gene expression including complement C3, *IL-1*β, *TNF-*α, *IFN-*γ and *hsp-70* in the Nile tilapia liver and it was found that these gene expression was significantly higher ($p < 0.05$) for fish fed 5 g/kg of FOS as well as for fish that received *B. subtilis* supplementary feeds (Panase et al., 2022). In addition, Olive flounder (*Paralichthys olivaceus*) fed *Bacillus* sp. and β-glucooligosaccharides combination for 8 weeks showed an improvement in transcription of IL-6 and TNF-α (Hasan et al., 2018).

2.10 Prebiotic and probiotic in aquaculture

2.10.1 Prebiotic in aquaculture

Prebiotics are non-digestible food ingredients that usefully influence the host by enhancing the growth performances and interacting with bacteria in the gastrointestinal tract and thus improve host health (Gibson and Roberfroid, 1995). Prebiotic may have the role of increasing growth rate, improve immune system as well as change the community of bacterial in gastrointestinal track (Yousefian and Amiri, 2009). The common prebiotics used in aquaculture include mannanoligosaccharide (Rahmani et al., 2020), fructooligosaccharide (Baroiller et al., 1995), inulin, and galactooligosaccharide (Grisdale-Helland et al., 2008). FOS, short and medium chains of β-D-fructans, is one of the most studied prebiotics in fish. It improved immune responses, growth rate, and survivability. Tilapia fed with 20 – 30 g FOS/ kg supplemented diet could enhance immune responses, reduce oxidative stress, and increase survivability after challenging with *A. hydrophila* (Abd El-Gawad et al., 2015). Caspian roach (*Rutilus rutilus*) fry fed 2% and 3% FOS improved digestive enzyme activities, enhanced growth performances, significantly elevated resistance to a salinity stress challenge (Soleimani et al., 2012). Tambaqui (*Colossoma macropomum)* fed 0.1 and 0.5% FOS presented a better growth performance (de Lima Paz et al., 2019). Dietary supplementation of FOS at a dose of

1% increased growth performance and stimulated immune responses of stellate sturgeon (*Acipenser stellatus*) juvenile (Akrami et al., 2013). Significance of prebiotics in aquaculture prebiotics are essential dietary supplement which enhance growth performance as well as microbial activities of digestive tract, also boost immune system and improve stress resistance that are discussed below.

Effects of prebiotics on microbes of gastrointestinal track

In the gastrointestinal track of all invertebrates and vertebrates, the bacterial community is affected by the substances and vice versa. On the other hand, there are positive and/or interaction between the bacterial and substance in gastrointestinal track (Fuandila and Yuhana, 2019). Various prebiotic oligosaccharides such as inulin and oligofructose are fermented in the colon where they stimulate the growth of bacterial populations related with a well-functioning colon and this stimulation occurs because oligosaccharides are readily fermented by beneficial colonic bacteria and are not used effectively by pathogenic bacterial species (Yousefian and Amiri, 2009).

Properties of fructooligosaccharides (FOS)

Fructooligosaccharides (FOS) are oligosaccharides that occur naturally in plants such as onion, chicory, garlic, asparagus, banana, artichoke, among many others. They are composed of linear chains of fructose units, linked by beta (2-1) bonds. The number of fructose units ranges from 2 to 60 and often terminate in a glucose unit. Dietary FOS are not hydrolyzed by small intestinal glycosidases and reach the cecum structurally unchanged. There, they are metabolized by the intestinal microflora to form short-chain carboxylic acids, L-lactate, CO_2, hydrogen and other metabolites. FOS have a number of interesting properties, including a low sweetness intensity; they are also calorie free, non-cariogenic and are considered as soluble dietary fibre (Sabater-Molina et al., 2009).

2.10.2 Probiotic in aquaculture

Probiotics shows a new dimension in disease resistance and improving water quality in aquaculture industry. Probiotics are used in aquaculture to enhance growth performance, amplify nutrition utilization, decrease disease prone, and improve immune system (Nayak et al., 2007). Bacteria have been broadly used as probiotic strains which are usually present in the intestine of healthy fishes such as the *Lactobacilli* and *Bifidobacteria*. Some gram positive bacteria like *Bacillus*, *Enterococcus*, *Streptococcus* act as common probiotic strains which are the main gastrointestinal microbiota (Prasad et al., 2003). Probiotics use in aquaculture show great impact on aquatic organisms. Probiotics decrease accumulation of organic load and maintain water quality in an efficient way.

Properties of *Bacillus subtilis*

Bacteria of the *Bacillus* genus are among the most widespread microorganisms in nature, they can be found in soil, water and air. Bacillus constitutes a diverse group of rod-shaped, Gram-positive bacteria, characterized by their ability to produce a robust spore (Sonenshein et al., 1993). In shrimp/fish aquaculture, feed represents the most expensive production cost. The quantity and quality of diets are primary factors influencing shrimp/fish growth, health status, disease prevention, pound contamination and expenses. Utilization of probiotic bacteria has emerged as a solution with enormous applications in the aquaculture feeding industry. *Bacillus* species principally *B. subtilis* are one of the most investigated bacteria for animal probiotic development due to, versatility of growth nutrients utilization, high level of enzymes production, secretion of antimicrobial compounds, spore producers, develops in aerobic and anaerobic conditions, and *B. subtilis* is Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA). Functional feeds development with alternative-economic nutrient vegetable sources of proteins, carbohydrates, lipids and Bacillus subtilis probiotic strains, must be considered in shrimp/fish aquaculture production systems; as an option to eliminate animal feed ingredients, improves digestion-assimilation, reduce water pollution and diseases, and to increase yields and profits (Olmos and Paniagua-Michel, 2014).

2.11. The application of probiotics, prebiotics, and synbiotics as feed additives for Tilapia

Probiotics and prebiotics are one of the feed additives in tilapia farming to improve growth performance and disease resistance. Prebiotics are nondigestible additives that improve the utilization of feed by encouraging the growth and activity of bacteria in the digestive tract that enhance fish health. Examples of these prebiotics include oligosaccharides, resistant starch, and specific non-starch polysaccharides. Probiotics on the other hand are live microorganisms which when added to the fish diet, can improve the intestinal microbial balance. Probiotics help in enhancing the zootechnical performance of tilapia fish, their immune response, and growth. These probiotics can consist of microbes such as *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp. and *Lactobacillus* sp. One of the most used probiotics in aquaculture is *B. subtilis.*

Prebiotics have been reported to have numerous beneficial effects in fish such as enhanced growth performance, increased disease resistance, improved non-specific immune response and immune-related genes. They can modify the gastrointestinal tract microbial community to enhance innate immune responses (Bailey et al., 1991).

Soleimani et al. (2012) studied the effects of fructooligosaccharide (Baroiller et al., 1995) prebiotic on the innate immune responses, stress resistance, digestive enzyme activities, growth factors, and survival of Caspian Roach (*Rutilus rutilus*) and found that 2% and 3% FOS additive feeds could improve the immune responses (Ig levels, lysozyme activity, and ACH50), whereas 1% dietary FOS only elevated serum lysozyme activity. All dietary FOS levels significantly increased resistance to a salinity stress challenge ($p < 0.05$) and highest survival was observed in the 3% FOS group.

The effects of dietary FOS on bacterial Infection, oxidative stress, and histopathological alterations in Nile Tilapia (*Oreochromis niloticus*) were investigated by Abd El-Gawad et al. (2015). Feeding fish with FOS supplemented diet increased

survivability after challenging with *A. hydrophila*. Dietary supplementation of FOS (20 and 30 g FOS/ kg diet) could minimize the physiological alterations, increase host immune system, and relieve oxidative stress as well as renewal of tissue histological structures.

Abd El-Gawad et al. (2016) reported that 2% dietary FOS was the most suitable for the antioxidant activity, non-specific immune response, and growth performance improvement in *Oreochromis niloticus*. The administration of 0.75% honey as a prebiotic was effective in improving the growth performance, immune response, and resistance of shrimp to *V. parahaemolyticus* infection (Fuandila and Yuhana, 2019).

Panase et al. (2022) investigated the influences of fructooligosaccharide on growth performances, immune responses, and disease resistance of Nile tilapia, *Oreochromis niloticus.* The results showed that lysozyme and respiratory burst activities were significantly increased in fish fed with 5 g/kg FOS. Immune-related genes including complement *C3*, *IL-1*β, *TNF-*α, *IFN-*γ, and *hsp-70* in the tilapia liver were significantly higher in fish fed 5 g/kg of FOS.

Probiotics are live microorganisms that can survive, proliferate, and colonize the animal gut, when applied in the appropriate doses. *Bacillus*, *Lactobacillus*, and *Lactococcus* are widely known as the main probiotics in aquaculture (Balcázar et al., 2006; Kotzent, 2017). There are many beneficial effects of probiotics including the exclusion of invading microbes, increased feed utilization, enhanced growth performances, improved non-specific immune responses, increased animal survival.

Dietary *B. subtilis* C-3102 supplementation (10^5 CFU/g) significantly increased the total amounts of adhesive viable bacteria, induced upregulation of intestinal cytokine expression (IL-1b, TGF-β and TNF-α) and downregulation of intestinal HSP70; however, it had no effects on growth performances of hybrid tilapia (He et al., 2013).

The application of *B. subtilis* strains NZ86 and O14VRQ (10^7 CFU/ g) as probiotics in tilapia feeding for 51 days stimulated several innate immune responses. Different strains provided different levels of nonspecific immune responses (Galagarza O. A. et al., 2018). *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2, isolated from Nile tilapia were used as probiotics and it was found that there were no significant effects on the average weight, average daily growth, specific growth rate, or feed conversion ratio of tilapia fry after an 8-week feeding trial; however, immune parameters including lysozyme, phagocytic activity and respiratory burst activity of juvenile fish treated with probiotics were significantly higher than those of the control (Sookchaiyaporn et al., 2020). Panase et al. (2022) investigated the effects of commercial *Bacillus subtilis* product (Greentech Aquaculture co., LTD., Thailand) on growth performances, immunity improvement, and disease resistance of Nile tilapia (*Oreochromis niloticus*. The results had no significant effect ($P > 0.05$). on growth performance and survival rate. Lysozyme activity was significantly greater in the $1x10^9$ CFU/g (G5), $3x10^9$ CFU/g (G6) and $5x10^9$ CFU/g (G7) of *B. subtilis* after 56 days. Also, all fish fed *B. subtilis* showed significantly ($p < 0.05$) higher respiratory burst activity than other groups. The expressions gene of complement *C3, IL-1*β, *TNF-*α, *IFN-*γ and *hsp-70* in the liver were significantly higher for fish fed any concentration level of *B. subtilis* ($p < 0.05$) used in the study. These *inconsistent research results* possibly were due to different *B. subtilis* strains, dosage, and duration application as well as different environments.

The supplementation of probiotic (*Bacillus subtilis* – BS, C-3102 strain) 2 g BS/ kg for six weeks performed better in average daily gain, feed conversion rate, specific growth rate, protein efficiency ratio, carcass yield, total and standard length, and body height than those maintained on control diets. This probiotic supplementation resulted in higher villus height and intestinal perimeter ratio than the control diet (Azevedo et al., 2016).

Synbiotics, a combination between prebiotics and probiotics, have been used in aquaculture for over 10 years. However, the mechanisms of how synbiotics work as growth and immunity promoters are far from being unraveled. Synbiotics may indirectly and directly promote the growth of aquatic animals through releasing extracellular bacterial enzymes and bioactive products from synbiotic metabolic processes (Huynh et al., 2017).

Widanarni andTanbiyaskur (2015) reported that synbiotic dietary supplementation of 10^6 CFU/ml *Bacillus* sp. and 2% oligosaccharides from sweet potato var. sukuh in Nile tilapia for 14 days enhanced the growth and feed conversion ratios as well as survival rate against *S. agalactiae* infection challenge.

Application of micro-encapsulated probiotic *Bacillus* NP5 RfR, prebiotic MOS and synbiotic for 40 days were able to increase growth performance, immune response and survival rate of tilapia infected by *S. agalactiae* (Agung and Yuhana, 2015).

The dietary combination of *Bacillus circulans* PB7 (BCPB7) and fructoligosaccharide (Baroiller et al., 1995) improved ($p < 0.05$) growth performance (weight gain, specific growth rate, feed conversion ratio and protein efficiency ratio) and immune-physiological (hematological indices, serum biochemistry, lysozyme, NBT activity), antioxidative status in the form of antioxidant enzyme (catalase, superoxide dismutase, glutathione-S-transferase), acetylcholine esterase (Cadelis et al.), Na+ K+ ATPase and stress biomarkers (cortisol, glucose and HSP-70) function for *Labeo rohita* (rohu) juveniles (Singh et al., 2019).

Kumar et al. (2018) reported dietary supplementation of synbiotic (*B. subtilis* and Mannan oligosaccharide) had a synergistic effect on improving innate immunity (Lysozyme activity and respiratory burst activity) and disease resistance against *Aeromonas hydrophilla* infection of *Cirrhinus mrigala* juveniles.

The combination with *Bacillus* sp. SJ-10 and β-glucooligosaccharides supplement not only improved growth performance and innate immunity such as respiratory burst, superoxide dismutase, and lysozyme activity but also protected olive flounder (*Paralichthys olivaceus*) from infectious streptococcosis (Hasan et al., 2018).

The inclusion of symbiotic MOS and probiotics (3.50×10^9 CFU/g *L. acidophilus,* 3.50×10^9 CFU/g *Bifidobacterium* sp, and 3.50×10^9 CFU/g *E. faecium*) in diets for 63 days had a positive influence on the growth performance and immunity of Nile tilapia (Cavalcante et al., 2020).

The different results are probably caused by the types of prebiotics and probiotics, administration dosages, duration, and conditions of experiment, as well as fish ages and species.

Chapter 3

Methodology

3.1. Materials

3.1.1 Instruments and equipment

1. Micropipette
2. 96 well plate
3. Cage 2 x 2 m
4. Syringe 1 ml
5. Digital Scales
6. Centrifuge
7. Spectrophotometer
8. Thermal Cycler
9. Hemocytometer
10. Beaker
11. Eppendorf

3.1.2 Chemicals

1. Culture media (RPMI)
2. NaCl
3. KCl
4. Na_2HPO_4
5. KH_2PO_4

6. Distilled water

7. Bacteria suspension *Micrococcus lysodeikticus* (Sigma, USA)

8. NBT (Sigma Aldrich)

9. Methanol 100%

10. Methanol 70%

11. 2N KOH

12. DMSO (Dimethyl Sulfoxide)

13. Lysis Buffer

14. RNase-Free Water

16. DNase

17. PureLink RNA Mini Kit

3.1.3 Prebiotic

1. Fructooligosaccharide (Baroiller et al., 1995)

3.1.4 Probiotic

1. *Bacillus subtilis*

3.1.5 Pathogenic bacteria

1. *Streptococcus agalactiae*

3.2. Methodology

3.2.1. Fructooligosaccharide and *Bacillus subtilis* preparations

The fructooligosaccharide (Baroiller et al., 1995) used in this study was supported by Quantum Hi-Tech Biological Co., Ltd, China. FOS appearance was white or light-yellow powder without visible impurity. The product composition was more than 50% including 1-kestose (1-kestotriose; GF2), nystose (GF3), 1F-fructofuranosylnystose (GF4). The other undesirable components including bacterial, moulds, and yeast were not more than 10 CFU/g.

The commercially available probiotic product (Greentech Aquaculture co., LTD, Thailand) contains *Bacillus subtilis* 1x10 ^{9} CFU/g.

3.2.2. Diet preparation

The basal diet (HiGrade 9951, CPF) was commercially available containing 30% crude protein and 3% lipid, which has been shown to be sufficient to support the optimal growth of Nile Tilapia. This basal feed was used as a control diet. The basal diet was supplemented with three levels of FOS (1, 3, and 5 g /kg), three levels of *B. subtilis* (1x0^{9}, 3x10^{9}, and 5x10^{9} CFU / g and three levels synbiotic (1 g /kg FOS +1x10 ^{9} CFU/g *B. subtilis* supplemented diet, 3 g /kg FOS + 3x10 ^{9} CFU/g *B. subtilis* supplemented diet and 5 g /kg FOS + 5x10 ^{9} CFU/g *B. subtilis* supplemented diet.

The FOS and *B. subtilis* at a particular concentration were sprayed into 1 kg of the basal diet. These diets were coated with 20 mL of fish oil and air-dried at room temperature for 24 h, and then stored in sealed plastic bags at 4 °C for further use.

3.2.3 Fish and experimental design

Apparently healthy Nile tilapia (average body weight 24.5±1.6 g) were obtained from a local fish farm and acclimated for two weeks in (2 m x 2 m) cage. The fish were fed to satiation with a commercial diet two times per day at 08.00 AM

and 16.00 PM. After acclimation, four hundred twenty fish were randomly divided into seven groups and stocked in 2mx2m cage in triplicate at a rate of 20 fish per cage. The experiment was conducted for 56 days. Fish were fed two times per day at a rate of 5% of the body weight and the fish were weighed every two weeks to adjust the feeding amount.

3.2.4. Growth performance and survival measurement

At the end of the feeding trial, fish in each cage were weighed for growth performance and survival rate measurements. The growth parameters were calculated according to the following formula:

Weight gain (WG, %) = (final weight−initial weight) × 100

Average daily gain (ADG g day-1) = 100 x (final body weight – initial body weight) / experimental period

Feed Conversion Ratio (FCR) = quantity of feed offered/weight gain

Survival (%) = (final number of fish/initial number of fish) X 100

3.2.5. Blood collection

Three fish from each cage (9 fish per group) were randomly selected for blood collection at the termination of the feeding trial (56 days). Fish were randomly selected, anesthetized, and cleaned using alcohol with special care around the anus in order to avoid any contamination. Then, 1 mL of blood was taken from the caudal vein using plastic syringe. An amount of 0.5 mL blood sample was placed into a heparin tube for the determination of respiratory burst activity. Another 0.5 mL blood was transferred into Eppendorf without anticoagulation and allowed to clot at room temperature for 4 h. The serum was then separated and moved into new tubes and stored at -80 °C for lysozyme activity.

3.2.6. Immunological assays

3.2.6.1 Lysozyme activity

The lysozyme activity was measured following the method of Parry Jr et al. (1965) with slight modifications. Briefly, 25 µL of fish serum were loaded into a 96-well plate in triplicate. Then, 175 µL of *Micrococcus lysodeikticus* suspension [0.2 mg / mL in sodium phosphate buffer (pH 6.2)] were added to each well. The reaction was determined by a spectrophotometer at 540 nm and the absorbance recorded every 1 min for 10 min. The activity of lysozyme in fish serum was calculated as the reduction in A540 of 0.001 /min and expressed as unit/mL.

3.2.6.2 Respiratory burst activity

Superoxide anion (O^{2-}) was used to determine respiratory burst activity through nitroblue tetrazolium (NBT) reduction reaction performed by modifying the protocol. Briefly, white blood cells (6 x 10^6 cells) were added to 96-well plate in triplicate. Then, 25 µL of NBT were added to each well and incubated at room temperature for 2 h. After incubation, the supernatant was discarded and 150 µL of 100% methanol were added to fix the cell. The well was then washed with 70% methanol twice. A 150 µL of potassium hydroxide (2M KOH) and 100 µL of dimethyl sulfoxide (DMSO) were added to each well. The mixture was thoroughly mixed, and the reaction was measured at an absorbance of 655 nm (A655) via spectrophotometer.

3.2.7. Gene expression

3.2.7.1 RNA extraction and cDNA synthesis

Total RNA derived from Nile tilapia liver tissues were collected from a total of three fish per group after a feeding trial for 56 days. Tissues were transferred into

RNAlater solution (4 °C) for gene expression analysis. Tissues were extracted using PureLink RNA Mini Kit (Ambion, USA) according to the manufacturers' protocol. The quality of total RNA was measured spectrophotometrically (NanoDrop 2000, Thermo scientific) and by electrophoresis on 1% agarose gel. First-strand cDNA from RNA of the best quality (absorbance 260/280 > 1.8 and 260/230 > 1.8) and cDNA Synthesis by qScript cDNA Synthesis Kit (Quantabio, USA).

3.2.8. Quantitative real-time Polymerase Chain Reaction (qRT-PCR) analysis

Complementary DNA (cDNA) was synthesized via the use of SensiFAST ™ SYBR® No-ROX Kit (Bioline, UK) following the manufacturer's protocol. An amount of 20 ng μL^{-1} for liver and 50 ng μL^{-1} for spleen cDNA were used. The primer sequences of C3, *IL-1*β, *TNF-*α, IFN-γ, *HSP70* genes and β-actin housekeeping gene are shown in Table 1. The quantitative qPCR (PCRmax Eco 48 Real-time qPCR System, PCRmax, UK) was used for gene expression. SYBR green method was applied to determine the gene expression via RT-PCR (SensiFast SYBR Lo-Rox kit, Bioline). The amplification conditions were as follows: 45 cycles, (95 °C for 10 s, and 63 °C for 30 s and 72 °C for 30 s). Afterwards, the relative expression levels of target genes were analysed by the $2^{-\Delta\Delta CT}$ method.

Table 1 Primers used for detection of a target gene.

Gene	FWD or REV	Sequence (5′–3′)	Product size (bp)	References
Actin	Forward	TGG CAA TGA GAG GTT CCG	95	Phumyu et al. (2012)
	Reverse	TGC TGT TGT AGG TGG TTT CG		
C3	Forward	TGT GAG TCT ACA GTG AGG AGC	196	Phumyu et al. (2012)
	Reverse	CCC AGA TCT AAA GCC ATT CTG C		
IL-1β	Forward	TGCTGAGCACAGAATTCCAG	60	Kayansamruaj et al. (2017)
	Reverse	GCTGTGGAGAAGAACCAAGC		
IL-8	Forward	GCACTGCCGCTGCATTAAG	85	Ming et al. (2013)
	Reverse	GCAGTGGGAGTTGGGAAGAA		
TNF-α	Forward	GAGGTCGGCGTGCCAAGA	119	Chen et al. (2016)
	Reverse	TGGTTTCCGTCCACAGCGT		
IFN-γ	Forward	TGACCACATCGTTCAGAGCA	128	Chen et al. (2016)
	Reverse	GGCGACCTTTAGCCTTTGT		
HSP70	Forward	TGGAGTCCTACGCCTTCAACA	238	Chen et al. (2016)
	Reverse	CAGGTAGCACCAGTGGGCAT		

3.2.9. Challenge test

S. agalactiae was freshly prepared by inoculating a single colony of the bacteria into NB broth and cultured at 32 °C for 24 h. It was harvested by centrifugation at 5,000 rpm at 4 °C for 10 min, followed by washing and resuspending in 0.85% NaCl. The *S. agalactiae* suspension was adjusted to 10^8 CFU/ml with 0.85% NaCl before injection. At the end of feeding trial, ten fish were randomly collected from each group and intraperitoneally injected with 0.1 ml of *S. agalactiae* (10^8 CFU/ml) and mortality was recorded for 14 days.

3.2.10. Statistical analysis

Results are expressed as the mean values ± standard deviation (SD). Differences between treatments were determined using a one-way analysis of variance with the statistical software package SPSS Version 15.0. A *post-hoc*, Duncan test was applied to examine significant differences between treatments. Significant differences were accepted at $p < 0.05$.

CHAPTER 4
Results and discussion

4.1. Effect of prebiotic and probiotic on growth performances and survival rates

The growth performances of Nile tilapia after a 56-day feeding trial with FOS and *B. subtilis* are presented in Figure 2. The average weight (Figure 2A), weight gain, WG (Figure 2B), average daily gain, ADG (Figure 2C), feed conversion ratio, FCR (Figure 2D), and survival rates (Figure 2) were not significantly different from the control group ($P > 0.05$). Neither the supplementation of FOS (1 – 5 g/kg feed) or *B. subtilis* (1 – 5 x10^9 CFU/g) did not promote growth performances and survival rates in this study**.** Although prebiotic and probiotic feed additive applications have been considered as promising alternative approaches for preventing diseases in fish and shellfish aquaculture, the significant results were not observed in this study. The better feed utilization, promoted growth performances, improved survival rate, boosted immunological responses, and enhanced animal welfare were reported in (Carbone and Faggio, 2016; Dawood, Mahmoud AO et al., 2020; Ringø, 2020; Ringø et al., 2020). *B. subtilis* supplementations resulted in superior growth performances, as has been reported in Dabry's sturgeon, *Acipenser dabryanus*; hybrid Hulong grouper, *Epinephelus fuscoguttatus* × *E. lanceolatus*; and tongue sole, *Cynoglossus semilaevis* (Wang et al., 2021). Probiotics possibly regulate the various autochthonous bacteria in a gastrointestinal tract that help to improve digestion or increase appetite of host organisms thus leading to better nutrient absorption and improved growth. There are several studies that have reported the improvement of growth performances in Tilapia after *B. subtilis* feeding in Nile tilapia. For example, Nile tilapia fed a basal diet supplemented with *B. subtilis* MRS11 at 1 × 10^8 CFU/g of feed for 60 days improved growth performances, intestinal morphology, immunity, and the survival rate after challenge with *Streptococcus iniae* (Büyükdeveci et al., 2023). The dietary supplementation of mixed Bacillus strains (Sanolife® PRO-F) to Nile tilapia, *O. niloticus* at 0.5–1 g/kg diet improved the growth, feed utilization, antioxidant property

and immune parameters (El-Son et al., 2022). A dietary supplement of *B. subtilis* HAINUP40 can effectively improve the growth performance, immune responses, and disease resistance of Nile tilapia (Liu et al., 2017). However, the present study revealed no significant improvement in growth and feed utilization. Similarly, the application of *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2 in tilapia fry produced no effect ($P > 0.05$) on average weight, average daily growth, specific growth rate or feed conversion ratio after an 8-week feeding trial (Sookchaiyaporn et al., 2020). The possible reasons for this difference may be due to the difference in probiotic activities, beneficial bacteria interactions in the fish guts, the amount of the probiotic products added, strain/species composition, its viability, as well as types of feeds, feeding durations, and experimental conditions. Prebiotics can increase feed utilization efficiency by promoting growth of gut microbiota in fish leading to lower feed conversion and increase growth rates. Unfortunately, the supplementation of FOS (1–5 g/kg feed) did not promote growth performances and survival rates in the present study. These results were in agreement with previous investigations reported, where juvenile large yellow croaker was used, *Larimichthys crocea* (0.2–0.4% FOS) (Ai et al., 2011) and Atlantic salmon (*Salmon salar*) (1% FOS). However, the results of this study did contrast with studies on Caspian roach (Rutilus rutilus) fry (1–3% FOS), tambaqui (*Colossoma macropomum*) (0.1 and 0.5% FOS), stellate sturgeon (*Acipenser stellatus*) juveniles (1% FOS), and blunt snout bream (*Megalobrama amblycephala*) (0.4–0.8% FOS) (Zhang, C. N. et al., 2014). The distinction between these and the current findings may be because of FOS additive levels, the fish species used, and the experimental conditions.

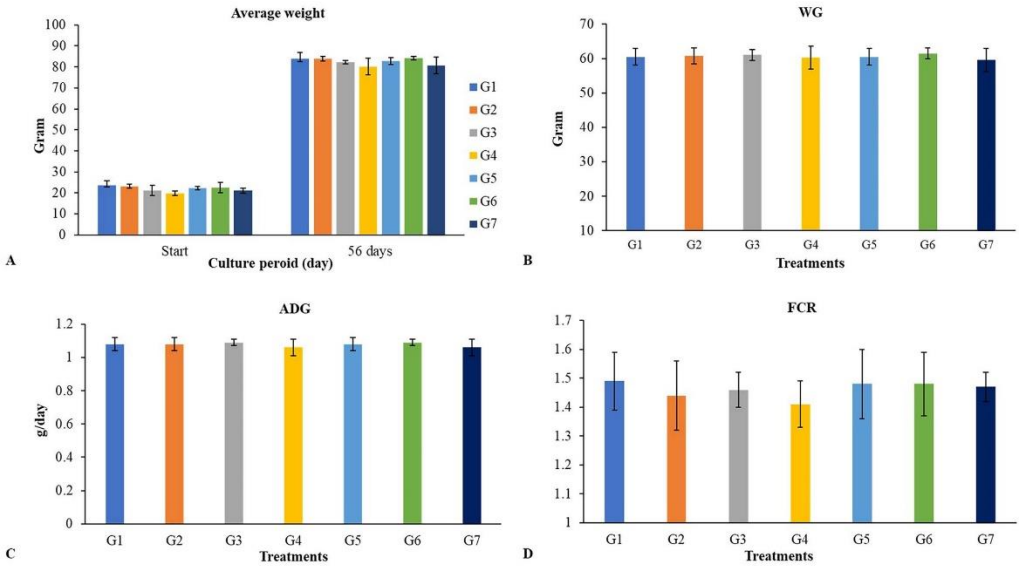

Figure 2 Growth performances of Nile tilapia fed a control feed and diets supplemented with different concentrations of prebiotic and probiotic for 56 days (n=60): average weight, average daily growth (ADG), weight gain (WG), Feed Conversion Ratio (FCR) and survival rate (SR) of Nile tilapia.

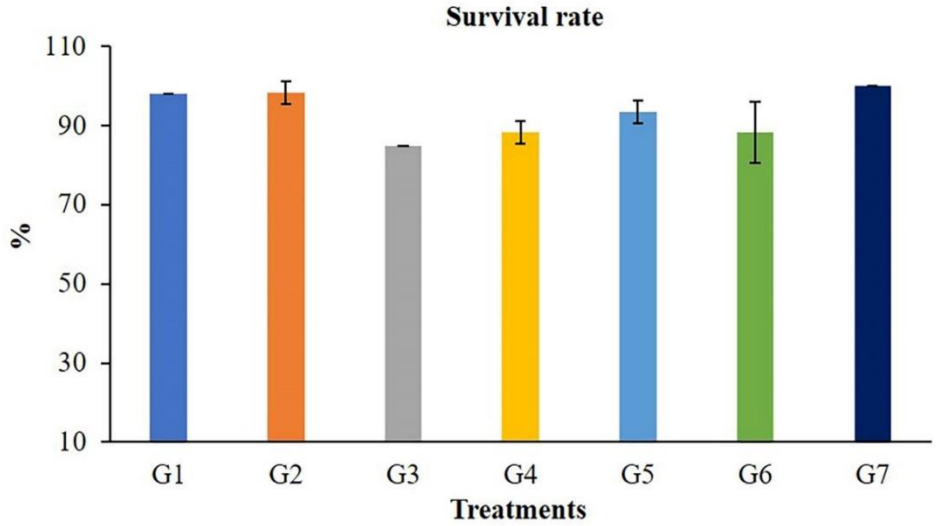

Figure 3 Survival rate of Nile after fed a control feed and diet supplemented with different concentrations of prebiotic and probiotic for 56 days.

4.2. Effect of prebiotic and probiotic on Immune parameters

4.2.1 Lysozyme activity

Lysozyme is a hydrolase enzyme produced by leucocytes, predominantly neutrophils and macrophages. It is an essential parameter in the innate immune defense of both invertebrates and vertebrates. In fish, this enzyme can be found in the mucus, the lymphoid tissues, plasma, and other fluid components of a body (Magnadóttir, 2006). The highest values of lysozyme were found in fish fed G4 and G7 diets (Figure 4). Lysozymes might be enhanced in fish fed with 5 g/kg of FOS (G4) or 5 x 10^9 CFU/g *B. subtilis* (G7) but those in G2, G3, and G5 treatments were not different from the control. In addition, significant differences ($P < 0.05$). In this study, lysozyme activity significantly increased in Nile tilapia supplemented with 5 g/kg of FOS, 3 × 10^9 CFU/g *B. subtilis*, 5 × 10^9 CFU/g *B. subtilis*. The dose, feeding time, composition, and source need to be considered for prebiotic probiotic and synbiotic feed addition because responses may vary depending on species, size, age, and physiological status. Previous studies have reported that prebiotics and probiotics, either singly or in combination, can stimulate an increase in lysozyme levels or stimulate macrophages, which are the primary producers of lysozyme in fish. Caspian roach fry fed 2% and 3% FOS for 7 weeks had significantly greater lysozyme activity than the 1% FOS and the control group (Akhter et al., 2015). The effects of FOS on various innate immune responses, including phagocytosis, lysozyme activity, and the complement system activity in *Sparus aurata* and *Dicentrarchus labrax*, were reported (Carbone and Faggio, 2016). In addition, the dietary supplementation with 1×10^4 and 1×10^6 CFU/g *B. amyloliquefaciens* spores significantly improved lysozyme activity in Nile tilapia after 15 and 30 days of feeding (Selim and Reda, 2015). Thus, prebiotic and probiotic supplementation at an appropriate concentration possibly enhanced lysozyme activity in fish. The additional parameters need to be examined to make sure which ones are the key indicators for immune response determination for healthy fish.

4.2.2 Respiratory burst activity

A respiratory burst is an indication of the oxidative potential of reactive oxygen species including hydrogen peroxide, superoxide anions, and hydroxyl radicals. These reactive oxygen species are produced by activated phagocytic cells and they are responsible for killing engulfed pathogen. Reactive oxygen species have been widely used to evaluate the ability of the host to defend against pathogens (Abbas et al., 2014). Significant differences ($P < 0.05$) in a respiratory burst activity were observed in tilapia fed with 1 and 3 g /kg of FOS, 5x10 ^{9} CFU/g *B. subtilis*, after 56-day feeding trial (Figure 5).

Probiotics could enhance phagocytic activity in many aquatic animals. The respiratory burst activity of Nile tilapia treated with *Bacillus* sp. KUAQ and *Bacillus* sp. KUAQ2 containing 3×10^8 CFU/g of feed (Sookchaiyaporn et al., 2020) and Nile tilapia fed with *B. subtilis* at a dose of 1×10^7 CFU/g of feed was significantly higher than in those of the control. In addition to probiotics in aquaculture, prebiotics FOS, MOS, β-glucan, and GOS are also used as feed additives to stimulate immune responses. According to previous reports, Caspian roach (*Rutilus rutilus*) fed 2% and 3% of FOS and Common carp (*Cyprinus carpio*) fed with 2% FOS (Hoseinifar et al., 2017) showed significantly increased levels of respiratory burst activity compared to a control group ($P < 0.05$). The mechanism of immune responses starts when bacterial cell wall components such as lipopolysaccharides or peptidoglycans have adhered to the binding proteins in a host, and the binding complexes are then recognized by recognition proteins. After these reaction processes, the immune function, such as phagocytosis, can be activated (Vargas-Albores and Yepiz-Plascencia, 2000). In addition, *Bacillus* sp. can synthesize various vitamins, which may affect the leucocytes and enhance lysozyme and respiratory burst activity (Hoseinifar et al., 2018).

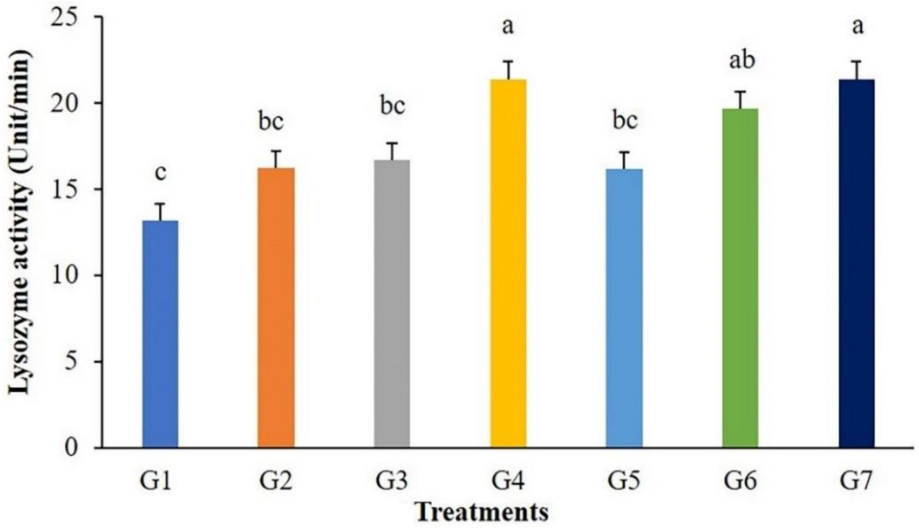

Figure 4 Lysozyme activity of Nile tilapia fed with prebiotic and probiotic for 56 day (n = 5). Bars with different letters indicate significant difference (p < 0.05).

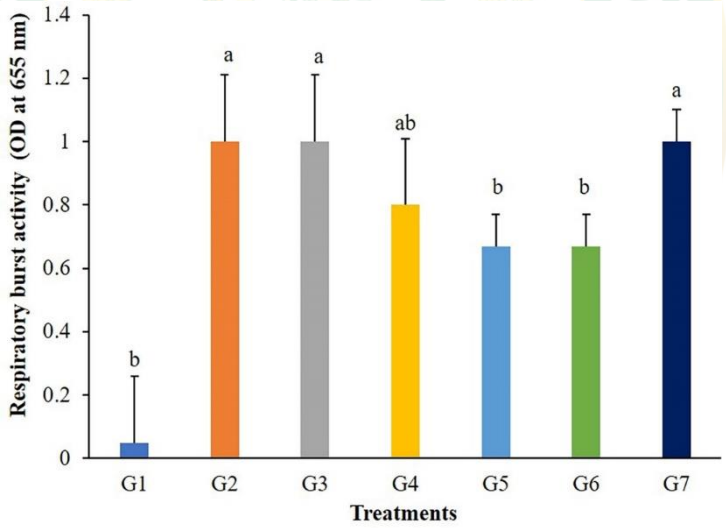

Figure 5 Respiratory burst activity of Nile tilapia fed with prebiotic and probiotic synbiotic for 56 day (n = 5). Bars with different letters indicate significant difference (p < 0.05).

4.3. Effect of prebiotic and probiotic on gene expression in the liver of Nile tilapia

A transcript of the immune-related gene expression for the liver of the tilapia is given in Figure 5. Complementary C3 and IL-1β were significantly up-regulated in

the liver of tilapia fed with 5 g FOS/kg feed (G4) and those fed with *Bacillus* additive diets (G5, G6, and G7) ($P < 0.05$). The TNF-**α** gene expression levels in fish fed with 5 g FOS/kg feed (G4) and all *B. subtilis* treatment groups were significantly higher compared with the control group and other treatment groups (G2 and G3) ($p < 0.05$). Furthermore, higher TFN-**γ** gene expression was found in the fish fed with 5 g FOS/kg feed (G4) over the control group and other treatment groups ($P < 0.05$). In addition, a higher level of hsp70 gene expression was found in the 5 g FOS/kg feed (G4) and all concentrations of *Bacillus* additive feeds (G5, G6, and G7) over those of the control group and other groups ($P < 0.05$). The complement system is a major component of innate humoral immunity modulation and has a vital role in host homeostasis, inflammation, antibody opsonization, and defense against pathogens. It consists of three activation pathways: the classical pathway, lectin pathway, and alternative pathway (Noris and Remuzzi, 2013). The complement component 3 (C3) gene is responsible for producing a protein that plays an essential role in immune system regulation and pathology (Meng et al., 2019; Ricklin et al., 2016). Probiotic *B. subtilis* and FOS could stimulate complement C3 gene expression levels in livers and spleens (Zhang, Q. et al., 2014), which are the main organs for C3 synthesis. In this study, the enhancement of C3 expression in livers was noticed in fish fed with 5 g FOS/kg feed and those fed with *Bacillus* supplementary diets. This result agrees with previous reports on teleost C3, which pointed out that the liver and spleen are generally considered the prime organs involved in C3 synthesis (Fu et al., 2019). C3 levels in groupers (*Epinephelus coioides*) fed with *Bacillus* spp. were significantly higher than that of the control after 30 days of feeding (Sun et al., 2010). In addition, after 3 weeks of *B. subtilis* supplementary feeding, complement activity in Gilthead seabream (*S. aurata* L.) improved compared with controls (Salinas et al., 2008). The expression of C3 was significantly up-regulated in the liver and spleen after challenging the southern catfish (*Silurus meridionalis*) with *A. hydrophila* (Fu et al., 2019). Greater C3 levels can help grass carp better cope with secondary infections of *A. hydrophila*, allowing them to survive. Prebiotic and probiotic metabolites could

stimulate C3 complement after being directly activated by bacterial lipopolysaccharide and subsequently this resulted in the direct killing of pathogens by lysis (Nayak, 2010).

IL-1β and TNF-α are cytokines required for activating the innate immune response, mediating the recruitment, activation, and adherence of circulating phagocytic cells, responsible for inflammation activity, neutrophil activation, and microbial killing of both gram-positive and negative bacteria (Tort et al., 2003). The results of this study showed that expression of IL-1β and TNF-α was significantly affected by the application of 5 g FOS/kg feed and *Bacillus* additive diets. IFN-γ is one of the antiviral cytokines and functions as the primary activator of macrophages. The expression of IL-1, IFN-γ and TNF-α genes in the head kidney of *C. auratus* fed with *B. velezensis* at a density of 10^9 CFU/g was shown to be increased (Yi et al., 2018). This was also true for Japanese seabass fed with *B. pumillus* SE5 fermented soybean (Rahimnejad et al., 2019), and for Nile tilapia fed *A. oryzae* at 1×10^6 or 1×10^8 CFU/g (Dawood, Mahmoud AO et al., 2020).

Administering FOS and *B. subtilis* enhanced the expression levels of liver hsp70 gene in fish, potentially strengthening their tolerance to environmental stressors such as heat, disease, parasitic infection, and chemical exposure. The hsp70 gene expression level was higher for fish fed with FOS 5 g/Kg feed and for all *B. subtilis* addition groups ($p < 0.05$) in this study. The results were similar to those of previously reported studies, Nile tilapia fed with *B. subtilis* and *B. licheniformis*, mixed in a ratio of 1:1 w/w at 10 g/kg showed the greater expression of the hsp70 gene in the head-kidney (Abarike et al., 2018). In addition, the liver hsp70 expression of blunt snout bream fed 0.4 % FOS was significantly enhanced under high heat stress, ambient temperature +8 °C (Zhang et al., 2015). High levels of hsp70 possibly indicated high levels of protein damage and increased tolerance to subsequent stress and others (Zhang et al., 2013). Hsp70 is an effective tool for helping in the

survival rates of cells through stress protection, cures, and environmental pressure relief (Lindquist and Craig, 1988).

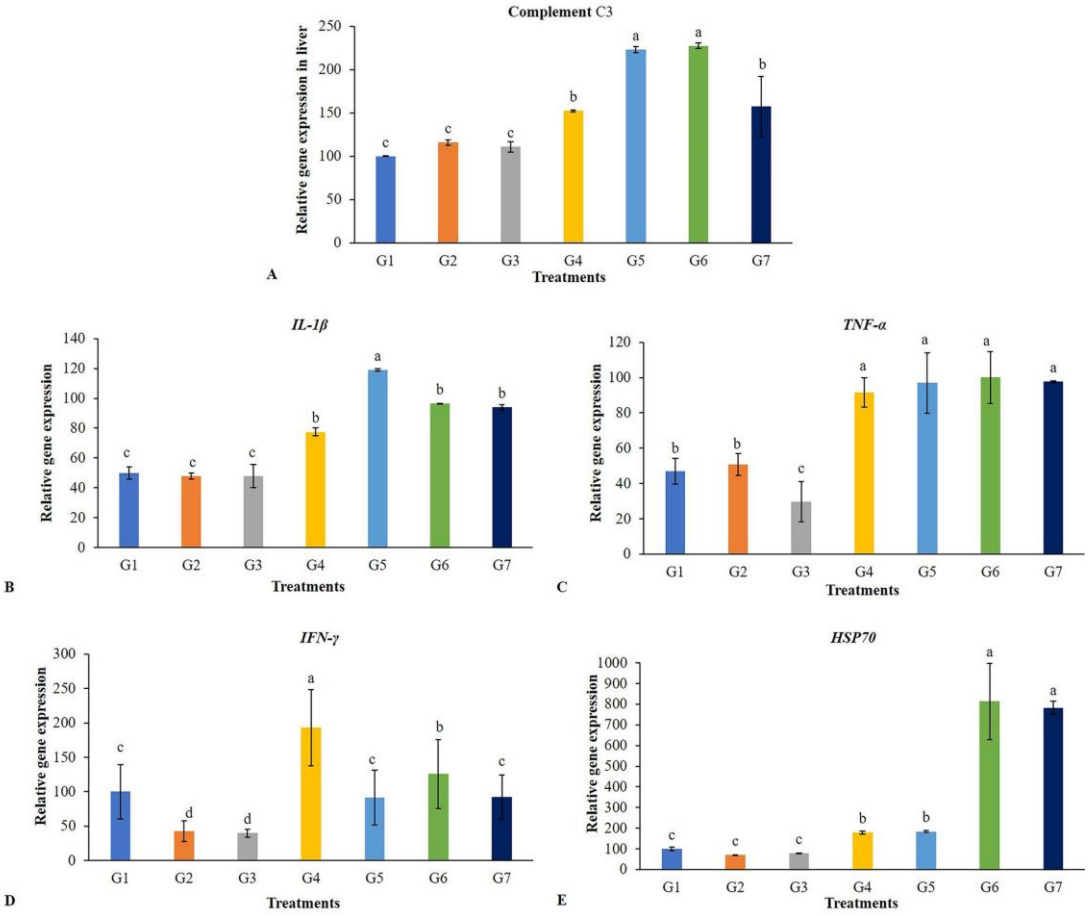

Figure 6 Gene expression in the liver of complement C3, interleukin 1beta (IL-1β), tumor necrosis factor (TNF-α), interferon gamma (IFN-γ) and heat shock protein 70 (hsp70) of Nile tilapia fed with FOS and B. subtilis for 56 days. (A) Complement C3, (B) Beta (IL-1β), (C) tumor necrosis factor (TNF-α), (D) interferon gamma (IFN-γ), and (E) heat shock protein 70 (hsp70).

4.4. Challenge test

The 14-day challenge test indicated that the highest survival rate was found in the G6 group, whereas the lowest survival rate was observed in the control group (Figure 7). However, there were no significant differences in survival rates ($P > 0.05$) between the control and the supplemented diet groups. Clinical signs of infected fish included abnormal swimming, darkened color and less of an appetite. In addition, hemorrhages on the surfaces of their bodies and on their livers were found to be larger than those found in normal fish. Clinical signs of infected fish included abnormal swimming, darkened color and less of an appetite. In addition, hemorrhages on the surfaces of their bodies and on their livers were found to be larger than those found in normal fish. *S. agalactiae* is considered a critical bacterial disease causing high mortality rates and economic losses in tilapia. The challenge test is used as an ultimate assay to assess the fish immune response. Although the highest survival rate was noticed in tilapia fed with 3×10^9 CFU/g *B. subtilis* group; however, there were no significant differences. Similarly, fish were fed with probiotics, this did not increase the survival rate of tilapia challenged with *S. agalactiae* (Sookchaiyaporn et al., 2020). In addition, the combined feeding with *B. subtilis* strains SB3086 and SB3615 did not result in any significant difference in reducing mortality due to *S. iniae* infection in juvenile Nile tilapia (Addo et al., 2017). FOS and *B. licheniformis*, used as prebiotic and probiotic, did not significantly influence ($P > 0.05$) the survival rate of triangular bream after *A. hydrophila* challenge (Zhang et al., 2013). On the other hand, 10 g/kg of a mix of *B. subtilis* and *B. licheniformis* application results in significantly greater survival of tilapia against *S. agalactiae* (Abarike et al., 2018). The differences in pathogen prevention may be due to FOS additive levels, purity, sources, the fish species used, pathogen virulence, and the experimental conditions. Moreover, a non-significant increase in the protection level of FOS and *B. subtilis* supplemented groups against *S. agalactiae* although immunity was improved. The possible explanation could be all immune-related gene expression applied in this study was the first line of non-specific defense, possibly

this expression or defense mechanism was not strong enough to protect the fish from deadly pathogens or maybe this pathogen was very virulent.

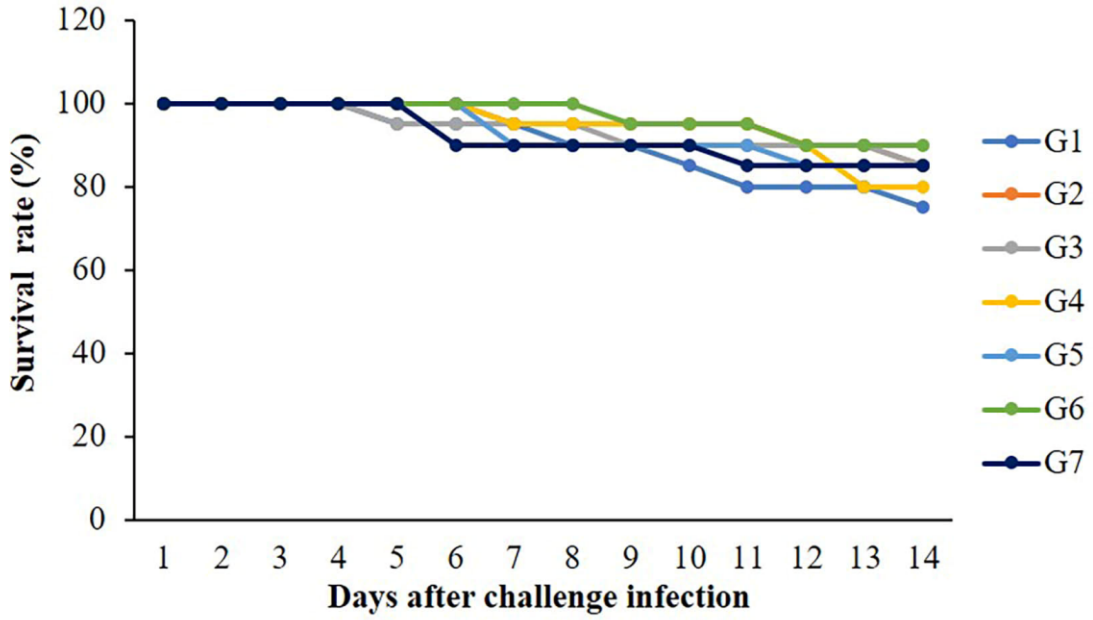

Figure 7 Survival rates (%) of Nile tilapia fed with FOS and B. subtilis after challenge with S. agalactiae 1 × 10^8 CFU/ml (n = 20) for 14 days.

4.5. Effect of synbiotic on growth performances and survival rates

Recently, the application of probiotics and prebiotics is an important part of good aquaculture practices that improve growth performances and disease resistance in aquatic organisms. In principle, probiotics and prebiotics have been combined to provide the survival of lived microbial nutritional supplements at the digestive tract of the fish (Gibson and Roberfroid, 1995) leading to the growth enhancement. Several studies showed the growth performance in fish improvement by using synbiotic feed additive (Dawood, Mahmoud AO et al., 2020; Mehrabi, 2012). However, our study had no significant improvement in growth and feed conversion ratio compared to fish fed a control diet. The growth performances of Nile tilapia after 56-day feeding trial with

synbiotic between FOS and *B. subtilis* were presented in Figure 1. The average weight (Figure 1A), weight gain, WG (Figure. 1C) and feed conversion ratio, FCR (Figure 1D) were not significantly different compared with the control group (p>0.05). However, the average daily gain, ADG of fish in G2 and G3 groups was significantly higher compared with the control group and other groups (Figure 1B). The survival rate of tilapia in G4 group was significantly different compared with the control group and other groups (P <0.05) (Figure 7)**.** Our results agree with (Addo, 2013). Addo (2013), who combined the probiotic strains and prebiotics used as feed additives and showed the reduced mortality in Nile tilapia and channel catfish under the conditions of laboratory. The synbiotic additive feed did not provide any significant improvement in growth rate and feed utilization in juvenile barramundi, *Lates calcarifer*. The different results are probably caused by the types of prebiotics and probiotics, administration dosages, duration, and conditions of experiment, as well as fish ages and species. Most of the experiments have not been further conducted on farms. The low mortality rates possible are due to the expression of the non-specific immunity which is discussed in the immune parameter section. Synbiotics are the combination of probiotics and prebiotics in a form of synergism (Cerezuela et al., 2011). The application of synbiotic is considered as an alternative approach for the disease prevention in fish and shellfish aquaculture. The synbiotic dietary supplementation can result in better growth performances, survival rate, and immunological responses, as well as an increased area of intestinal absorption, which enhanced general animal welfare (Dawood, M. A. et al., 2020).

The results of our study indicated that dietary synbiotic could significantly boost the growth performance, survival rate of Nile tilapia (Figure 8). These results may be correlated with the high enzymatic activities provided by the synergistic action of prebiotic and probiotic and subsequently promote the growth. Prebiotics in the form of synbiotics are hydrolyzed to their respective sugars in the intestinal tract of the host and are then utilized as a source of carbon to increase the biomass of

bacteria and prebiotics are utilized by intestinal bacteria under diverse mechanisms dependent on sugar linkages and the bacterial strains (Goh and Klaenhammer, 2015). Among the prebiotics and probiotics widely adopted, both oligosaccharides and *Bacillus* have been successfully used in aquaculture. It was noted that the supplementation of synbiotics yielded significantly better results than individual applications. Thus, the investigation of the potentially interactive effects between prebiotics and probiotics in aquaculture is of great significance. In the present study supplementation of synbiotic has shown significant enhanced growth performance, survival rate and disease resistance defense against *S. agalactiae* infection.

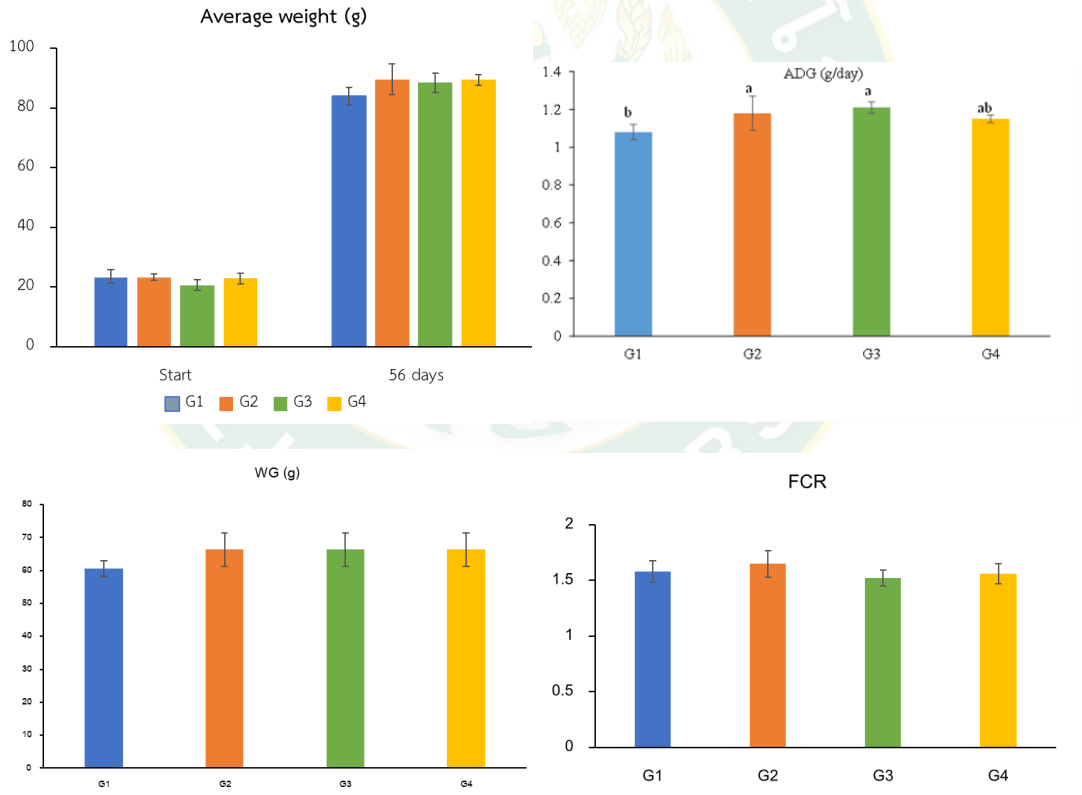

Figure 8 Growth performances of Nile tilapia fed a control feed and diets supplemented with different concentrations of synbiotic for 56 days (n=60): average weight, average daily growth (ADG), weight gain (WG), Feed Conversion Ratio (FCR) and survival rate (SR) of Nile tilapia.

4.6. Effect of synbiotic on immune parameters

4.6.1. Lysozyme activity

Lysozyme is an enzyme that degrades the peptidoglycan in bacterial walls and plays an important role in controlling infectious fish pathogens by an opsonization that activates the complement system and phagocytosis. It is found at the mucus, serum, and intestines of aquatic animals. In the present study, lysozyme activity showed the significantly higher in fish fed Diet G3 (3 g /kg FOS + 3x10 ^{9} CFU/g *B. subtilis*) and G4 (5 g /kg FOS + 5x10 ^{9} CFU/g *B. subtilis*) compared with control group (Figure 9) ($P< 0.05$). Like our results, Zebrafish (*Danio rerio)* fed with polysaccharide gel extracted from the rind of durian fruit which encapsulated with *Bacillus subtilis* and co-inoculation with *Artemia nauplii* showed a positive effect in lysozyme activity (Priya et al., 2021). Moreover, several studies reported the elevation of serum lysozyme activity in the dietary administration of synbiotics such as low molecular weight sodium alginate and *Lactobacillus plantarum* (Van et al., 2016). Japanese eel, *Anguilla japonica* fed with *B. subtilis* at 0.5 × 10^{7} CFU/g and mannanoligosaccharide at 5 g/kg provided significantly higher than those fed with other diets (Lee et al., 2018). Thus, prebiotic and probiotic supplementation at an appropriate concentration possibly enhanced lysozyme activity in fish.

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4.6.2 Respiratory burst activity

A respiratory burst is an indication of the oxidative potential of reactive oxygen species including hydrogen peroxide, superoxide anions, and hydroxyl radicals. These reactive oxygen species are produced by activated phagocytic cells and they are responsible for killing engulfed pathogen. Reactive oxygen species have been widely used to evaluate the ability of the host to defend against pathogens (Abbas et al., 2014). Significant differences ($p < 0.05$) in respiratory burst activity were observed in G3 and G4 after 56 days of the feeding trial (Figure 10).

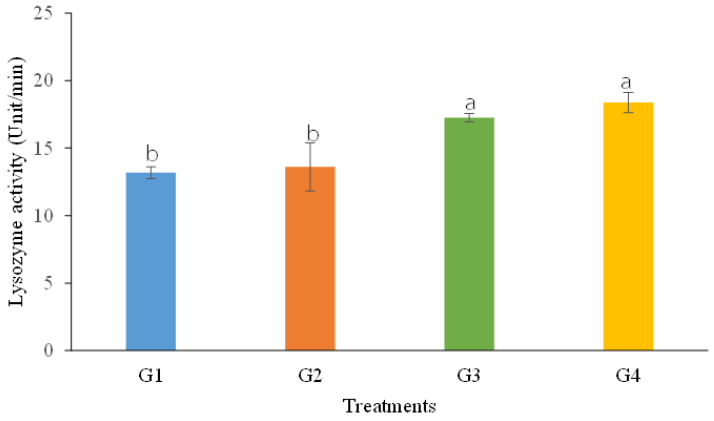


Figure 9 Lysozyme activity of Nile tilapia fed with synbiotic for 56 day (n = 5). Bars with different letters indicate significant difference (P < 0.05)

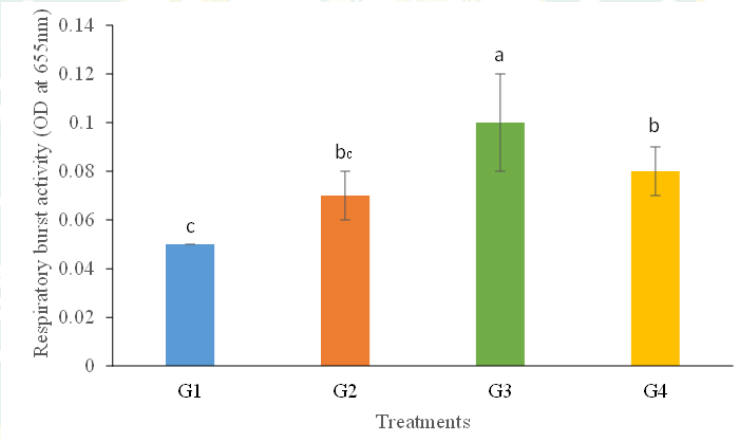


Figure 10 Respiratory burst activity of Nile tilapia fed with synbiotic for 56 day (n = 5). Bars with different letters indicate significant difference (P < 0.05).

4.7 Effect of synbiotic on gene expression in the liver of Nile tilapia

The health status of tilapia fed synbiotic diets was investigated by determining the expression of Complement C3, *IL-1β*, *IL-8*, *IFN-γ*, and *HSP-70* genes in the liver, the results are shown in Figure 11. Complement C3, *IL-1β*, *IL-8*, *IFN-γ*, and *HSP-70* gene expression were significantly up-regulated in the liver of tilapia fed

with 5 g FOS/kg feed and $5x10^9$ CFU/g of *B. subtilis* (G4) and fish fed with 3 g/kg FOS + $3x10^9$ CFU/g *B. subtilis* (G3) additive diets ($p<0.05$).

The complement system is a vital innate immune barrier in pathogen prevention and regulates humoral immune responses (Beutler, 2004). The complement component C3 gene is responsible in an inflammatory response and monocyte/macrophage phagocytosis. After activation or pathogen infection, the C3 molecule is decomposed into C3a and C3b, and modulates the inflammatory response to defend against pathogen infection. The present study indicates that increased the serum Complement C3 may be significantly beneficial in the healthy and infected fish fed with 3 g/kg FOS + $3x10^9$ CFU/g *B. subtilis* (G3) and 5 g FOS/kg feed and $5x10^9$ CFU/g of *B. subtilis* (G4).

The interleukin 1β (*IL-1β*), interleukin 8 (*IL-8*), and tumor necrosis factor α (*TNF-α*) are cytokines required for activating the innate immune responses, mediating the recruitment, activation, and adherence of circulating phagocytic cells, responsible for inflammation activity, neutrophil activation, microbial killing, and they can further stimulate the B-cells and T-cells (Cerdo and García-Santos, 2019). The increased expression of *IL-1β*, *IL-8*, and *TNF-α* genes was noticed in the synbiotic fed tilapia in this study. The expression levels of *IL-1β*, *IL-10*, and *TNF-α* in tilapia fed pistachio hulls derived polysaccharide and *Pediococcus acidilactici* were significantly modulated. The expression of IL-1β, IL-8, and TNFα of carp fed β-glucan, mannan oligosaccharide and *Lactobacillus casei* synbiotic for 60 days was a significant increase prior and after challenging by *Aeromonas hydrophila*.

IFN-γ is one of antiviral cytokines and functions as the primary activator of macrophages. The *IFN-γ* gene expression of tilapia fed with 3 g FOS/kg feed and $3x10^9$ CFU/g of *B. subtilis* (G3) were significantly higher compared with the control group and other groups (G2 and G4) ($p<0.05$).

The *hsp-70*, heat shock protein, plays a crucial role in the cellular response to stress and cellular homeostasis maintenance (Lackie et al., 2017). In the present study, *hsp-70* levels were elevated with synbiotic fed fish when compared to control fish.

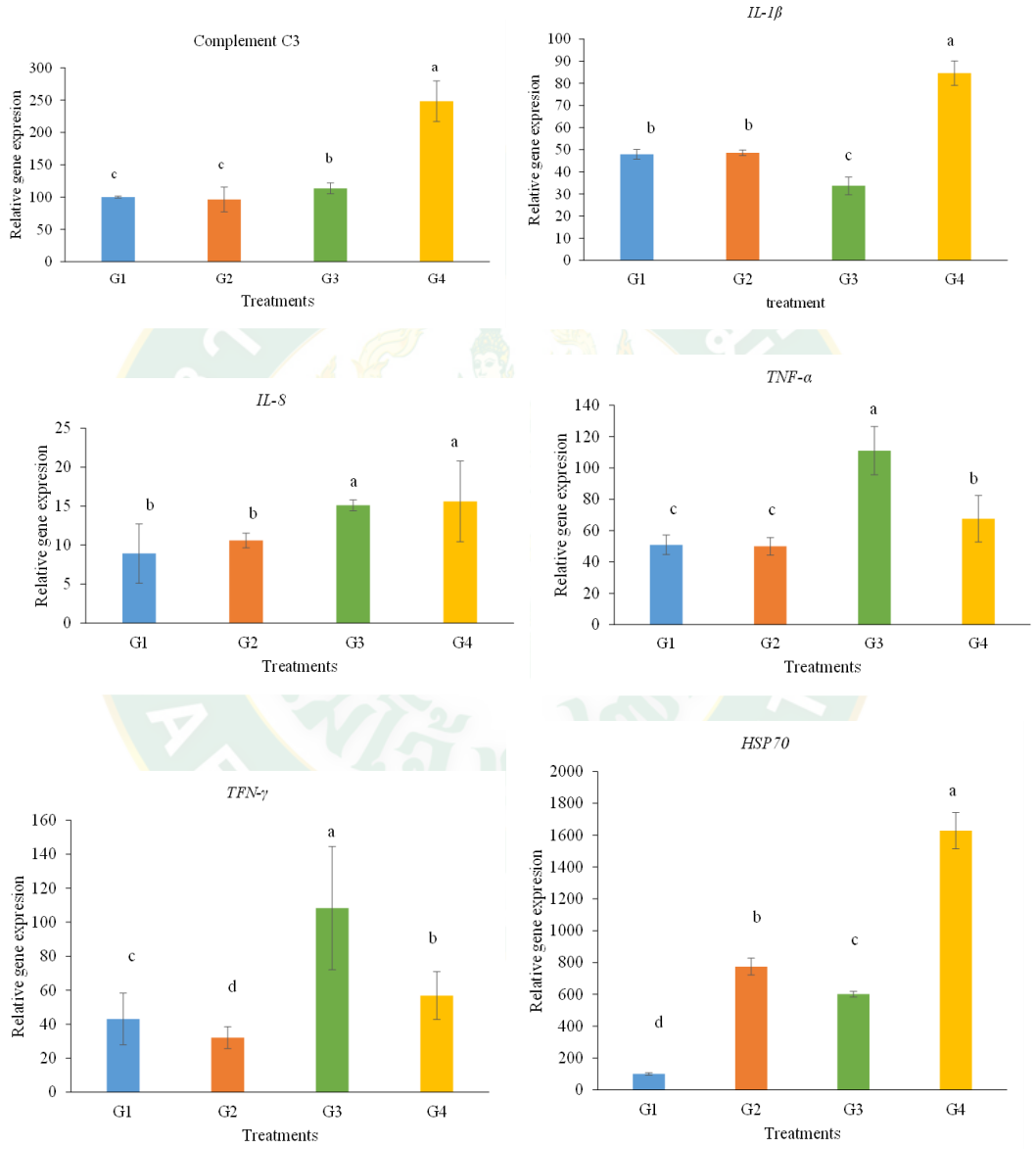


Figure 11 Gene expression in the liver of complement C3, interleukin 1beta (IL-1β), interleukin-8 (IL-8) tumor necrosis factor (TNF-α), interferon gamma (IFN-γ) and heat shock protein 70 (hsp70) of Nile tilapia fed with synbiotics for 56 days.

4.8. Challenge test with *S. agalactiae*

S. agalactiae is pathogenic bacteria causing high mortality and economic losses in tilapia. The challenge test is used as an eventual assay to assess the fish immune responses. At the end Nile tilapia feeding trial with synbiotic for 56 days, fish were challenged with *S. agalactiae*, the cumulative mortality rates of Nile tilapia were recorded for 14 days (Figure 12). At the end of the 14-day challenge test, the highest survival rates were found in the G4 group while the lowest survival rates were observed in a control group. Clinical signs of infected fish included abnormal swimming, darkened color, less appetite, hemorrhage on the surfaces of the body and the livers were larger than normal fish were noticed. The combination of mannan oligosaccharides (Rahmani et al., 2020) and commercial probiotic DBA® (*Bifidobacterium* sp, *Lactobacillus acidophilus* and *Enterococcus faecium*) reduced the mortality of Nile tilapia infected with *A. hydrophila* (Cavalcante et al., 2020). The synergetic effect of *Bacillus subtilis* and the prebiotic Previda®, a commercial hemicellulose extract was reported in Nile tilapia (8 weeks of feeding) against *A. hydrophila* infection (Addo et al., 2017). The higher survival of these fish to higher innate immune responses which was noticed from enhanced immune parameter.

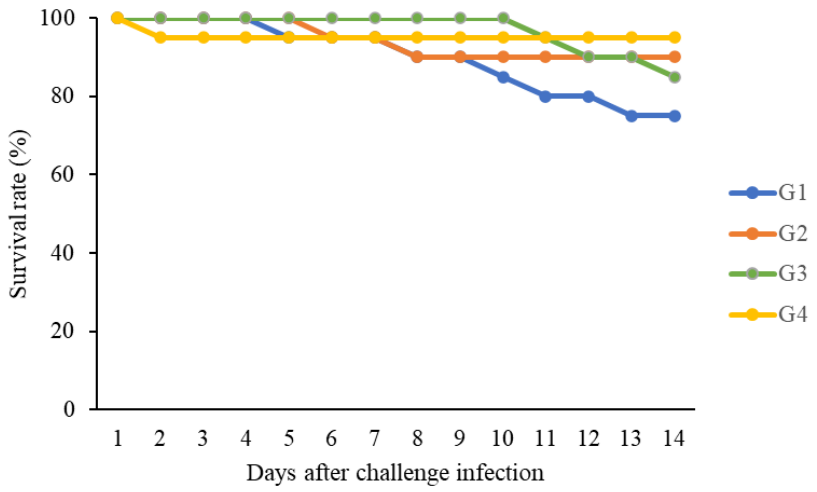


Figure 12 Survival rates (%) of Nile tilapia fed with synbiotic after challenge with S. agalactiae 1 × 10^8 CFU/ml (n = 20) for 14 days.

4.9. Cost of the use of prebiotics, probiotics, and synbiotics in fish farming

Tilapia are able to grow fast in cages because of the good water quality and running water. Most fingerlings are stocked at 50 - 80 g and then reach a market size of 800 g in 4 months. The costs of Nile tilapia cage culture were estimated (Table 2). The main cost is feed so feeding is very important to maintain the operating cost. Input costs were dominated by feed (70%) and seed stocked (16%) (Lebel et al., 2013) The marketable sale prices at farms range from 65 to 75 THB/kg. The difference in the cost is due to the stocking rate. The cage size for this model was 3X3 m^2 while 1,000 fish/cage (~111 fish/m^3) were initially stocked. Moniruzzaman et al. (2015) reported that based on growth and economic return, 50 fish/m^3 was the best stocking density (compared with 75, 100 and 125 fish/m^3) for monosex tilapia cultured in cages in Kaptai Lake of Bangladesh. This estimated cost and return did not include labor, cage construction, fuel, and transportation costs. These cages usually last for two years or four production cycles. The data were randomly selected from 2 farmers who have raised fish in Nan River, Thailand. In this prediction, we calculated the prebiotic and probiotic cost without the change in fish survival and production. The profitability of cage culture depends on input costs, growth rate, survival rate, production, and selling prices. Succeed fish farmers must manage a combination of market, climate and environment-related risks (Lebel et al., 2013). The difficulty of achieving sustainability of cage culture, especially for a small-scale aquaculture due to climate change effects can be minimized by stocking larger-sized fish, good site selection, and suitable culture period.

Table 2 Costs and profits of Nile tilapia cage culture

Farmers 1 & 2	Cage No.	Estimated Survival (%)	Fish Production (kg)	seed cost (THB)	Chemical Cost (THB)	Probiotic cost (THB)	Probiotic cost (THB)	Feed (bags)	FCR	Fish cost/kg (THB)	Total main cost (THB)	Rough profit (THB/month)
1T1	10	60	6,000	50,000	3,000	0	0	600	2.0	57.83	347,000	10,750
1T2						600	480				348,080	10,480
1T3						1,800	1,440				350,240	9,940
1T4						3,000	2,400				352,400	9,400
2T1	30	70	7,000	150,000	6,000	0	0	2,100	2.0	56.43	1,185,000	45,000
2T2						700	560				1,186,260	44,685
2T3						2,100	1,680				1,188,780	44,055
2T4						3,500	2,800				1,191,300	43,425

Remarks: Probiotic (G biotic) 500 THB/liter, Prebiotic (FOS) 400 THB/kg, Cost excludes labor, cage construction, fuel, and transportation costs.

Chapter 5
Conclusion

5.1. Growth performances and survival rates

A feed containing 1, 3 and 5 g/kg of FOS and $1x10^9$ CFU/g $3x10^9$ and $5x10^9$ CFU/g of *B. subtilis* showed no significant effects on overall growth performances in Tilapia. However, the average daily gain of fish in 1 g /kg FOS + $1x10^9$ CFU/g *B. subtilis* supplemented diet (G8) and 3 g /kg FOS + $3x10^9$ CFU/g *B. subtilis* (G9) groups was significantly higher compared with the control group and other groups. In addition, the survival rate of tilapia in G10 group was significantly different compared with the control group and other groups (P <0.05). The positive effect of using synbiotic feed additives was due to the improved action of probiotic bacteria by prebiotics.

5.2. Immune parameters

5.2.1. Lysozyme activity

Lysozyme activity significantly increased in Nile tilapia supplemented with 5 g/kg of FOS, 3×10^9 CFU/g *B. subtilis*, 5×10^9 CFU/g *B. subtilis*, 3 g /kg FOS + $3x10^9$ CFU/g *B. subtilis* and 5 g /kg FOS + $5x10^9$ CFU/g *B. subtilis*.

5.2.2. Respiratory burst activity

The significant differences ($p < 0.05$) in Respiratory burst activity were observed in 1 and 3 g /kg of FOS, $5x10^9$ CFU/g *B. subtilis*, 3 g/kg of FOS+ $3x10^9$ CFU/g *B. subtilis* and 5 g /kg of FOS+ $5x10^9$ CFU/g of *B. subtilis* after 56 days of the feeding trial.

5.3. Gene expression in the liver of Nile tilapia

Complementary C3 and IL-1β were significantly up-regulated in the liver of tilapia fed with 5 g FOS/kg feed, those fed with *Bacillus* additive, 1 g/kg FOS + $1x10^9$ CFU/g and 5 g/kg FOS + $5x10^9$ CFU/g ($p < 0.05$). The Il-8 gene expression levels in fish fed with 5 g FOS/kg feed, $1x10^9$ CFU/g *Bacillus* and 3 g/kg FOS + $3x10^9$ CFU/g *Bacillus* and 5 g/kg FOS + $5x10^9$ CFU/g *Bacillus* ($p < 0.05$) additive diets. The TNF-α gene expression levels in fish fed with 5 g FOS/kg feed (G4) and all *B. subtilis* treatment groups and 5 g/kg FOS + $5x10^9$ CFU/g were significantly higher compared with the control group and other treatment groups ($p < 0.05$). Furthermore, higher TFN-γ gene expression was found in the fish fed with 5 g FOS/kg feed (G4), $3x10^9$ CFU/g *Bacillus* and 3 g/kg FOS + $3x10^9$ CFU/g *Bacillus* over the control group and other treatment groups ($p < 0.05$). The hsp70 gene expression level was higher for fish fed with FOS 5 g/kg feed and for all *B. subtilis* and synbiotic addition groups ($p < 0.05$) in this study.

5.4. Challenge test

There were no significant differences in survival rates ($P > 0.05$) between the control and the supplemented diet groups. The feed additives lead to additional expenditures, fish farmers have to concern before application and also the use of new microbial strains has to safely conduct to avoid potential negative side effects. Thus, further investigation of other prebiotics or herbs in combination with *B. subtilis* is encouraged at molecular levels and screening for beneficial metabolites that may stimulate digestive enzymes, growth, and health benefits in tilapia.

5.5 Cost of the use of prebiotics, probiotics, and synbiotics in fish farming.

The application of prebiotics, probiotics, and synbiotics in tilapia feed results in the increased operating cost. As a result, the farmers must concern and control this additive cost in case some time there is no effect on fish growth, survival and final production.

APPENDIX

APPENDIX A

Solution and pathogenic bacteria preparation

1. Lysozyme activity

Preparation of reagents for lysozyme activity analysis

1.1 Preparation 1 M Sodium phosphate buffer saline (PBS)

NaCl 8 g KCl 200 mg

Na2HPO4 1.44 g

KH_2PO_4 240 mg and adjust the volume to complete 100 ml of distilled water

To adjust the pH, use NaOH or HCl 0.1 N.

1.2 Bacteria suspension

Micrococcus lysodeikticus (Sigma, USA) at concentration 3 mg/ml (PBS 0.05 M pH 7.8)

1.3 Calculation of lysozyme activity (units/min)

One unit (1 U) of lysozyme activity was defined by the manufacturer as decreasing a 0.001 absorption value at 450 nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 25 ° C in a 250 ml reaction mixture using 96 well microtiter plate.

2. Respiratory burst activity

Preparation of reagents for Respiratory burst activity analysis

2.1 chemicals

- NBT (Sigma Aldrich)
- Methanol 100%
- Methanol 70%
- 2N KOH
- DMSO (Dimethyl Sulfoxide

2.2 Method

1. Approximately 6 x 10^6 cells of white blood cells (WBC).

2. White blood cells were added to 96-well plate, mixed with 25 µl of NBT, and incubated at room temperature for 2 h.

3. The supernatant was then decanted, and the WBC fixed with 125 µl of 100% methanol for 5 min and were removed 125 µl by a micropipette by autopipette.

4. Followed by washing with 125 µl of 70% methanol for 5 min and were removed 125 µl by a micropipette by autopipette.

5. Potassium hydroxide (2 M KOH) and dimethyl sulfoxide (DMSO) were added to the dried WBC on 96-well plate.

6. Mixed well and the reaction of the superoxide anion measuring absorbance at 655 nm (A655) by spectrophotometer (Multiskan go, Thermo scientific).

3. Challenge test

Preparation of pathogenic bacteria

Method

1. Preparation of *Streptococcus agalactiae* pathogenic bacteria at concentration 10^8 CFU/ml by measuring absorbance at 610 nm (A610) by spectrophotometer (Multiskan go, Thermo scientific).

2. At the end of feeding trial, fish were randomly collected from each group and intraperitoneally injected with 0.1 ml of *S. agalactiae* (10^8 CFU/ml).

3. Mortality was daily recorded for 14 days after injection.

APPENDIX B

Juvenile Nile tilapia and cages preparation

1. Cages preparation

Preparation 30 cages

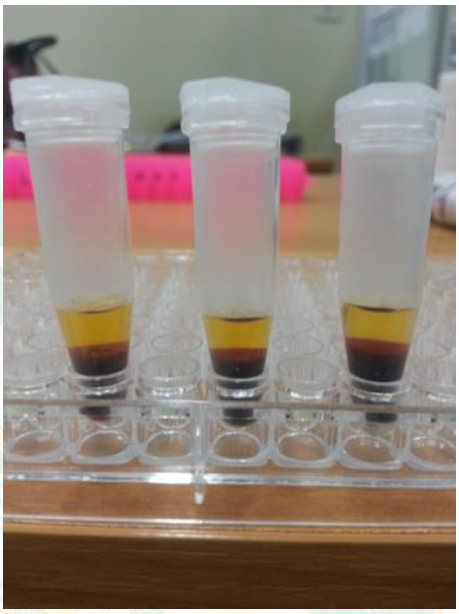
2. Juvenile Nile tilapia preparation

Nile tilapia (average body weight 24.5±1.6 g) were obtained from a local fish farm

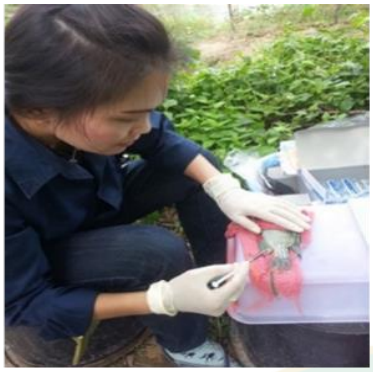
APPENDIX C

Lysozyme and white blood cells preparation

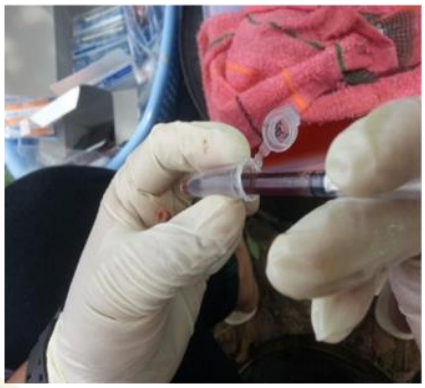
1. Lysozyme preparation



1. Lysozyme preparation

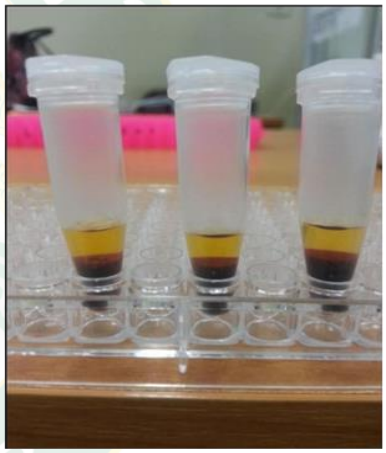


Blood samples

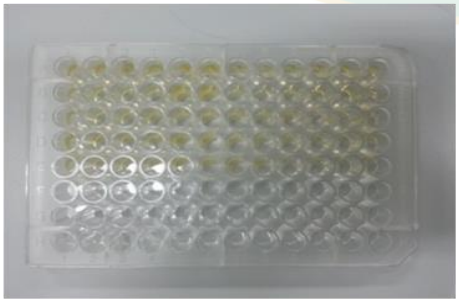


Eppendorf

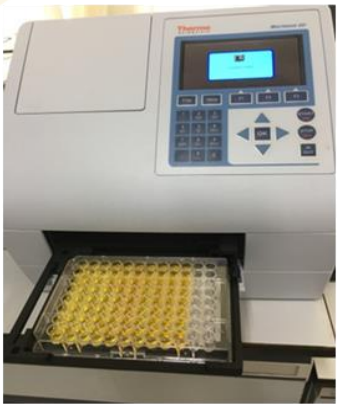
Serum



Serum



Serum in 96-well microplate and added *Micrococcus lysodeikticus*

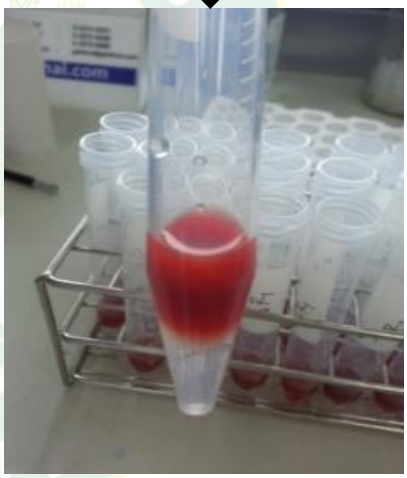


spectrophotometer at 450

2. White blood cells preparation

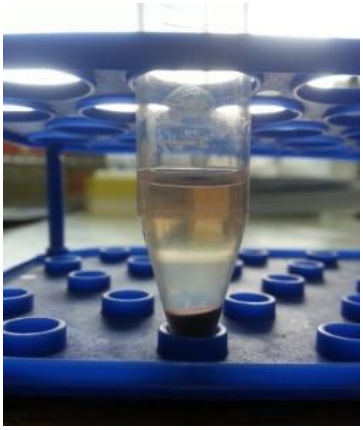
Blood samples

Heparinized blood samples

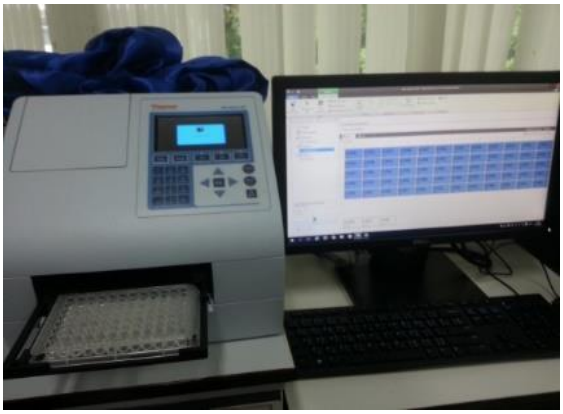


RPMI

Centrifuge at 4,000 rpm/minutes for 30 min



WBC middle layer were removed in the tube were harvested



Spectrophotometer at 655 nm

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