EFFECT OF SACHA INCHI SEED ON GROWTH PERFORMANCE, IMMUNE RESPONSES, AND EXPRESSION OF GENES RELATED TO IMMUNITY IN NILE TILAPIA

BUNTHEOURN KHEN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Agricultural Biotechnology)
2020

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has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Agricultural Biotechnology of Naresuan University

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Title EFFECT OF SACHA INCHI SEED ON GROWTH PERFORMANCE, IMMUNE RESPONSES, AND EXPRESSION OF GENES RELATED TO IMMUNITY IN NILE TILAPIA

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ABSTRACT

This present study was conducted to determine the effects of substituting fishmeal with sacha inchi seed on growth performance, the whole-body composition, apparent coefficient digestibility, hematological parameters, histological characteristics, and expression of genes related to immunity in Nile tilapia (*Oreochromis niloticus*). Two control diets including control diet 1 (fish oil-based diet) and control diet 2 (soybean oil-based diet) and other six experimental diets were formulated to replace fishmeal protein source with different inclusion protein levels of sacha inchi seed from 8%, 15%, 25%, 50%, 75%, and 100%. The experiment was divided into eight treatments and each treatment had 4 replicates. Moreover, Nile tilapia fingerlings with an initial mean weight approximate 11 g were selected and transferred to 32 fiber tanks at a density of 15 fish per fiber tank. Fish were fed up to apparent satiation with the experimental diets twice daily at 9:00 am and 16:00 pm for 10 weeks. After 10 weeks trial, the results indicated that Nile tilapia fed SIS25 diet had the highest value in term of average body weight (ABW), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), productive protein value (PPV), and apparent digestibility coefficient of dry matter and protein. Depending on the growth parameters, Nile tilapia fed SIS15 diet had the highest weight gain, followed by fish fed SIS25 diet which did not show significant differences with control diets.

The whole-body fish composition showed significant differences ($p < 0.05$) after substituting FM with SIS in diets with exception to moisture content. Nile tilapia receiving SIS25 diet had the highest value of the whole-protein content. No significant difference was detected in terms of the whole-fat content of fish fed SIS25 diet compared to control diet 1, but significantly higher compared to control diet 2. However, its values of both whole-protein and -fat content showed significant decreases after fish fed SIS between 50%-100% in diets. Fish fed SIS25 diet had the highest value in terms of the apparent digestibility coefficient of dry matter and protein which did not show significant differences with control diets. These values were significantly decreased when fish fed SIS between 50%-100% in diets. Additionally, significant differences ($p<0.05$) were observed in terms of blood biochemistry parameters in fish fed the experimental diets. Fish fed with SIS up to 25% had higher values of hemoglobin concentration, hematocrit, RBCs, and WBCs compared to fish fed SIS substituted between 50%-100%. The highest intestinal villus height was observed in fish fed SIS25 diet and it started decreasing after receiving SIS between 50%-100%. The staining liver cells sections showed that nuclei of all treatment fish were damaged. Furthermore, the liver tissues of Nile tilapia receiving SIS between 75%-100% protein levels showed intra cytoplasmic round circumscribed fat vacuoles. There were no significant differences in expression levels of target genes (TNF-α, IL-1β, and TGF-β gene) when SIS were used to replace fishmeal. However, the expression levels of TNF-α, IL-1β, and TGF-β gene tend to increase when substituting FM with SIS up to 25%. The increased levels of target genes were the same trend with the WBCs value. According to economic analyses of experimental diets showed that the SIS25 diet offers the best economic viability, taking into consideration the cost of feed fed over the trial period. These responses suggest that substituting FM with inclusion levels up to 25% of SIS can be a consequence of improved immune response and showed the best growth performance.

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CHAPTER 1

INTRODUCTION

1. Background and Significance of the Study

Fishmeal is one of the main protein sources in the conventional aquaculture sector due to its high protein content (30-72%), being a good source of essential amino acids (EAA), essential fatty acids (EFA) as well as it is highly digestible and palatable to most fish (El-Sayed and Tacon, 1997; NRC, 1993; NRC, 2011) . Most of the fishmeal produced is incorporated into commercial diets fed to fish, shrimp, swine, poultry, dairy cattle, and other animals (Table 1). It is unlikely that supplies of commercially available fishmeal and oil will be able to keep pace with the projected increase in worldwide production of aquaculture and terrestrial animal feeds. In most recent years, aquaculture has used approximately 46% of the total annual fishmeal production, a figure that is expected to rise as demand for aquaculture products increases in the next decade. Optimal use of fishmeal in practical aquaculture diets is necessary to minimize feeding costs which can account for 40% or more of operating expenses. The concentration of high-quality nutrients, especially, protein, makes fishmeal one of the most sought and expensive feedstuffs. The cost of high-quality fishmeal (65% protein) has ranged from approximately $385 to $554 per ton since the year 2000, or 2.0 to 3.5 times the price of soybean meal (Miles and Chapman, 2006). However, the global supply of fishmeal is decreasing since the world's capture fisheries have passed the peak in the amount of wild fish caught at sea. Together with an increased demand of fishmeal, it has resulted in a rise in the price of fishmeal influencing not only the finfish culture, but also the husbandry of crustacean, pets, and livestock production (FAO, 2014). With the increasing price of fishmeal and fish oil it may no longer be considered as a low-values product anymore (Olsen *et al.*, 2014).

Approaches have been made to reduce the fishmeal in aquaculture diets by replacing it with an alternative, less expensive animal, or plant protein products. Sacha inchi seed is a plant protein product which has a high content of oil (54%) and protein (27%). This plant is recognized because of its high content in oleic,

linoleic and alpha-linoleic acids and vitamin E (tocopherols and tocotrienols) compared to values in other kinds of seeds (Gonzales *et al.*, 2018; Sathe *et al.*, 2002). According to oil content, sacha inchi seeds predominantly contain essential fatty acids including omega 3, 6, and 9 fatty acids. Omega 3 and omega 6 polyunsaturated fatty acids are essential fatty acids which provide important health benefits to humans (Pietrowski *et al.*, 2011; Rymer and Givens, 2005). Since consumption of omega-3 fatty acids, whether from fish oil, flax or supplements, can protect against cardiovascular disease, the consumption of sacha inchi oil, as a novel vegetable source of omega-3 fatty acids, may become an important alternative for the prevention or protection against cardiovascular disease (Maurer *et al.*, 2012; Rodeiro *et al.*, 2018). Ortiz-Chura *et al.* (2018) studied the apparent digestibility of dry matter, organic matter, protein and energy of kañiwa (*Chenopodium pallidicaule* Aellen) , kiwicha (*Amaranthus caudatus* L) , quinoa (*Chenopodium quinoa* Willd) , beans (*Phaseolus vulgaris* L.) , Sacha inchi, (*Plukenetia volubilis* L.) and jumbo squid (*Dosidicus gigas*) meal in juvenile rainbow trout. The results suggested that quinoa meal and jumbo squid meal have an acceptable digestibility and can be used in feed formulation, but sacha inchi meal is a potential ingredient for the feeding of juvenile rainbow trout. In consequence of the previous researches rarely study about sacha inchi seed in aquatic diets, further researches are needed to evaluate the influence of sacha inchi seed on growth performance and feed utilization in fish diets. So, this study carries out in order to determine the optimum level of protein utilization from fishmeal replacing with sacha inchi seed in diets on growth performance and blood compositions of Nile tilapia. Moreover, this study also examines the expression of the innate immune-related genes.

In the tropical regions, Tilapia aquaculture is widespread more than 100 nations. Tilapias rank second in terms of global aquaculture production, after carp species (El-Sayed *et al*., 2003; El-Sayed *et al*., 2005). Among cultured tilapia species, Nile tilapia (*Oreochromis niloticus*) has recognized as the most important farmed tilapia in the world (El-Sayed *et al*., 2005). General hardiness, incredible adaptability and ability to reproduce in a wide range of physical and environmental conditions, excellent growth rates on a wide variety of natural and prepared diets, resistance to

handling and disease-causing agents, and their broad consumer appeal as a food fish has made it better candidate for aquaculture (Pullin and Lowe-McConnell, 1982).

Table 1: Use of fishmeal in agriculture

Sector	Year 2002 - 2010 (%)	
Aquaculture	46	56
Pigs	24	20
Poultry	22	12
Ruminants	1	< 1
Other	7	12

Source: Miles and Chapman (2006)

2. Purposes of the Study

This present study is aimed to determine the effects of using different protein levels from full-fat sacha inchi seed as replacement of fishmeal protein in diets for Nile tilapia on growth performances, immune responses, and expression of genes related to immunity.

CHAPTER 2

LITERATURE REVIEWS

1. Sacha inchi

1.1 Taxonomic Classification

Order: Malpighiales

Family: Euphorbiaceae

Tribe: Plukenetieae

Subtribe: Plukenetiinae

Genus: *Plukenetia*

Species: *Plukenetia volubilis* L.

1.2 Distributions

Sacha inchi (*Plukenetia volubilis*) is identified as incha inchi, inca peanut, mountain peanut or sacha peanut. It is an oleaginous plant grows in tropical rainforest of the Amazon region which is originated from the parts of Peru at altitudes between 200-1500 meters (Guillén *et al.*, 2003; Gutiérrez *et al.*, 2011; Hamaker *et al.*, 1992). Moreover, it is distributed in the Latin American tropics from the South of Mexico, the Indies Western countries, the Amazon and the Acre in Bolivia (Valles, 1993). Recently, it is also being cultivated commercially in South East Asia, most remarkably in Thailand (Saengsorn and Jimtaisong, 2017).

1.3 Environmental aspects

Sacha inchi can acclimate to the temperature between 10 and 36 ºC, however, the optimal temperature ranges from 25 and 30 ºC. High temperatures are unfavorable because it causes abortion in flowers and the formation of small seeds. Besides that, light is another important ecological aspect; while more light receives the vegetation cover, the greater the population of buds, flowers, and fruits. This plant prefers an environment in which water is continuously available, and well-drained acidic soil **(**Hanssen and Schmitz-Hübsch, 2011; Silva *et al.*, 2016**).**

1.4 Botanical characteristics

Sacha inchi is a climbing, monoecious or bisexual, and deciduous plant. The leaf blade is triangular-ovate with a length of 6-13 cm and a width of 4-10 cm, with a truncate or cordate base. A margin is crenate or serrulate; there is one glandular protuberance on the adaxial side at the tip of the petiole. Sacha inchi is a flowering plant (inflorescence), 5 cm to 18 cm long, is racemose, elongated, and monoecious (bisexual). Flowers with pistils are solitary at base nodes; the stylar column is partially or totally connate, 15 mm to 30 mm long; numerous sub-globular masculine flowers are grouped on distal nodes; there are 16 to 30 conical stamens (0.5 mm long) with visible filaments. Capsules are tetramerous or pentamerous, glabrous, and 2.5 cm to 6 cm in diameter. Sacha inchi seeds are lenticular and laterally compressed. As the fruit matures, the colour of seed turns from green to blackish brown. Furthermore, the capsules contain edible dark brown oval seeds 1.5-2 cm (Figure 1) (Dostert *et al.*, 2009; Wang *et al.*, 2018).

Figure 1: Sacha inchi characteristics

Source: (Fu *et al*., 2014; Wang *et al*., 2012)

1.5 Nutritional characteristics

Sacha inchi seeds and oil have been consumed in Peru as part of the Inca diet for 3000 years. The sacha inchi seeds are valued for their high oil and protein content. (Dostert *et al.*, 2009; Gillespie, 1993; Rodeiro *et al*., 2018). Gutiérrez et al., (2011) revealed the main chemical composition of sacha inchi seeds from Colombia. The

macronutrient and micronutrient content were found. Sacha inchi consisted of a high oil content up to 41.4% and protein (24.7%). The main minerals present in sacha inchi seeds were potassium, magnesium, calcium, and other minerals which are shown in Table 2.

Sacha inchi seeds consist of oil (35-60%) including omega 3, 6, and 9 fatty acids, protein 25-30% **(**including essential amino acids such as cysteine, tyrosine, threonine, and tryptophan), vitamin E, polyphenols, minerals, and others (Cai *et al.*, 2012; Cai *et al.*, 2011; Chirinos *et al.*, 2013; Do Prado *et al.*, 2011; Fanali *et al.*, 2011; Sathe *et al.*, 2012). The protein and amino acids contents of sacha inchi seeds were approximately comparable to soybean, peanut, cottonseed, and sunflower which is shown in Table 3 (Hamaker, *et al***.**, 1992)**.** Moreover, sacha inchi seeds have an irreplaceable fatty acids composition comprising a large amount of unsaturated fatty acids that are predominantly polyunsaturated fatty acids, phytosterols, and tocopherols**.** The major compounds found in these three chemical categories are α-linoleic acid, β-sitosterol, and γ and δ-tocopherols, respectively**.** The oil is rich in omega-3 linoleic acid at about 45%–53%, omega-6 linoleic acid 34%–39%, and non-essential omega-9 about 6%–10% of fat content, a lesser extent saturated fatty acids and very low levels of trans-fatty acids**.** The amount of monounsaturated oleic acid (9**.**6%) in sacha inchi lipids is lower compared to the corresponding amounts in soybean, peanut, cottonseed, and sunflower seed oils with quantities of 22.3%, 41.3%, 18.7%, and 29.3% respectively. (Gutiérrez *et al***.**, 2017; Hamaker *et al***.**, 1992; Sathe *et al***.**, 2012; Wang *et al***.**, 2018). Condensed and hydrolysable tannins, lignans, flavonoids, and phenolic acids are the bio-actives in sacha inchi seed and the shell. Terpenoids, saponins, phenolic compounds (flavonoids) are the bioactive components in the leaves (Follegatti-Romero *et al.*, 2009; Guillén *et al.*, 2003; Gutiérrez *et al.*, 2017; Zanqui *et al.*, 2016). De Souza *et al.* **(**2013**)** reported that the sacha inchi nut contain a high lipid content about 48.5%, and low lipid content in the shell with the amount of 1**.**2%. Low contents of saturated fatty acids were discovered in both nut and shell, indicating anti-atherogenic, anti-thrombogenic and hypercholesterolemic effects**.** The n-3 fatty acids content with amount of 438**.**7 mg g^{-1} of total lipids found in the nut, while it was found in the shell about 329**.**4 mg g^{-1}**.** Concerning with tocopherol, the total content was greater than other oilseeds**.** The abundant amount of

α-tocopherol existing in the shell is highlighted since this is considered responsible for the metabolic activity of vitamin E**.** The values of dietary reference intake enable the evaluation of the nutritional potential of sacha inchi fractions**.**

The presence of secondary metabolites occurring in sacha inchi seed has been determined qualitatively indicating presence of saponins (abundant) and coumarins **(**moderate**)** in the seed and alkaloids (abundant) in both the aqueous and ethanolic extracts of the seed (Pariona Mendoza, 2008), but Ruiz *et al.* (2013) determined the composition, amino acid profile, fatty acid profile and anti-nutrients contents of sacha inchi seed and cakes (cake is a sub-product in the extractions processes of the oil), and showing the presence of low levels of saponins and tannins in this product, which could explain at least in part the absence of toxicity**.**

Table 2: Chemical composition of the sacha inchi seeds

Sacha inchi seed component	Value
Moisture (%)	3.3 ± 0.3
Fat (%)	42.0 ± 1.1
Protein (%)	24.7 ± 0.5
Ash (%)	4.0 ± 0.7
Total carbohydrate (%)	30.9 ± 0.6
Potassium (mg/kg)	5563.5 ± 6.4
Magnesium (mg/kg)	3210.0 ± 21.2
Calcium (mg/kg)	2406.0 ± 7.1
Iron (mg/kg)	103.5 ± 8.9
Zinc (mg/kg)	49.0 ± 1.1
Sodium (mg/kg)	15.4 ± 0.5
Cooper (mg/kg)	12.9 ± 0.3

Source: Gutiérrez *et al*. (2011)

1.6 Benefits of the sacha inchi

The benefits of sacha inchi are evident due to its high omega content compared to the oils of all the oilseeds used in the world for human consumption; within the benefits, the following:

1. Improves the activities of the nervous system.
2. Speeds up different brain functions.
3. Fortifies the bones.
4. Improves digestive and fatty metabolism.
5. Helps to lose weight.
6. Improves the immune system.
7. Strengthens the heart
8. Reduces the probability of vascular diseases.
9. Improves blood circulation.
10. Improves intestinal metabolism.
11. Reduces bad cholesterol or LDL.
12. Reduces triglycerides (Escobar Artieda, 2014).

Table 3: Amino acid profile of sacha inchi protein compared to other oilseed proteins.

Amino acids	Sacha inchi	Soybean	Peanut	Cottonseed	Sunflower
Total protein (%)	27	28	23	33	24
EAA[1]					
His	26	25	24	27	23
Ile	50	45	34	33	43
Leu	64	78	64	59	64
Lys	43	64	35	44	36
Met	12	13	12	13	19
Cys	25	13	13	16	15
Met + Cys	37	26	25	29	34
Phe	24	49	50	52	45
Tyr	55	31	39	29	19
Phe + Tyr	79	80	89	81	64
Thr	43	39	26	33	37
Trp	29	13	10	13	14
Val	40	48	42	46	51
Nonessential AA[2]					
Ala	36	43	39	41	42
Arg	55	72	112	112	80
Asp	111	117	114	94	93
Glu	133	187	183	200	218
Gly	118	42	56	42	54
Pro	48	55	44	38	45
Ser	64	51	48	44	43

[1] EAA (Essential amino acid), His (Histidine), Ile (Isoleucine), Leu (Leucine), Lys (Lysine), Met (Methionine), Cys (Cysteine), Phe (Phenylalanine), Tyr (Tyrosine), Thr (Threonine), Trp (Tryptophan), and Val (Valine).

[2] Nonessential AA (Nonessential amino acid), Ala (Alanine), Arg (Asparagine), Asp (Aspartate), Glu (Glutamate), Gly (Glycine), Pro (Proline), and Ser (Serine).

Source: Hamaker *et al*., (1992)

Sacha inchi seeds are predominantly oil including omega 3, 6, and 9 fatty acids. Polyunsaturated fatty acids (Omega 3 and omega 6) are essential fatty acids which deliver vital health benefits to humans. There is an increasing study on the fortification of food products with ω–6 polyunsaturated fatty acids (ω–6 PUFAs) and especially ω–3 polyunsaturated fatty acids (ω–3 PUFAs). The ω–3 PUFAs decrease inflammatory markers, blood pressure, and triglycerides, which are established risk factors for cardiovascular disease (Pietrowski *et al.*, 2011; Rymer and Givens, 2005). Eidhin *et al.* (2003) provided a comprehensive review of the health benefits associated with ω-3 PUFAs differentiating specific a–linoleic (ALA, C18:3, ω–3), eicosapentaenoic (EPA, 20:5, ω–3) and docosahexaenoic (DHA, 22:6, ω–3) acids and demonstrated that EPA and DHA can be produced in humans by the sequential elongation and desaturation of ALA. Considering the important physiological functions of ω–3 PUFAs, dietary intake of these long–chain fatty acids is indispensable. Fish oil is the primary source of these essential polyunsaturated fatty acids as it contains ω–6 (0.9–12 g.100 g^{-1} oil) and ω–3 (11.9–35.3 g.100 g^{-1} oil) (Liu *et al.*, 2014; Rubio-Rodríguez *et al.*, 2010; Venegas-Calerón *et al.*, 2010).

Fanali *et al.* (2011) highlight the presence of antioxidant compounds such as flavonoids and tocopherols in the nut; the latter may reduce the risk of heart disease, type 2 diabetes, and cancer (Köksal *et al.*, 2006; Yang, 2009). Tocopherols and tocotrienols are fat-soluble compounds and fractions of vitamin E, which are identified by the prefixes α, β, γ, and δ. They are compounds with different activities of vitamin E and the isomer α-tocopherol is the most biologically active. These compounds are found only in plants (De Souza *et al.*, 2013; Yada *et al.*, 2011).

1.7 Use of sacha inchi seed meal in aquatic diets

Araujo-Dairiki *et al.* (2018) studied the use of SIS as an ingredient in the diets of juvenile Tamaqua (*Colossoma macropomum*), and matrinxã (*Brycon amazonicus*) which the experiment was assessed in a two-factorial completely randomized design (fish species x feed), include a group of 12 juvenile tambaquis (29.8 ± 1.0 g initial weight and 11.0 ± 1.4 cm initial length) and a group of 8 juvenile matrinxãs (34.8 ± 1.3 g initial weight and 13.7 ± 1.0 cm initial length) by using SIS of three different proportion (0, 15 and 30%) in fish feed. Fish were fed with the preparation feeds for

60 days, receiving two daily meals (8 h and 15 h) until satiety. At the end of the experiment showed that both tambaquis and matrinxãs accepted the feeds including SIS, and there were no significant differences in survival, growth, and body composition of fish from feeds without sacha inchi (0%). The results indicated that sacha inchi does not affect negatively nor significantly improve the development of tambaqui and matrinxã over commercially available feed.

2. Nile tilapia

2.1 Taxonomy

Nile tilapia is taxonomically categorized as follows**:**

Phylum**:** Chordata

Class**:** Actinopterygii

Order**:** Perciformes

Family**:** Cichlidae

Genus**:** *Oreochromis*

Species**:** *Oreochromis niloticus*

Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) is a freshwater fish species in the Cichlids family of order Perciformes. It is a deep-bodied fish with the large, oblong laterally compressed body, and scales cycloid. Its mouth is large, and the lips are thick. The forehead and back slope evenly up to near the base of the dorsal fin. The colours body bars vary from silver to olive/grey/black. Colour in spawning season, pectoral, dorsal and caudal fins become reddish; caudal fin is full of numerous black bars. Dorsal fin has 16 – 17 spines, 11 to 15 branched dorsal-fin rays and lacking a red margin. First gill arch is countable between 27 to 33-gill rakers. Lateral line is interrupted. Anal fin has 3 spines and 10-11 rays. Caudal fin is truncated. Around 9 years of age, Nile tilapia grows to a maximum length of 62 cm, weighing 3.65 kg and the normal size of *O. niloticus* is 20 cm. Nile tilapia characteristic is represented in Figure 2. (Bwanika *et al.*, 2004; Gilpin, 2011; Picker and Griffiths, 2011; Rainboth, 1996).

Figure 2: Morphological characteristic of Nile tilapia.

2.2 Distributions and habitats

Nile tilapia is a tropical freshwater and estuarine species. Its natural habits are the shallow waters, still waters on the edge of lakes, wide rivers with sufficient vegetation, irrigation channels and sewage canals as well (Gilpin, 2011; Picker and Griffiths, 2011). It is native to the Nile River basin; the coastal rivers of Israel; the south-western middle east; the Niger, Benue, Volta and Senegal rivers, and the lakes Chad, Tanganyika, Albert, Edward, and Kivu**.** It has been introduced for farming purposes to over 50 countries on all the regions except Antarctica. Nowadays, it is found in nearly every country within the tropics (Daget *et al.*, 1991; Gilpin, 2011; Pullin *et al.*, 1997; Trewavas, 1983).

2.3 Nutritional requirements of Nile tilapia

Nutritional requirement of tilapia depends on various factors such as species, size, protein sources and quality, non-protein energy level in the test diets, feeding rate, water quality variables, the presence and density of natural food (Nguyen, 2008; NRC, 1993). Nile tilapia are recognized to feed on phytoplankton, periphyton, aquatic plants, invertebrates, benthic fauna, detritus, bacterial films, worms, fish and fish eggs (FAO, 2012; Gilpin, 2011). Abari *et al.* **(**2015**)** reported the feed and feeding habit of Nile tilapia (*Oreochromis niloticus*) in Doma Dam, Nasarawa state, Nigeria. This study revealed the percentage of food items in *O. niloticus* stomach which majority of fish behind the dam fed on phytoplankton (16.5%) and leaf parts (7.5%) while a few

fish fed on desmids (2%) and insect pupae (1%). The most plentiful foodstuff in the Dam is plant ingredients (31%), followed by animal ingredients (14%) and the least is detritus (11%), but few food ingredients (29%) obtained in the stomach content of fish could not be recognised. The ingredient requirements consist of protein, lipids, energy, vitamins, and minerals. These are so important for growth, reproduction, and other physiological functions. Nutrients for fish derived from various food sources including plankton, bacteria, insects, and other fish or from organic matter and processed feeds added to the ecosystem (Hancz, 2011). Complete diets were used to culture fish which require ingredients such as protein (18-50%), lipid (10-25%), carbohydrate (15-20%), ash (< 8.5%), phosphorus (< 1.5%), water (< 10%), and trace amounts of vitamins, and minerals (Craig *et al.*, 2017).

The efficiency of feed influenced exceedingly by the size and sex of the experiment animals, their reproductive status, and the time of year at which the growth testing has completed**.** For instance, small-sized fish require the efficiency of feed for growth than the large-sized fish (Jobling, 1994) **.** The feed is usually manufactured from a range of raw ingredients, or feedstuffs, and is formulated to contain an array of essential and non-essential nutrients**.** A nutrient can be a chemical element or compound that an animal can utilize in metabolism or growth, an essential nutrient is one that the animal cannot synthesize de novo**.** Thus, an animal**'** s requirements for essential nutrients must be met via the diet**.** Feedstuffs may vary from simple compounds, such as common salt (NaCl), to meals of plant and animal origin that are made up of complex mixtures of compounds from several nutrient classes **(**Houlihan *et al.*, 2008**).**

2.4 Nutrients

There are three organic nutrients such as carbohydrate, fat, and protein, are called energy-yielding nutrients or macronutrients used to provide energy**.** Vitamins and minerals are known as micronutrients, the essential dietary elements that are required in small quantities **(**milligrams or microgram daily**).** They function as the components of enzyme cofactor or coenzymes in many biochemical reactions and metabolic processes vital for survival, growth, and reproduction (Whitney and Rolfes, 2005).

2.5 Proteins

The elemental protein compositions consist of carbon 50–55%, hydrogen 6–8%, oxygen 20–23%, nitrogen 15–18% and Sulphur 0–4%. Most of the amino acids have a chemical structure RCH(NH_2)-COOH, with a carboxyl (-COOH) and an amino (-NH_2) group affixed to the α carbon atom which is represented in Figure 3 (Houlihan *et al*., 2008). Whenever the body is growing, repairing, or replacing tissue, proteins are involved. Essential key functions of protein are described below:

1. Growth and maintenance.

Proteins form integral parts of most body structures such as skin, tendons, membranes, muscles, organs, and bones. As such, they support the growth and repair of body tissues.

2. Enzymes.

Proteins facilitate chemical reactions.

3. Hormones.

Proteins regulate body processes (Some, but not all, hormones are made of protein).

4. Antibodies.

Proteins inactivate foreign invaders, thus protecting the body against diseases.

5. Fluid and electrolyte balance.

Proteins help to maintain the fluid volume and the composition of the body fluids.

6. Acid-base balance.

Proteins help maintain the acid-base balance of body fluids by acting as buffers.

7.Transportation.

Proteins transport substances, such as lipids, vitamins, minerals, and oxygen around the body.

8. Energy.

Proteins are the majors' source of energy that provides some fuel for the body's energy needs (Fürst, 2009).

2.6 Protein requirements for tilapia

Nutrient requirements of aquatic animals are related to other animals, but they require higher in term of protein content, so the combinations of diet with proteins (25-45%) are regularly used for growth performance, reproduction and normal physiological functions **(**Davies and Gouveia, 2010; Stanković *et al.*, 2011**).** Many researches have been determined the protein requirement for fish, with estimated protein requirements ranging from 30% to 55% according to fish species, size, dietary protein sources and environmental conditions (Acosta *et al.*, 2009; NRC, 1993)**.** The study of Craig *et al.* **(**2017**)** reported that protein levels in aquaculture diet normally range from 30-35% for shrimp, 28-32% for catfish, 35-40% for tilapia, 38-42% for hybrid striped bass, and 40-45% for trout and other marine finfish (Table 4). Generally, protein requirements are usually lower for herbivorous fish and omnivorous fish than carnivorous fish. Similarly, fish reared in high-density system require protein higher than fish reared in low-density**.** In the same way, protein requirements are generally higher for smaller as well as early life stage fish**.** As mature fish, the requirement of protein commonly decrease. As the description above, protein requirement varies with the culturing environment, water quality, as well as the genetic structure and feeding rates of fish**.**

Table 4: Protein levels in aquaculture feeds

Aquatic animals	Protein level requirements (%)
Marine shrimp	18-20
Catfish	28-32
Tilapia	32-38
Hybrid striped bass	28-42
Trout and other marine finfish	40-45

Source: Craig *et al*., (2017)

For tilapia, the decreasing of protein requirement depends on stage of development and size. Tilapia fry requires higher dietary crude protein concentrations between 30–56% and juvenile about 30–40%, but lower protein levels (28–30%) for larger tilapia (Mjoun *et al.*, 2010). El-Sayed and Teshima **(**1992**)** described that

numerous species have been estimated the protein contents ranging from 20% to 56% and many other studies reported the requirement of protein which shows in Table 5. Moreover, Bahnasawy **(**2009**)** studied the effect of dietary protein levels on growth performance and body composition of mono-sex Nile tilapia reared in fertilized tanks. Fish were fed with four experimental diets in different crude protein levels including 17%, 25%, 30%, 35%, correspondingly. The result showed that weight gain and specific growth rate increased significantly with protein levels between 17% and 30%, however, fish received 35% crude protein level revealed an insignificant increase in growth parameters. This study recommended that a diet comprising 30% crude protein is considered as the optimum level for mono-sex tilapia growth under the current experimental environments.

Table 5: Protein requirement of Tilapia

Life stage	Size (g)	Requirement (% diet)
Fry	0.012-0.5	45
	0.51-0.8	40
Fingerling	2.4-3.5	30-35
Juvenile	16.5-20	30-35
Adult		
Bloodstock		30-45

Source: Sandamali, (2016)

2.7 Amino acids

Amino acids are essential for protein synthesis and have different functions in the body. Amino acids comprise carbon (alpha carbon), amino group (NH_2), a carboxylic group (COOH), hydrogen (H) and a side group attached (R). More than 300 amino acids, only 20 amino acids are in relation with protein synthesis. Essential amino acids (EAA) cannot be synthesized in the animal cells and it should be achieved from the diet. Conversely, non-essential amino acids are the amino acids that the body can synthesis and more than half of the amino acid are non-essential amino acids. Proteins in diets deliver these amino acids, but it is not essential that they do so

(Akram *et al.*, 2011). The amino acids requirements of Nile tilapia are presented in Table 6.

Table 6: Amino acid requirements of tilapia

Amino acid	Requirement (% of dietary protein)
Arginine	4.20
Histidine	1.72
Isoleucine	3.11
Leucine	3.39
Lysine	5.12
Methionine	2.68
Phenylalanine	3.75
Threonine	3.75
Tryptophan	1.00
Valine	2.80

Source: Sandamali (2016)

3. Protein sources in aquatic animal diets

Protein is the most expensive constituent in fish feeds. The nutritional value of fish feed is influenced by the quality of the protein ingredients used in feed formulation (Mohanta *et al.*, 2013). Generally, animal proteins quality is higher than plant proteins, mainly due to their superior complement of essential amino acids and almost of plant proteins are lacking in lysine and methionine (Robinson *et al.*, 2001). Animal protein sources (fishmeal, meat and bone meal, and blood meal) and natural feeds (algae, phytoplankton, and zooplankton) are valuable sources of protein with free amino acids and oligopeptides, fats and fatty acids, and vitamins; are essential substances for growth and development of fish (Kibria *et al.*, 1997; Stanković *et al.*, 2011).

3.1 Fishmeal

Fishmeal is manufactured by cooking, pressing, drying, and milling whole fish and fisheries by-product (Allen, 2016). Fishmeal has been used as a livestock feed for

many years and it has well-known the most expensive protein product recently (Tacon, 1993). Due to its high nutritional content value, it is acknowledged as a natural source of extraordinary quality protein for aquatic animal feed production, high levels of fatty acids (DHA or docosahexaenoic acid and EPA or eicosapentaenoic acid), essential amino acids such as lysine, which is often lacking in grain products that are the typical base for most animal feeds (Tantikitti *et al.*, 2016; Zinn *et al.*, 2009). Furthermore, fishmeal is abundant in methionine and cysteine content, high digestibility and biological value (Keller, 1990). Likewise, it comprises vitamins (vitamin A, D, E, and B12), choline, niacin, pantothenic acid, biotin, and riboflavin and many other sources such as calcium (Ca), copper (Cu), iron (Fe), phosphorous (P), and trace minerals like selenium and iodine. Conversely, it is low in fiber and easy to produce (Ariyawansa, 2000; Cho and Kim, 2011).

High-grade quality of fishmeal offers a balanced amount of all essential amino acids, phospholipids, and fatty acids for optimum development, growth, and reproduction, specifically larvae and bloodstock. The nutrients in fishmeal also aid in disease resistance by boosting and helping to maintain a healthy functioning immune system. Anyway, the oil in the fishmeal is protected against oxidation by 150 ppm ethoxyquin (Jensen and Keller, 1990; Miles and Chapman, 2006). Fishmeal and fish oil have been described to offer main benefits to animal health, including improved immunity against disease, higher survival and growth, and reduced incidences of deformities (Schipp, 2008). Addition of fishmeal to animal diets increases feed efficiency and growth through better food palatability and enhances nutrient uptake, digestion, and absorption (Miles and Chapman, 2006).

In general, high-quality fishmeal comprises 60-72% crude protein by weight. From a nutritional view, fishmeal is the favoured animal protein addition in the feeds of farm animals and often the main protein source in diets for fish and shrimp. In this case, typical diets for fish might cover 32% to 45% of total protein by weight, and diets for shrimp may contain 25% to 42% total protein. The percentages of inclusion rate of fishmeal in diets for carp and tilapia may be from 5-7%, and up to 40%-55% in trout, salmon, and some marine fishes. A typical inclusion rate of fishmeal in terrestrial livestock diets is usually 5% or less on a dry matter basis (Miles and Chapman, 2006). In Thailand, the protein content of fishmeal is characterised into

three grades such as premium grade, grade 1, and grade 2. **(**Thai Fishmeal Producer Association, 2001**)** which is shown in Table 7. The protein content ranges from 50->65% and fat content from 4-20%**.** Ash content is highly variable from about 11-12% in anchovy meal to >23% in whitefish meals made from filleting waste (Tantikitti *et al.*, 2016).

Table 7: Classification of fishmeal quality in Thailand

Grade	**Protein** (%)	**Ash** (%)
Premium grade	> 65%	
Grade 1	>60%	<26%
Grade 2	>55%	<28%

Source: Thai Fishmeal Producers Association (2001)

3.2 Plant protein sources

There are various plant protein sources were used in fish diets including soybean meal, cottonseed meal, rapeseed and canola meals, peanut meal sunflower meal and oilseed meals. Plant protein ingredients have reasonably low cost compared to animal protein sources. In general, properly processed plant products and by-products also have high protein digestibility. They can be used in combination to replace more expensive ingredients such as fishmeal (O'Keefe, 2003; Oramary *et al.*, 2016; Robinson *et al.*, 2001).

3.3 Soybean meal

Soybean meal is the most plentifully produced of all oilseed meals. It is named the leading protein source for animal diet as a consequence of its great protein quality, amino acids source, vitamins, minerals, and fats profile compared to alternatives (Chen, 2013; Govindarao, 2010).

Even if soybean meal may possibly not be a perfect replacement for fishmeal in all finfish diets, it is more economically stable. Compared to fishmeal, soybean meal is less expensive and displays less short and long-term price fluctuations (Allen, 2016). The processes of soybean to soybean meal and soy oil are presented in Figure 3. Splitting the hulls from the soybean cotyledons by cracking the seeds is the first

step of its process. After dehulling, the soybean is subjected to preconditioning at 65-70°C for 30 min to make the soybeans more pliable for flaking. Flaking is necessary to make oil extraction of the soybean more rapid and more efficient. In extraction, the oil is washed with a solvent, mostly hexane, in order to separate the oil from the flakes. Batch extractors, as well as continuous extractors, are used. Desolventization or toasting is normally performed at a temperature of 70-80 °C for about 20 minutes. This is the most critical stage in soybean processing. To retain high solubility of the protein, control of processing variables is essential with respect to temperature, pressure, the presence of moisture, and residence time. In the last step, grinding and classification are necessary to meet special standards e.g. defatted soybean meal or soy grits (Marsman, 1998).

3.4 Nutrient composition

Soybean meal is characterized a higher content of crude protein about 40-49%. Soybean meal standardized on 44-49% of protein there is on the feed market. The protein of soybean contains a considerable quantity of lysine, but the value of protein is limited by methionine and cystine content. The amino acid profile of soy protein is generally superior to other plant proteins; though compared to menhaden meal protein, it is deficient in lysine, methionine, threonine, and valine (Table 8). The increased level of cystine compensates for the deficiency of methionine to some extent. However, total sulfur-containing amino acids are still higher in menhaden protein (Banaszkiewicz, 2011; O'Keefe, 2003).

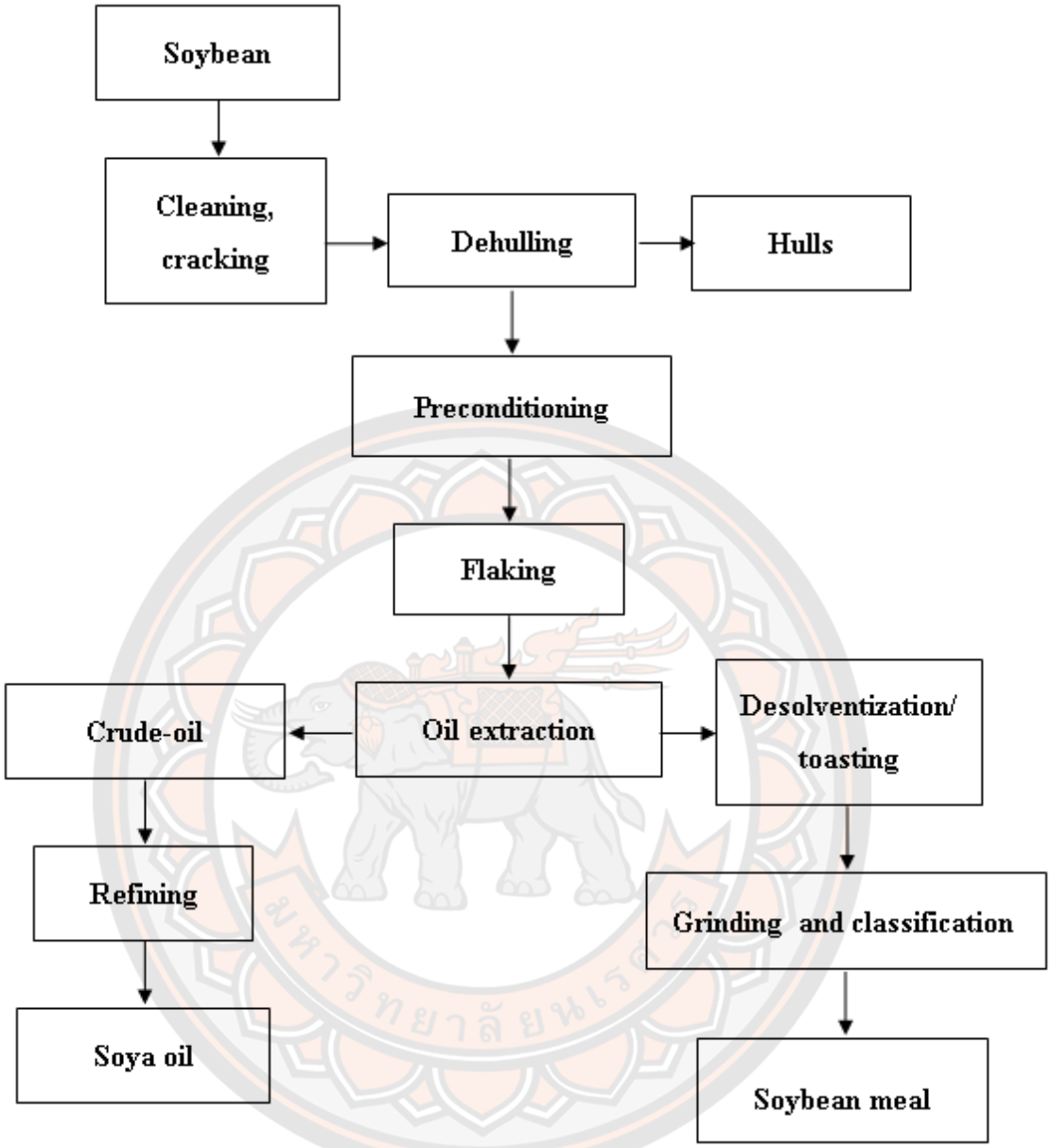

Figure 3: Soybean meal processing.

Source: Marsman, (1998)

Table 8: Essential amino acid content of protein sources commonly used in diets for fish

Name IFN	Amino acid content as % of protein					
	Menhaden	Soybean	Peanut	Cottonseed	Rapeseed	Corn Gluten
Arginine	6.1	7.4	9.5	10.2	5.6	3.4
Histidine	2.4	2.5	2.0	2.7	2.7	2.3
Isoleucine	4.7	5.0	3.7	3.7	3.7	4.2
Leucine	7.3	7.5	5.6	5.7	6.8	16.8
Lysine	7.7	6.4	3.7	4.1	5.4	1.7
Methionine	2.9	1.4	0.9	1.4	1.9	2.9
Cystine	0.9	1.7	1.5	1.9	0.8	1.7
Phenylalanine	4.0	4.9	4.2	5.9	3.8	6.6
Tyrosine	3.2	3.4	3.2	2.0	2.2	5.3
Threonine	4.1	3.9	2.4	3.4	4.2	3.6
Tryptophan	1.1	1.4	1.0	1.4	1.2	.5
Valine	5.3	5.1	3.9	4.6	4.8	5.1

Source: O'Keefe, (2003)

Relating to high protein content, soybean meal is mostly used in poultry and pigs' diet. In mixtures protein content of soybean meal in poultry diet can use up to 40%. Growing fish require the commercial diets with the high levels of protein between 25% and 45%. Consequently, high protein content plant feedstuffs are preferentially used in formulating diets for most species of fish (Banaszkiewicz, 2011; O'Keefe, 2003).

Soybean meal comprises 35-40% carbohydrates (Table 9). These carbohydrates could be divided into three groups such as soluble sugar, storage polysaccharides, and structural polysaccharides. Soluble sugar contains approximately 50% of total carbohydrates, mostly sucrose, raffinose, stachyose, and verbascose. However, free galactose, glucose, and fructose were not found in soybean meal because they were damaged during processing. In term of storage polysaccharide in

soybean meal, there is starch, constituting less than 1% of dry matter (Chen, 2013; Grieshop *et al.*, 2003; Liu, 1997).

Table 9: Chemical composition of soybean meal

Constituent	Dry weight basis (%)
Carbohydrates	35-40 (Liu, 1997)
Sucrose	6-9
Raffinose	1-1.5
Stachyose	5 -8
Starch	0 -5
non-starch polysaccharides	16-22
Crude protein	50-58
Fat	< 1
Ash	6

Source: Marsman, (1998)

3.5 Anti-nutritional factors in soybean meal

A number of anti-nutritional factors are found in soybean products comprising protease inhibitors, lectins, phytic acid saponins, phytoestrogens, antivitamins, and antigenic compounds (Francis *et al.*, 2001; Sindelar, 2014). Some anti-nutritional factors can be damaged or inactivated by proper heat treatment (Trypsin inhibitor) and some by supplemental enzymes (NSP enzymes, phytase), while others are unaffected by the methods applied now commercially (Yasothai, 2016).

3.6 Replacement of fishmeal by soybean meal in fish diets

Davis *et al.* **(**2005**)** studied the replacement of fish meal with soybean meal in the production diets of juvenile Red Snapper, *Lutjanus campechanus*. The replacement of menhaden fishmeal with solvent extracted soybean meal was performed in diets containing 10% poultry by-product meal and was formulated to be 40% protein, 8% lipid, and a total sulfur amino acid content of > 3.0% of the protein. The response of red snapper (mean initial weight 10.9 g) to diets containing graded levels of fishmeal (30, 20, 10, 0%) as well as the response to a low fishmeal diet

(10%) without poultry by-product meal were evaluated over a 6-week growth period. Significant ($p < 0.05$) differences in final mean weight, percent weight gain, and feed conversion were observed. The final weight gain ranged from 30.9 g for fish fed diets with 30% fish meal to 12.6 g for 0% fish meal. The feed conversion efficiencies ranged from 60.1% for fish fed diets with 30% fish meal to 7.7% for 0% fish meal. No significant differences were observed for survival between the treatment means. There was a reduction in performance as the fishmeal was replaced with soybean meal, the use of 10% poultry by-product meal or 10% fish meal resulted in a similar performance of the fish. The findings of the study suggest that replacing fish meal with high levels of soybean meal appears to reduce the palatability of the diet.

El-Saidy and Gaber **(**2002**)** studied the complete replacement of fishmeal by soybean meal with dietary L-Lysine supplementation for Nile tilapia *Oreochromis niloticus* (L.) fingerlings by using five experimental diets. Diet 1 was determined to be a high-quality commercial tilapia fish diet with hexane-extracted soybean meal (SBM) amount of 30% and menhaden fish meal 20%. Diets 2 to 5 contained 55%, 54%, 53%, and 52% Soybean meal and 0.5%, 1.0%, 1.5%, and 2.0% L-lysine HCl supplementation, respectively. 1% of L-methionine was added in all diets except for diet 1. All diets were formulated to be isonitrogenous (33.2% protein) and isocaloric (4.8 kcal gross energy/g of diet). The fishes were cultivated in 15 glass aquaria each containing 80 L of dechlorinated tap water for 10 weeks. The result showed that there was a significant difference in final individual weight, weight gain (%), SGR, FCR, PER, and total food intake among treatments after 10 weeks feeding trial. Diet 2 (55% SBM and 0.5% L-lysine) was recorded of having the highest values of final individual weight, weight gain (%), SGR, FCR, PER, total food intake, and the lowest values were recorded with fish fed all other diets.

Fagbenro and Davies **(**2004**)** studied the effect of replacing high percentages (50%, 75% or 100%) of dietary protein from the fish meal with soy protein concentrate on growth, diet utilization, and carcass composition of the African catfish, *Clarius gariepinus*, was evaluated. Supplemental methionine was added to the diet formulation in which soy protein concentrate replaced 100% of fish meal protein. The formulated diets were fed to catfish fingerlings twice daily for 70 days. Replacement of 50% and 75% of fish meal protein with soy protein concentrate did not affect

growth, feed utilization or carcass composition, which were similar ($p > 0.05$) to those fed with the control diet. At 100% replacement, growth and feed utilization were depressed and slightly inferior haematological characteristics were observed, compared with the other treatments. The results indicate that 75% of fish meal protein in catfish diets can be replaced with soy protein concentrate and that catfish can be replaced with soy protein concentrate and that catfish can effectively utilize supplemental methionine, thereby allowing up to 100% of the dietary protein from fish meal to be replaced by soy protein concentrate.

Freitas *et al.* (2011) studied the growth and feeding responses of the mutton snapper, *Lutjanus analis* (Cuvier, 1828) fed on diets with soy protein concentrate in replacement of Anchovy fish meal. The study evaluated the growth (first phase) and feeding responses (second phase) of juvenile mutton snapper fed four isonitrogenous and isoenergetic diets with increasing levels of soy protein concentrate (SPC) in substitution to fish meal (FM). The fish meal was replaced by soy protein concentrate at 0% (basal diet SPC000), 33% (SPC130), 57% (SPC214) and 77% (SPC300). After 95 days of rearing, fish fed soy protein concentrate 77% attained a significantly lower body weight compared with those fed diets 0%, 33%, and 57%. Fish yield increased significantly from 891 ± 36 g m^{-3} for fish fed soy protein concentrate 300 to an average of 1099 ± 111 g m^{-3} for other diets. A maximum fish body weight gains of 0.60 ± 0.05 g day^{-1} and a maximum specific growth rate of 1.47 ± 0.07 % day^{-1} were achieved for SPC 0% (000). Behavioural assays conducted for 10 days revealed the loss in fish growth with diets containing higher levels of SPC was due to a decline in feed intake. Low feed intake driven by poor feed palatability appeared to have been a major obstacle against higher inputs of SPC in diets for mutton snapper.

Jahan *et al.* (2007) studied the partial replacement of fishmeal protein with soybean meal protein mrigal, *Cirrhinus cirrhosus* (Ham.) fry diets. Fish were fed in 12 glass aquaria at density of 10 fishes per aquarium for 60 days. In this experiment, four isonitrogenous diets were used with 30 % protein and 11% lipid where the fish meal and soybean meal were the main sources of protein. Soybean protein was used to replace fishmeal protein with different levels including 0% (diet 1) used as a control diet, 25% (diet 2), 50% (diet 3) and 75% (diet 4), without amino acid supplementation. The fish were fed twice daily at 9:00 am and 15:00 pm with

formulated diets at a fixed feeding rate of 5% body weight per day during the entire experimental period. At the end of the experiment, livers from nine fish from each treatment were fixed in 10% neutral buffered formalin and all samples were stained with haematoxylin and eosin and periodic acid-schiff (PAS) for histological examination. The result of this study indicated that diet 3 (50% fishmeal and 50% soybean meal) had the highest attainment in fish body weight, average live weight gain and SGR whereas those of diet 4 were significantly lower than all other diets. The weight gains between diet 1 and 2 were no significant differences ($p > 0.05$). Diet 4 had the highest FCR (3.12) with a tendency to increase with increasing the proportion of soybean content in the diet. The lowest FCR and the higher protein efficiency ratio (PER), higher apparent net protein utilization (ANPU) were obtained in case of diet 3 and were not significantly different ($p > 0.05$) from those of all other dietary groups except diet 4. The result indicated that fish meal protein might be replaced 50% by soybean meal protein in the diet of *C. cirrhosus* fry for better growth. After that, histological studies of liver samples from different treatments were compared to the control group, which did not show any alteration from normal histology, slight irregularly arranged hepatocytes and some glycogen deposition was observed in the liver of fish fed with the 50% soybean meal in the diet, when the level of soybean meal was used up to 75% in the diets necrotic hepatocytes with huge empty spaces and some glycogen was present. Although, the liver histology of fish fed the 25% soybean meal in the diet was similar to the control. The histological changes in liver of the fishes were observed due to rearing in control condition and higher inclusion of soybean meal protein as soybean meal also possess additional factors other than trypsin inhibitor like phytic acid.

Sharma and Saini **(**2017**)** studied the replacement of fishmeal with soybean meal in Nile tilapia (*Oreochromis niloticus*) Diet. Nile tilapias were cultured in fifteen plastic tanks of 225 litters capacity for 42 days. The experiment was conducted in triplicate which each tank was stocked with 10 fish fries. Fish were fed 8% of body weight per day. In term of experimental diets, fish meal was replaced with soybean meal with 0% (control diet), 25 (T1), 50 (T2), 75 (T3) and 100% (T4) respectively. Maize flour (10%), rice bran (25%), groundnut oil cake (25%) and fish meal (40%) were used as the basal (control) diet. The three ingredients such as maize flour, rice

bran, and groundnut oil cake were taken in the same quantity as in basal diet in these experimental diets. During experiment, growth parameters of fish were analysed at weekly interval. The result displayed significant differences in net weight gain, percent weight gain, specific growth rate, food conversion ratio and gross conversion efficiency of fish fed with different diets. Control diet had the highest value in growth parameters compared to other diets such as 3.18g of net weight gain, 185.64% of percent weight gain, 0. 928% of SGR and 0.343 of GCE. Likewise, FCR value of fish fed control diet was significantly different ($p \leq 0.05$) from other treatments. Moreover, this study indicated that the increasing levels of soybean meal in the fish diet had a negative impact on growth and growth parameters. As the result, the growth significantly decreased with an increasing soybean levels in Nile tilapia diet. According to the founding in this experiment, it is concluded that 25% of fish meal can be replaced with soybean meal because there were no significant differences between a fish meal-based diet (100%), fish meal and soybean meal (75%+25%) based diet. However, it is recommended that replacing 25% of fish meal can be performed with soybean meal.

Yones **(**2005**)** studied the complete replacement of fish meal with soybean meal in the diet of Nile tilapia and Mullet raised in polyculture system by using four different diets with 25.3±0.16% crude protein and fish were fed for 240 days. Fishmeal was used as the main protein source in diet 1 and in diets 2, 3 and 4, fishmeal was replaced by soybean meal with the amounts of protein 50%, 75% and 100%, respectively. The treatments were performed in 4 earthen ponds each of total area of 2.1 ha. During the trial, experimental diets were given to fish two times a day at the rate of 3% body weight. Moreover, fish weights were detailed every month to adjust the amount of feed for each pond. The results revealed the significant differences in final weight of both species; the highest final weights were reported in T2 followed in decreasing order by T1, T3, and T4, respectively. The highest growth rate and specific growth rate values were observed in tilapia and mullet fed on the diet containing 50% soybean meal in replacement with fishmeal, and the lowest values were obtained by the groups of fish receiving 100% of soybean meal. The suggestion, 50% of soybean meal is possible for replacing of fishmeal without giving negative effects on growth performance or total fish yields.

4. Fish blood

Blood is a vital complex fluid that calculates in the cardiovascular system, which includes the fluid and a series of conducting blood vessel that transports it throughout the body **(**Sukhomlinova *et al***.**, 2016; Martini, 2006). Blood volume in birds and mammals is normally 7-10% of the body weight (7-22% in marine mammals**)**, 3-8% in reptiles and amphibians, and 3-7% in most elasmobranchs and teleost fish **(**Brill *et al.*, 1998; Olsen, 2011; Saengsorn and Jimtaisong, 2017; Saguer *et al.*, 2006; Williams and Worthy, 2002)**.**

4.1 Functions of Blood

The functions of blood are classified into three categories such as transportation, protection, and regulation. Based on the blood transportation, nutrients are transported from the digestive tract and storage sites to the rest of the body. Moreover, it transports gases by carrying oxygen (O_2) from the lung to external tissues and carbon dioxide (CO_2) from those tissues to the lungs and kidney. Waste products are absorbed to be detoxified or removed by the liver and kidneys. Besides that, blood carries hormones from the endocrine glands in which they are produced to their target cells.

Blood defends the body against invasion by pathogens in several ways. Certain blood cells are capable of engulfing and destroying pathogens, and other products and secrete antibodies into the blood. Antibodies incapacitate pathogens, making them subject to destruction, sometimes by white blood cells. It prevents excessive loss of blood when an injury occurs. Blood clotting involves platelets and a plasma protein, fibrinogen. Without blood clotting, it could bleed to death even from a small cut.

Blood helps regulate body temperature by picking up the heat, mostly from active muscles, and transporting it about the body. If blood is too warm, the heat dissipates from dilated blood vessels on the skin. The pH and ion composition of interstitial fluids are regulated. Diffusion between interstitial fluids and blood eliminates local deficiencies or excesses of ions such as calcium or potassium. Blood also absorbs and neutralizes acids generated by active tissues, such as lactic acid produced by skeletal muscles (Sobti, 2008; Mader, 2006).

4.2 Blood components

Blood is a fluid composed of the cardiovascular system such as fluid (plasma) plus cells and cellular elements (red blood cells, white blood cells, and platelets), shows in Figure 4. Plasma makes about fifty-five percent of the total blood volume and represents part of the extracellular fluid of the body. The remaining 45 percent solid mass consists of red blood cells covered by a thin layer of white blood cells. The ratio of red blood cells to blood plasma is expressed as haematocrit (Kibble and Halsey, 2009; Sobti, 2008; Sukhomlinova *et al.*, 2016).

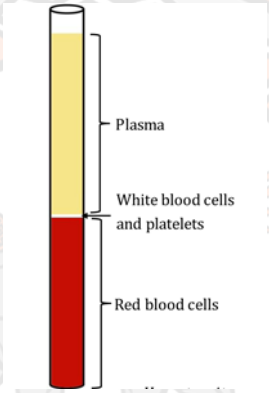

Figure 4: Blood components
Source: Mondal and Budh (2020)

4.2.1 Plasma

The liquid part of blood, the blood plasma, represents approximately 55% (by volume) of the entire blood. There are many types of components dissolved in blood plasma, which exhibit very different functions. The components of blood plasma can be divided into several groups:

1**.** Water

Water is far by the main component of blood plasma, approximately 90 percent**.**

2**.** Mineral salts and ions

There are many salts and ions dissolved in blood plasma, e**.**g**.** sodium chloride, buffer salts such as bicarbonate to guarantee a constant pH or metal ions such as calcium, copper or iron, which are essential in many biological processes and are contained in many blood plasma proteins **(**albumins, globulins, and fibrinogen**).**

Albumins are made in the liver and maintain the osmotic pressure of the blood. α and β globulins are utilized to transport molecules to facilitate the movement of other substances through circulation. The globulins provide most of the antibodies for immune processes of the body. Fibrinogen is involved in blood clotting and is synthesized in the liver.

3. Low molecular weight component

Blood plasma contains many types of low molecular weight compounds: carbohydrates such as glucose and fructose, the whole set of amino acids, nucleotides such as ATP and cAMP, many vitamins, hormones, fatty acids, lipids and triglycerides, bile acids, urea and ammonia, and many more components.

4. High molecular weight components

Peptides and (glyco)-protein, oligosaccharides and polysaccharides, oligonucleotides and polynucleotides.

5. Gases in soluble form

Many gases such as oxygen, carbon dioxide, and nitric oxide are dissolved in blood.

6. Metabolites

Blood plasma serves not only as the medium to transport the above-mentioned components but also the products of the metabolism (Schaller *et al*., 2008; Sobti, 2008).

4.2.2 Blood cells

There are three main types of blood elements including erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets) (Schaller *et al.*, 2008).

1) Erythrocyte, red blood cell

The term is derived from two Greek words: *erythros* (red) and *kytos* (hollow) (Schaller *et al*., 2008). In vertebrates, erythrocytes or red blood cells (RBC) are the most abundant cell type in circulation, that are nucleated cells in the majority of vertebrates with the exception of mammals. All nucleated non-mammalian red blood cells contain organelles in the cytoplasm and show different maturation stages including changes in the cytoplasmatic shape, staining, nuclear size and chromatin

density (Morera and MacKenzie, 2011; Shen *et al.*, 2018). The extensive cytoskeleton of red cells causes them to be extremely pliable and allow them to pass through the microcirculation. Red cells can also withstand large osmotic pressure differences, which are encountered when they pass through the renal medullary circulation. Fish blood has characteristically low concentrations of erythrocytes (1 to $5\times10^6/mm^3$) (Fig. 5 and 6a) and haemoglobin (Speckner *et al.*, 1989). When red cells leave the bone marrow, they contain several key cytoplasmic proteins:

1. Hemoglobin is the major protein present in red blood cells and is responsible for the large O_2-carrying capacity of blood.

2. Glycolytic enzyme is needed because red cells have no mitochondria and must synthesize adenosine triphosphate (ATP) via glycolysis.

3. Carbonic anhydrase is used to catalyze the following equilibrium reaction, which is essential for CO_2 carriage in the blood (Schaller *et al*., 2008).

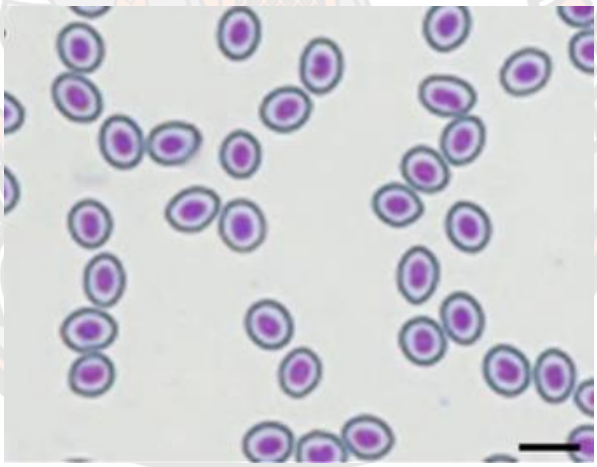

Figure 5: Tilapia red blood cells characteristics

Source: Shen *et al*., (2018)

2) Leucocyte, white blood cell

Leucocyte is derived from two Greek words: *leukos* (white) and *kytos* (hollow). Leukocytes are involved in immune defence and produce antibodies on one side and are differentiated into memory cells on the other side. White blood cells are classified into the granular leukocytes and the agranular leukocytes (mononuclear cell) (Schaller *et al*., 2008).

2.1) Agranular leukocytes (mononuclear cell)

Agranular leukocytes are characterized by the absence of granules in the cytoplasm and are manufactured in the lymph glands and spleen. There are two types of agranulocytes including monocytes which have a kidney-shaped nucleus and lymphocytes have a spherical nucleus.

Monocytes or macrophages (once they have entered tissue) are derived from two Greek words: *macro* (large) and *phagein* (eat). Monocytes are the largest of the white blood cells with an abundant blue-gray cytoplasm that lacks granules and are occasionally vacuolated (Campbell and Murru, 1990). As in mammals, after taking up residence in the tissues, monocytes differentiate into even larger macrophages. Macrophages phagocytize pathogens, old cells, and cellular debris. They also stimulate other white blood cells, including lymphocytes, to defend the body (Mader, 2006; Sobti, 2008; Schaller *et al*., 2008). In fish, melanomacrophage centres have been shown to trap and retain antigens and immune complexes, thus functioning as a primitive analog to the lymphoid germinal centre (Claver and Quaglia, 2009; Press and Evensen, 1999).

Lymphocytes represent approximately 25% of leukocytes and produce the antibodies, approximately 2000 molecules/second, and lymphocytes are of two types, B cells, and T cells. B cells descendants (plasma cells) protect us by producing antibodies that combine with antigens and thereby target pathogens for destruction. Some T cells (cytotoxic T cells) directly destroy any cell that has foreign antigens (Mader, 2006; Schaller *et al*., 2008). T-, B- and NK-lymphocytes are present in bony and cartilaginous fishes, but not in jawless fishes or invertebrates (Anderson ***et al.***, 2001). It is generally believed that jawless fishes only have innate immunity (Shintani ***et al.***, 2000). However, lymphocytes of lampreys have been shown to transcribe novel variable lymphocyte receptors, consisting of leucine-rich repeats that can generate diversity through a somatic rearrangement process (Pancer ***et al.***, 2004). In contrast to higher vertebrates, most fish hatch at the embryonic stage of life, with the innate immune system serving as their primary defence mechanism. The acquired immune system (B- and T-cells) arises later in life, generally after food consumption (Claver and Quaglia, 2009; Rombout ***et al.***, 2005).

2.2) Granular leukocytes or polymorphonuclear cell

The expression is derived from the Latin word *granula* (granule) . Granulocytes represent approximately 70% of leukocytes and contain a nucleolus and their lifetime is only a few hours**.** Three types of granulocytes exist, termed according to their staining properties**:**

Neutrophils are the most abundant of the white blood cells among granular leukocytes**.** They have a multilobed nucleus joined by nuclear threads; therefore, they are also called polymorphonuclear**.** They have granules that do not significantly take up the stain eosin, a pink to red acidic stain, or a basic stain that is blue to purple**.** Neutrophils are the first types of white blood cells to respond to an infection, and they engulf pathogens during phagocytosis**.** Neutrophils represent approximately 65 of leukocytes and are usually the first to respond to bacterial infection and are involved in smaller inflammatory processes (Mader, 2006; Schaller *et al*., 2008).

Eosinophil is described for some fish but are poorly understood**.** Granules in fish eosinophils may have bar-shaped crystalloids similar to those described in human eosinophils**.** There is a lack of knowledge regarding the full function of fish eosinophils, but they seem to function in a similar manner to mammalian mucosal mast cells (Ellis, 2001). The eosinophils are found frequently in the digestive tract and gills and have been associated with antigenic stimulation and parasitic infestations (Barnett *et al.*, 1996; Claver and Quaglia, 2009; Powell *et al.*, 1990).

Basophils are the most variable cell type in fish. They are apparently absent in certain species, such as zebrafish (*Danio rerio*) and sea bass (*Dicentrarchus labrax*), but present in others, like the sea bream (*Sparus aurata*) and carp (*Cyprinus carpio*). When present, basophils occur in very low numbers. Fish that do not have basophils also appear to lack mast cells, immunoglobulin E, and serotonin (Claver and Quaglia, 2009; Crowhurst *et al.*, 2004; Ellis, 1986; Esteban *et al.*, 2000; López-Ruiz *et al.*, 1992; Tripathi *et al.*, 2004).

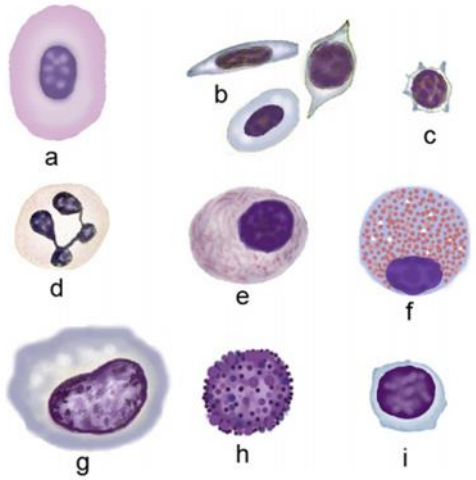

Figure 6: Blood cells of fish. Erythrocyte (a), normal (b) and activated (c) thrombocytes, neutrophil of trout (d), heterophil of shark (e), eosinophil (f), monocyte (g), basophil (h), and lymphocyte (i)

Source: Claver and Quaglia, (2009)

3) Platelets or Thrombocytes

Platelets contain no nucleus and exhibit a discoid shape with a diameter of approximately $1\text{-}3\times10^{-3}$ mm and their lifetimes are 8-10 days. Blood contains approximately $2\text{-}3\times10^{8}$ platelets/ml of blood. Blood platelets are essential in the healing process of vascular injuries by closing the site of injury via intercalation with the fibrin network during the coagulation cascade. During this process activated platelets undergo a dramatic shape change from discoid to spherical, exposing spines termed pseudopodia, which become sticky, thus annealing the injured blood vessel.

5. Growth hormone gene

Growth hormone (GH), also known as somatotropin, is a peptide hormone that is synthesized and secreted by the somatotrophs of the anterior pituitary gland (Reh and Geffner, 2010). The growth hormone gene is presented as a single copy in the genome of most vertebrates. The structure of the growth hormone gene in fish is typical of vertebrate (Kamenskaya *et al.*, 2015). Tilapias contain an extra intron compared with the mammalian GH structure. Moreover, *Tilapia nilotica* growth hormone gene was similar to the Salmonidae fish, Atlantic salmon and rainbow trout

which consists of six exons and five introns (Ber and Daniel, 1993). Growth hormone gene is more important in the regulator of metabolism, osmoregulation, reproduction and skeletal growth in Livestock (Rezaei and Akhshabi, 2011). GH is the only hormone known to produce a dose-dependent stimulation of postnatal growth. Most of the effects attributed to GH action appear to be the result of a direct effect of GH on cells in different peripheral tissues, including cartilage. In addition to the growth-stimulating effect, GH has the intrinsic properties of being able to exert both insulin-like and insulin-antagonistic effects in adipose tissue and skeletal muscle (Isaksson *et al.*, 1985). On the other hand, the indirect effects are affected primarily by the action of insulin-like growth factor-1, which is secreted primarily from hepatocytes in response to elevated GH binding to a surface receptor (Brinkman and Sharma, 2018). In accordance with its many functions, specific GH binding sites have been demonstrated in several different tissues in mammals and chicken as well as in fish, including liver, gills, kidney, muscle, brain, fat, and cartilage (Jönsson and Björnsson, 2002). In many species of fish, the growth hormone gene is represented by two non-linked functional paralogues, gh1, and gh2 (Kamenskaya *et al*., 2015). The growth hormone in fish plays a major role in the regulation of growth, development, physiological process, immune systems, reproduction function as well as the regulation of ionic and osmotic balance (Abdolahnejad *et al.*, 2015).

The study of Acosta *et al*., 2009 showed the differences of hormone receptor interaction and the structure-function relationship between fish and mammals, as well contribute to understanding the interactions of GH with its receptors in fish. Furthermore, due to the truncated tilapia growth hormone (tiGH) variant being supplied in *P. pastoris* culture supernatants showed to be a potent enhancer for growth, survival, quality of larvae and immune parameters, it can be used in aquaculture mainly for ornamental fish production. It allows having treated fish of increased growth rates and more resistant to stress conditions and infections and reducing production costs. Abdolahnejad *et al.* **(**2015**)** reported that GH mRNA could be observed in the eyed eggs and at unfertilized eggs of Siberian sturgeon. The highest amounts of GH mRNA were found at 25- and 50-days post-hatching larvae (dph), while the lowest levels were detected at 1 and 3 dph larvae stage. These study suggested that, the growth hormone mRNA functions essentially at the developmental

stages of Siberian sturgeon. Cruz and Manalad **(**2006**)** studied the insulin-like growth factor-I gene expression as a growth indicator in Nile tilapia (*Oreochromis niloticus* L) and showed that hGF-I level remained positively correlated to growth rate as affected by feeding regime, temperature and social stress and hIGF-I plays a key role in controlling growth in *O. niloticus* and indicates that IGF-I mRNA quantification could prove useful for the rapid assessment of growth rate in *O. niloticus*.

6. The innate immune system

The innate immune system is the only defense weapon in invertebrates and plays an instructive role in the acquired immune system of a higher vertebrate**.** Teleost fish serve a key role as the bridge between innate and adaptive immune responses in that they are the earliest class of vertebrates possessing the elements of both innate and adaptive immunity**.** In teleost, innate immunity occupies a more important position for the initial protection against pathogen invasion, due to the constraint on adaptive immunity in suboptimal environments **(**Gao *et al.*, 2012; Magnadóttir, 2006; Ullal *et al.*, 2008; Whyte, 2007**).** In fact, numerous immune-relevant genes for both innate and adaptive immunity, including those encoding cytokines, complements, lectins, immunoglobulins, and certain cell surface molecules, have been characterized by various fish species**.**

6.1 Fish cytokines

Cytokines are secreted proteins with growth, differentiation, and activation functions that regulate the nature of immune responses. Cytokines are involved in several steps of the immune response, from induction of the innate response to the generation of cytotoxic T cells and the production of antibodies. In higher vertebrates, the combination of cytokines that are secreted in response to an immune stimulation induces the expression of immune-related genes through multiple signalling pathways, which contributes to the initiation of the immune response. Cytokines can modulate immune responses through an autocrine or paracrine manner upon binding to their corresponding receptors (Wang *et al.*, 2011). Cytokines have overlapping and sometimes contradictory pleiotropic functions that make their classification difficult. Cytokines are produced by macrophages, lymphocytes, granulocytes, DCs, mast cells,

and epithelial cells, and can be divided into interferons (IFNs), interleukins (ILs), tumour necrosis factors (TNFs), colony stimulating factors, and chemokines (Savan and Sakai, 2006). They are secreted by activated immune-related cells upon induction by various pathogens, such as parasitic, bacterial, or viral components (Salazar-Mather and Hokeness, 2006). Fish appear to possess a repertoire of cytokines similar to those of mammals. To date, several cytokine homologues and suppressors have been cloned in fish species. Some cytokines described in fish are TNFα, IL-1β, IL-2, IL-4, IL-6, IL-8 or IFN (Reyes-Cerpa *et al.*, 2013; Zhang *et al.*, 2018).

6.1.1 Tumour necrosis factor alpha (TNF-α)

Tumour necrosis factor alpha (TNF-α) belongs to a large family of structurally related cytokines called Tumour necrosis factor superfamily TNFSF, an ancient family of structurally related cytokines whose orthologs can be traced back to protostomian invertebrates (Secombes *et al.*, 2016). TNF-α is produced and secreted by many cell types including immune cells, endothelial cells, epithelial cells, smooth muscle cells, and cardiac myocytes. TNF-α can bind to two receptors; TNF receptor 1 (TNF-R1) (55-kDa) and TNF-R2 (75-kDa) (Santee and Owen-Schaub, 1996; Schall *et al.*, 1990). Two distinct TNF-α receptors are also widely expressed on multiple cell surface: TNF-R1 assisting most of the activity of TNF-α, is a ubiquitous membrane receptor that is found in most cell types, however, TNF-R2 is primarily expressed by T cells and endothelial cells (Kim and Teh, 2004; Turner *et al.*, 2004; Valencia *et al.*, 2006). TNF-R1 can be activated by either sTNF-α or tmTNF-α, however, TNF-R2 is preferentially activated by tmTNF-α. Even though there are some overlap and cross-talk between TNF-R1 and TNF-R2, they are structurally different; therefore, TNF-α signalling through TNF-R1 and TNF-R2 can elicit distinct cellular responses depending on the cell. Binding of TNF-α to TNF-R1 can activate the transcription factor NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), regulate cytokine production, and mediate inflammation and/or apoptosis (Bouwmeester *et al.*, 2004; Howerton and Tarzami, 2017; Hsu *et al.*, 1996; Jiang *et al.*, 1999; Saltzman *et al.*, 1998). TNF-α (tumour necrosis factor alpha) is a pro-inflammatory cytokine that plays an important role in diverse host responses, including cell proliferation, differentiation, necrosis, apoptosis, and the induction of other cytokines. TNF-α can

induce either NF-kB mediated survival or apoptosis, depending on the cellular context (Rahman and McFadden, 2006). It is one of the early immune genes expressed at an early stage of infection in fish (Zou and Secombes, 2016). Studies have also shown that the main target of fish TNF-α are endothelial cells, suggesting that TNF-α is mainly involved in the recruitment of leukocytes to the inflammatory foci rather than in their activation (Roca *et al.*, 2008). TNF-α is currently one of the most well-studied fish cytokines, has been identified, cloned, and characterized in several bony fish, including Japanese flounder (Hirono *et al.*, 2000), rainbow trout, *Oncorhynchus mykiss* (Laing *et al.*, 2001), gilthead seabream (García-Castillo *et al.*, 2002), common carp, *Cyprinus carpio* (Saeij *et al.*, 2003), Nile tilapia, *Oreochromis niloticus* (Praveen *et al.*, 2006), turbot, *Psetta maxima* (Ordás *et al.*, 2007), channel catfish, *Ictalurus punctatus* (Zou *et al.*, 2003), and goldfish (Grayfer *et al.*, 2008). In addition to its roles in acute infection, TNF-α can be associated with oocyte maturation (Crespo *et al.*, 2010) and liver development in fish (Qi *et al.*, 2010), as well as with pathogenesis of several chronic fish diseases (Ronza *et al.*, 2015; Xu *et al.*, 2012). TNF-α also represents one of the major cytokines secreted by adipose tissue (Liu *et al*., 2015).

6.1.2 Interleukins

The interleukins are included to cytokines. They are small proteins involved in cell signalling, mostly in the immune systems (Kishimoto *et al.*, 1994; Paul and Seder, 1994). They are modulators of immune responses; besides, they possess the ability to regulate differentiation of lymphocytes and hemopoietic stem cells, cell proliferation and motility (Sherbet, 2011). Interleukin 1 (IL-1) is a polypeptide with diverse roles in immunity and inflammation having both protective and pro-inflammatory effects. IL-1 is synthesized by many cell types but particularly monocytes and macrophage which have been activated. Cloning of the genes for IL-1 revealed that there are two related gene products corresponding to these pIs, known as IL-1α and IL-1β (Hamblin, 1993). IL-1 α and IL-1 β can induce mRNA expression of hundreds of genes, including themselves (a positive-feedback loop), and their gene regulatory actions are conducted via a conserved signalling pathway (Weber *et al.*, 2010). Signal propagation mainly depends on mitogen-activated protein kinases

(MAPKs), MAPK kinases (MKK/MAP2Ks), MKK kinases (MKKK/MAP3K/MEKKs) and the downstream proteins of MAPKs, finally leading to activation of transcription factors that regulate the expression of host defence proteins (Ozbabacan *et al.*, 2014) (Figure 7).

IL-1β is the best characterized and most studied cytokine amongst the 11 mammalian IL-1 family members, one of the earliest expressed pro-inflammatory cytokines with overlapping functions with TNFα, enabling organism to respond promptly to infection (Zou and Secombes, 2016). IL-1β is produced by a variety of cells, but mainly blood monocytes and tissue macrophages. It affects almost every cell type playing a central role in the initiation of systemic and local responses to infection or injury by activating macrophages, T and B lymphocytes and NK cells (Dinarello, 2011; Netea *et al.*, 2010). It has been characterized in various fish species including: rainbow trout, *Oncorhynchus mykiss* (Zou *et al.*, 1999), common carp, *Cyprinus carpio* (Fujiki *et al.*, 2000), sea bass, *Dicentrarchus labrax* (Scapigliati *et al.*, 2001), gilthead seabream, *Sparus aurata* (Pelegrín *et al.*, 2001), small spotted catshark, *Scyliorhinus canicula* (Bird *et al.*, 2002), channel catfish, *Ictalurus punctatus* (Wang *et al.*, 2006), Nile tilapia, *Oreochromis niloticus* (Lee *et al.*, 2006), haddock, *Melanogrammus aeglefinus* (Corripio-Miyar *et al.*, 2007), orange-spotted grouper, *Epinephelus coioides* (Lu *et al.*, 2008), southern bluefin tuna, *Thunnus maccoyii* (Polinski *et al.*, 2014).

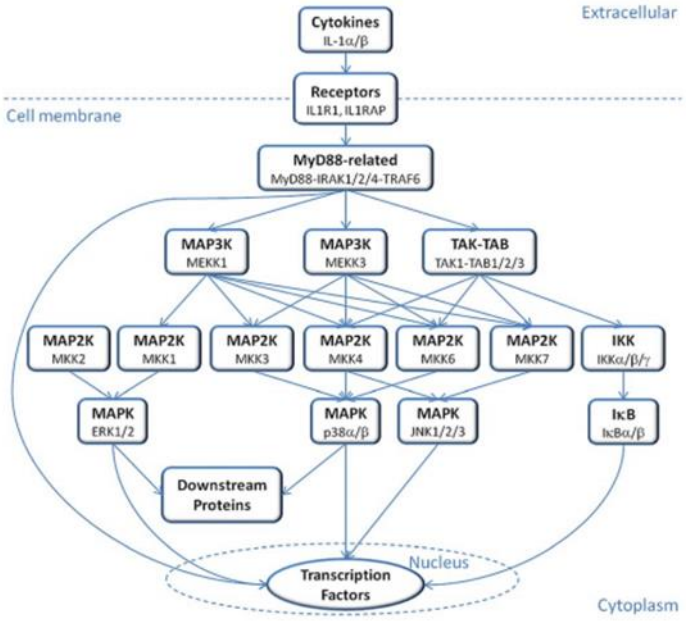

Figure 7: IL-1 signalling pathway diagram. In this simplified diagram of the IL-1 signalling pathway, the signal initiates by the recognition of cytokines by IL-1 receptors and propagates via multiple sub-pathways involving family homologs or alternate pathways to activate transcription factors downstream

Source: Ozbabacan *et al*., (2014)

6.1.3 Transforming growth factor-β (TGF-β)

Transforming growth factor-β, a pleiotropic polypeptide, regulates multiple biological processes, including embryonic development, adult stem cell differentiation, immune regulation, wound healing, and inflammation (Kastin, 2013). The family of TGF-β contains the three closely related isoforms, namely TGF-β1, TGF-β2 and TGF-β3, which are synthesized as large latent, inactive complexes where proper folding, interaction with critical interacting partners such as the latent TGF-β binding proteins or fibronectin and secretion/release from storage sites is controlled by disulphide bonds and many different activation factors (Dennler *et al.*, 2002). TGF-β immunoregulatory properties are primarily suppressive. Immune functions down-regulated by TGF-β include the following: major histocompatibility complex and Fc receptor expression, some cytokine production, thymocyte proliferation, T- and B-cell proliferation, IgG and IgM production, IL-2 receptor expression, cytotoxic

T-cell generation and function, lymphokine-activated killer and natural killer cell activation and function, macrophage activation, macrophage respiratory burst activity, neutrophil adhesion to endothelium, and haematopoiesis. Three isoforms of TGF-β (-β1, -β2, and -β3) are expressed by mammals (Harms *et al.*, 2000a).

Although cytokines are less well characterized in fish than in mammals (Secombes *et al.*, 1996), the ability to detect and measure mRNA of a teleost TGF-β that is closely related to the TGF-β1 isoform of mammals, was recently developed (Harms *et al.*, 2000b). A quantitative polymerase chain reaction (PCR) assay for TGF-β mRNA has been used to demonstrate an inverse relationship between macrophage bactericidal activity and TGF-β transcription in triamcinolone-treated hybrid striped bass (*Morone saxatilis* x *M. chrysops*) (Harms *et al.*, 2000c) and rainbow trout (*Oncorhynchus mykiss*) (Daniels and Secombes, 1999; Hardie *et al.*, 1998) grouping with Xenopus TGF-β5, chicken TGF-β4, and mammalian TGF-β1, were recently reported. Homology with TGF-β1 suggests that the fish TGF-β isolates may also be important in regulating immune responses in these species. Evidence for the conserved nature of TGF-β activity in fish is provided by both biologic cross-reactivity and antigenic cross-reactivity (Harms *et al*., 2000a).

TGF-β was first isolated and characterized from platelets (Assoian *et al.*, 1983), but most cell types can produce it (Sporn and Roberts, 1990): cells from the immune system, including lymphocytes (Brown *et al.*, 1987), macrophages (Wahl *et al.*, 1991) and mast cells (Pennington *et al.*, 1991), but also structural cells in various tissues, including rat cardiac fibroblasts (Lee *et al.*, 1995), human umbilical vein endothelial cells (Hannan *et al.*, 1988) and corneal epithelial cells (Wilson *et al.*, 1992).

As shown in vivo by immunohistochemistry and in situ hybridization, TGF-β is also produced in the airways by various cell types (Fig. 8): fibroblasts and epithelial (Magnan *et al.*, 1994), endothelial (Coker *et al.*, 1996) and smooth muscle cells (Magnan *et al*., 1994), (Aubert *et al.*, 1994). It is also expressed by infiltrated inflammatory cells, such as eosinophils (Ohno *et al.*, 1996), lymphocytes (Magnan *et al*., 1997) and mast cells (Gordon and Galli, 1994). Its primary location in normal human lungs appears to be the bronchial epithelium (Magnan *et al*., 1997) .

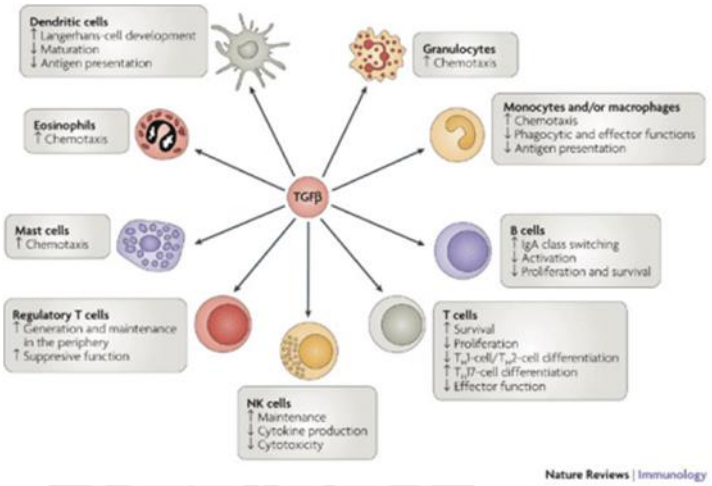

Figure 8: Cellular sources of and targets for transforming growth factor-β (TGF-β). TGF-β may be expressed and released by numerous cell types in the airways: structural and inflammatory cells infiltrated within the bronchial mucosa

Source: Duvernelle *et al.* **(**2003**)**

CHAPTER 3

MATERIALS AND METHODS

1. Materials

1.1 Specimen

Nile tilapia

1.2 Chemicals

1.2.1 Chemicals used in diet preparation (Appendix).

1.2.2 Chemicals used in chemical composition analysis of Nile tilapia and experimental diets (Appendix).

1.2.3 Chemicals used in chromic oxide analysis in fish faces and experimental diets (Appendix).

1.2.4 Chemicals used in blood composition analysis of experimental fish (Appendix).

1.2.5 Chemicals used in fish anesthetizing (Clove oil).

1.2.6 Chemicals used in gene expression (Appendix).

2. Instruments

2.1 Equipment used in fish trial

2.1.1 200 L-plastic tank.

2.1.2 Aerator equipment including rubber tube, aquarium air stone, an aquarium air pump.

2.1.3 Water discharge equipment in glass aquarium including rubber tube and submersible pump.

2.1.4 Moving fish equipment including landing net and a plastic bowl.

2.2 Equipment used in experimental diet

2.2.1 Hobart A-200T feed mixer

2.2.2 Feed weighing scales including beaker, Sartorius basic analytical balance scale, precision balance scale, and cylinder

2.2.3 Stainless steel food pans and hot air oven

2.2.4 Freezer

2.3 Equipment used in chemical composition analysis of diets

2.3.1 Protein analysis equipment including Gerhardt® Kjeldatherm GB8S distillation apparatus, Gerhardt® Vapodest 20 distillation apparatus, digestion tube, cylinder, beaker, burette, and Erlenmeyer flask.

2.3.2 Lipid analysis equipment including Soxtec System HT6 lipid extractor, filter papers, glass extract beaker, drying oven, desiccator, and precision balance scale.

2.3.3 Moisture analysis equipment including crucible, hot air oven, desiccator, and the precision balance scale.

2.3.4 Ash analysis equipment including precision balance scale, crucible, desiccator, and muffle furnace.

2.3.5 Fiber analysis equipment including fiber extractor, glass crucible, drying oven, desiccator, and the precision balance scale.

2.4 Equipment used in chromic oxide (Cr_2O_3) analysis in diets and fish faeces

2.4.1 Faecal collecting equipment including landing net, forceps, rubber tube, plastic storage box, and straining cloth.

2.4.2 Chromic oxide analysis equipment including drying oven, desiccator, mortar and pestle, precision balance scale, heating pad (0-300 °C), Erlenmeyer flask, 25 ml volumetric flask, and wash bottle.

2.4.3 UV-vis spectrophotometer.

2.5 Equipment for monitoring fish growth

Including Sartorius basic analytical balance scale, plastic bowl, and landing net.

2.6 Equipment used in blood composition analysis

2.6.1 Blood collection instruments such as 25G needle, 1 ml syringe and 1.5 ml microtube.

2.6.2 Serum separator including Beckman Avanti^{TM} 30 centrifuge

2.6.3 Shimadzu UV-1201 Spectrophotometer

2.6.4 Blood cells counting instruments including Haemocytometer, microscope.

2.7 Equipment used in histopathology

2.7.1 Surgery set

2.7.2 Autotechnicon Mono MOD. 2A automatic tissue processor

2.7.3 Microtome (Jung AG Heidelberg), (Leica, Disposable Microtome Blades), a warm bath, and microscope slides.

2.7.4 Equipment for permanent slides preparation including an incubator, stain set, and coverslip (40 × 60 ml).

2.7.5 Embedding centre.

2.7.6 Hot plate.

2.7.7 Olympus C-35 AD microscope and a cooled CCD camera (Olympus DP 71).

2.8 Equipment used in gene expression

2.8.1 PCR machine.

2.8.2 Real-time PCR machine.

3. Experimental methods

3.1 Experimental equipment preparation

Thirty-two plastic tanks with a volume of 200 L were prepared and cleaned. Together with aerator equipment were set up comprising rubber tube, aquarium air stone, and an aquarium air pump. After instrument preparing, 180 L of dechlorinated tap water were filled to the plastic tanks. Subsequently, each plastic tank was closed with the opaque colour of the plastic sheet with the aim of protecting the disturbance from an external environment. Furthermore, the experimental plastic tanks were cleaned and discharged the dechlorinated tap water every day about 13.00 pm.

3.2 Preparation of fish trial

Nile tilapia fingerlings (average weight approximately 0.5 g) were purchased from a private farm in Phitsanulok province. After arrival at the experimental site, fish were kept under quarantine condition in the two 500L fiber tanks and fed up to apparent satiation with commercial diet twice daily at 9:00 am and 16:00 pm for one month until fish gain weight up to 11 g per fish. Apparent healthy and similar size fish were randomly distributed into 32 200L-fiber tanks at a density of 15 fish per fiber tank. At that time, fish were trained to adapt to the condition of experimental fiber tank. After acclimatization to the experimental fiber tanks and diets, fish were fed up to apparent satiation with all the experimental diets twice daily for 10 weeks. During trial, fish were weighted and collected the body weight by anesthetizing with clove oil.

3.3 Experimental diet preparation

All experimental diets were formulated to be isonitrogenous (29-30% protein) and isolipidic (7-16% lipid) and their chemical composition are shown in Table 10. Two fishmeal-based diets including control diet 1 (fish oil-based diet) and control diet 2 (soybean oil-based diet) and other six experimental diets were formulated to replace fishmeal protein with different protein levels of sacha inchi seed from 8%, 15%, 25%, 50%, 75%, and 100% which named as SIS8 diet, SIS15 diet, SIS25 diet, SIS50 diet, SIS75 diet, and SIS100 diet, respectively. The ingredients of the experimental diet including fishmeal, soybean meal, sacha inchi seed, corn gluten, rice flour, fish oil, soya oil, vitamins, and minerals were weighed and calculated for feed formulation. All ingredients of each diet were ground through a 30 mesh, completely mixed by the feed mixer, and then pelleted into 3 mm diameters.

3.4 Procedures of experimental diet preparation

3.4.1 In this study, AOAC (1990) method was used to analyse the nutritional values of the feed ingredients. Experimental diets were formulated to be an equal level of protein, and energy content, except for fat content (7-15%). Nutritional composition of ingredients is represented in Table 10.

3.4.2 All ingredients were weighed by using a 30-mesh filter. Then, all raw ingredients were weighed at the desired rate. Moreover, other ingredients such as fish oil, soybean oil, methionine, lysine, vitamin, and mineral were roughly mixed into the plastic bags of each experiment diet.

3.4.3 Mixed ingredients above except fish oil and soybean oil were mixed by 20 kg kidchen mixer for 15 minutes. During the first five minutes, fish oil was added, thereafter drinking water was added 350 ml (35% v/w). Then all mixed ingredients were mixed for 5 min to become homogeneity (Table 11).

3.4.4 The homogenous experimental diets were brought to the aqua pellet feed machine and the diets should have similar pellet size.

3.4.5 The experimental diets were incubated at 60 °C until completely dry.

3.4.6 An obtained dried feed was packed in plastic bags and stored at 4 °C until being used for experimentation.

3.4.7 The experimental diets were subjected to analyze the nutritive values including moisture, crude protein, fat, ash, and fibre content analysis following AOAC, (1990) method. After that, the carbohydrate content (nitrogen-free extract, NFE) were calculated following by the formula below.

Nitrogen free extract, NFE = 100 – (moisture + crude protein + fat + ash + fibre).

Table 10: Nutritional composition of ingredients

Ingredients	Moisture	Protein	Fat	Ash	fibre
FM	5.84±0.21	61.32±0.11	7.04±0.10	19.84±0.05	2.85±0.87
SIS	2.01±0.08	28.74±0.56	44.05±0.53	2.88±0.06	23.27±1.17
SBM	10.63±0.14	42.51±0.24	0.53±0.07	6.08±0.06	7.41±0.44
CG	6.91±0.02	20.88±0.00	4.12±0.00	6.11±0.16	10.41±0.19
RF	10.39±0.19	6.03±0.02	0.20±0.00	0.19±0.04	0.13±0.12

* FM (Fishmeal), SIS (Sacha inchi seed), SBM (Soybean meal), CG (Corn gluten), and RF (Rice Flour).

Table 11: Chemical composition of eight experimental diets

Ingredients	Experimental Diets							
	Control 1	Control2	SIS8	SIS15	SIS25	SIS50	SIS75	SIS100
FM	17	17	15.65	14.45	12.75	8.5	4.25	0
SSM	0	0	2.9	5.4	9.1	18.1	27.2	36.3
SBM	37	37	37	37	37	37	37	37
CG	19.35	19.35	19.35	19.35	19.35	19.35	19.35	19.35
RF	17.35	17.35	17	16.7	16.2	13.55	8.7	3.85
FO	5.8	0	0	0	0	0	0	0
SBO	0	5.8	4.6	3.6	2.1	0	0	0
Vit[1]	1	1	1	1	1	1	1	1
Min[2]	1	1	1	1	1	1	1	1
Meth	1	1	1	1	1	1	1	1
Lysine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100	100	100	100

* Abbreviations: FM (Fishmeal), SIS (Sacha inchi seed), SBM (Soybean meal), CG (Corn gluten), RF (Rice Flour), FO (Fish oil), SBO (Soybean oil), Vit (Vitamin premix), Min (Mineral premix), and Meth (Methionine).

[1] Vitamin premix (mg or IU/kg diet): A, 5,000 IU; D3, 1,000 IU; E, 5,000 mg; K, 2,000; B1, 2,500 mg; B2, 1,000 mg; B6, 1,000 mg; B12, 10 mg; inositol, 1,000 mg; pantothenic acid, 3,000 mg; niacin acid, 3,000 mg; C, 10,000 mg; folic acid, 300 mg; biotin, 10 mg.

[2] Mineral premix (g/kg diet): calcium phosphate, 80; calcium lactate, 100; ferrous sulphate, 1.24; potassium chloride, 0.23; potassium iodine, 0.23; copper sulphate, 1.2; manganese oxide, 1.2; cobalt carbonate, 0.2; zinc oxide, 1.6, magnesium chloride, 2.16; sodium selenite, 0.10.

Table 12: Chemical composition of experimental diets.

Diets	Chemical composition of experimental diets.				
	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Fiber (%)
Control1	4.45±0.08	30.87±0.63	7.01±0.19	7.73±0.08	6.05±0.14
Control2	3.70±0.06	29.51±0.37	6.98±0.21	7.77±0.10	6.17±0.10
SIS8	3.91±0.10	29.97±0.73	7.00±0.36	7.55±0.08	6.41±0.24
SIS15	3.93±0.25	29.43±0.76	7.69±0.03	7.53±0.18	6.24±0.14
SIS25	4.03±0.11	30.77±0.74	7.73±0.02	7.15±0.03	6.77±0.37
SIS50	3.76±0.23	29.65±0.47	8.01±0.16	6.67±0.04	8.08±0.24
SIS75	4.44±0.15	30.73±0.73	10.43±0.01	6.18±0.03	8.95±0.36
SIS100	4.52±0.20	29.41±0.10	15.66±0.00	5.55±0.05	11.52±0.54

3.5 Experimental Design

In this study, the effects of SIS were used to replace fishmeal on growth performance, feed efficiency, and chemical composition of Nile tilapia with 6 different protein levels of SIS (8%, 15%, 25%, 50%, 75%, and 100%) into the diets. Completely randomized design (CRD) were used in this experiment. The experiment was divided into eight treatments which each treatment has 4 replicates. Then, AOAC (1990) method was used to analyze the nutritional component of specimens including moisture, crude protein, fat, ash, and fibre content before starting the experiment. Moreover, fish (11 g mean weight) were randomly selected and transferred to 32 fibre tanks at a density of 15 fish per fibre tank. During experiment, fish were fed up to apparent satiation with the experimental diets twice daily at 9:00 am and 16:00 pm. Furthermore, 100% of the water in each tank was cleaned and replenished for 75% every day.

3.6 Data collection

3.6.1 Monitoring behaviours of fish

During the trial, fish behaviours were monitored in all treatments including swimming, feed acceptability, and external appearances such as the colour of fish, fin and bone corrosion, and the wound on various organs.

3.6.2 Growth studies

Fish were weighed in each replicate in every 2 weeks interval in order to determine weight gain and the remaining fish were counted throughout the experiment. Afterward, all data were analysed growth rate, survival rate, weight gain, specific growth rate (SGR), feed conversion ratio (Dupree and Sneed, 1966), and the rate of fish intake follow the method of Yone and Fujii **(**1975**)** according to the equation below.

$$\text{Survival rate (\%)} = \frac{\text{Final number of surviving fish}}{\text{Initial number of fish}} \times 100$$

$$\text{Weight gain (\%)} = \frac{\text{Final body weight of fish} - \text{initial body weight of fish}}{\text{initial body weight of fish}} \times 100$$

Specific growth rate (SGR) was calculated using the following formulae (Ridha, 2006):

$$\text{SGR} = \frac{\ln(\text{final body weight of fish}) - \ln(\text{initial body weight of fish})]}{\text{number of days}} \times 100$$

Feed conversion rate (FCR) was calculated using the amount of feed intake of the fish in gram (g) and body weight gained in gram (g) during the experiment. Feed conversion ratio and gross fish yield were calculated using the following formulae (Ridha, 2006):

$$\text{Feed conversion rate (FCR)} = \frac{\text{Feed intake}(\text{g})}{\text{Weight gain}(\text{g})}$$

$$\text{The rate of feed intake} = \frac{F \times 100}{\frac{w_0 + w_1}{2} \times \frac{N_0 + N_1}{2} \times t}$$

F = dried feed weight of fish intake (g)

W_0 = initial weight of fish (g)

W_1 = final weight of fish (g)

N_0 = initiate the number of fish

N_1 = final number of fish

t = the period that fish received diets

3.6.3 Chemical composition determination of fish

Before starting the experiment, twenty fish samples were randomly collected to analyse the moisture content and taken to incubate at 100 °C until dry. The specimens were kept into the desiccator until the specimens cooling down and then, specimens were weighed to determine the moisture content. Thereafter, the specimens were ground thoroughly to examine the chemical compositions of fish including crude protein, fat, and ash in the method of AOAC (1990). At the end of the experiment, two fishes were randomly sampled from each glass aquaria to analyse moisture, protein, and fat content. Protein content was calculated the protein efficiency ratio (PER) and PPV.

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain} \times 100}{\text{Protein intake}}$$

$$\text{Protein productive value (PPV)} = \frac{\text{Protein gain}}{\text{Protein intake}}$$

3.6.4 Apparent digestibility analysis

The apparent digestibility was analyzed by using chromic oxide (Cr_2O_3) as the indicator and added 0.5% into the experimental diets. Fish were fed from week 9 to 10 of the feeding trial. Then, feces were collected on week 10 following Boonyaratpalin and Phromkunthong (2000) method. The feces (approximately 100 g) were incubated at 60 °C and ground into homogenous. After that, it was brought to analyse chemical compositions (such as protein, fat, and ash) follow method of AOAC (1990), chromic oxide (Cr_2O_3) in diets and feces follow by the method of

Furukawa **(**1966**)** and calculate the apparent digestibility following a method of De Silva and Anderson **(**1994**)** as the equation shown below.

ADC (%) = 100× [1 - (dietary Cr_2O_3 x faeces Cr_2O_3) × (faeces nutrients x dietary nutrient)].

3.6.5 Blood collection and haematological examination

At the termination of the feeding trial, three fish of each treatment (12 fish per dietary treatment) were taken for sampling blood parameters. Fish were anesthetized in 50 ppm of clove oil prior to dissection or blood sampling. Blood samples were collected from caudal peduncle by using 1 ml syringes and 25G needle that has been treated with anti-coagulant such as heparin to prevent clotting into small sampling bottles containing Ethylene diamine tetra-acetic acid (EDTA). After the collection, the blood samples were taken to the laboratory of the Department of Agriculture, Natural Resources and Environment, Naresuan University where the haematological analysis was carried out.

1) Packed cell volume (PCV)

Blood samples were collected into the capillary tube which was sealed with critaseal at one end. Then, the sampled tubes were centrifuged for 5 minutes at 12,000 rpm using Hawksley microhematocrit centrifuge. Microhematocrit reader were used to read the haematocrit value. A mean of two readings was recorded as a percentage for the fish haematocrit (Kefas *et al.*, 2015).

2) Determination of Red blood cells counts

A standard hemocytometer were used in the counting of the red blood cells in relation to the method of Blaxhall and Daisley **(**1973**)**.

$$\text{RBC } (10^6/\text{ml}) = \frac{(C \times D \times 100 \text{ x } 4000)}{(S \text{ x } 80)}$$

Where, C = Number of cells counted

D = Diluting factors

S = Number of 1 mm square counted

3) Determination of White blood cells counts

White blood cells were determined using the method described by Blaxhall and Daisley **(**1973**)**.

$$\text{WBC } (10^3/\text{ml}) = \frac{(\text{C x D x 100 x 10})}{(\text{S x 4})}$$

Where C = Number of cells counted

D = diluting factor

S = Number of 1mm square counted

4) Determination of haemoglobin

The hemoglobin concentration was determined with cyanmethemoglobin (Dacie and Lewis, 1977).

5) Determination of mean corpuscular hemoglobin (MCH)

The mean corpuscular hemoglobin (MCH) was calculated using the formula described by Dacie and Lewis (1977).

$$\text{MCH (pg)} = \frac{\text{Packed cell volume /dL x 10}}{\text{RBC/uL}\,(\text{in 106})}$$

6) Determination of mean corpuscular volume (MVC)

The mean corpuscular volume (MCV) was determined as described by Dacie and Lewis (1977).

MCV (dL) = Packed cell volume x 10 / red blood cells

7) Determination of mean corpuscular hemoglobin concentration (MCHC)

The mean corpuscular hemoglobin concentration (MCHC) was calculated using the formula described by Dacie and Lewis (1977).

$$\text{MCHC (g/dL)} = \frac{\text{Hb/dL} \times 100}{\text{PCV}(\%)}$$

3.6.6 Histological changes in fish

At the termination of the feeding period, tissues (liver, and intestine) of two fish of each replicate were selected for this study.

3.6.6.1 Fixation

The tissues were excised out and rinsed with deionized water. Preservation was carried out using 10% neutral buffered formalin as the compound fixative for histological processing.

3.6.6.2 Tissue processing

An automatic tissue processor was employed. The selected tissues were dehydrated, cleared, impregnated, and embedded in paraffin wax following standard procedures. Dehydration from aqueous fixatives such as formalin is usually initiated in 70% alcohol before progressing through 90-95% to absolute alcohol before proceeding to the clearing stage. The clearing was carried out to achieve de-alcoholization. The tissues were passed through two changes of chloroform for an hour in each change. During impregnation, the clearing agent was eliminated from the tissues while molten paraffin wax replaced it. This was achieved by passing of the cleared tissue through changes of paraffin wax molten at 54 ^{o}C for an hour in each case. The final processing stage was the embedding of tissues in paraffin wax. This was necessary to hold the tissue in position and ensure that tissues were not crumbled during sectioning. The tissues so processed were rendered stiff enough for sectioning.

3.6.6.3 Tissue sectioning

The tissue blocks were sectioned using a microtome. The thickness of the sections was set at 15 μm. The microtome was set to produce a cutting rhythm that formed a ribbon of about 1.5 cm long. The sections were spread out in a water bath at 45 ^{o}C. The sections were successfully attached to the slide using bovine albumen adhesive. Prepared slides were incubated at 50 ^{o}C to dry and fix the sections. The slides were ready for staining after three hours.

3.6.6.4 Staining of sections

The sectioned tissues were stained with Erhlich's Haematoxylin and Eosin (H and E) stains. The stained slides were then examined for histopathological lesions (Udotong, 2015).

3.6.7 Gene expressions of fish

3.6.7.1 Total RNA extraction

At the end of the experiment, liver tissue samples were used to extract the total RNA. The tissue samples were subjected to frozen in liquid N_2 immediately after removing from fish to prevent RNA from degradation. Approximately 50-100 mg of each tissue samples were homogenized in 1 ml of Qiazol lysis reagent and incubated at room temperature for 5 minutes. The volume of the tissue should not exceed 10% of the Qiazol lysis reagent. A volume of 200 μl of chloroform was added to each sample, covered the sample tightly, shook vigorously for 15 s, allowed to stand at room temperature for 2-3 minutes and centrifuged the resulting mixture at 12,000 x g for 15 minutes at 4 °C. The colourless upper aqueous phase containing RNA was transferred to a fresh tube and precipitated by mixing with 500 μl of isopropyl alcohol. The mixture sample was incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 15 minutes at 4 °C. The RNA precipitate will form a pellet on the slide and bottom of the tube. Total RNAs were isolated using a RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity and purity of RNA were determined using the absorbance at 260 nm and 280 nm electrophoresed on 1% formadehyde-agarose gel.

3.6.7.2 cDNA synthesis

First-strand cDNA was synthesized using a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen, Carslbad, CA, USA). The reaction mixture (20 µL) contained approximately 1–5 µg of the total RNA; 10 pmol of oligo-d(T) primer; 1 µL of dNTP mixture (10 mM); 10.8 µL of distilled water; 4 µL of 5× First-strand buffer; 1 µL of 0.1 M dithiothreitol (DTT); and 1 µL of SuperScript III RT (200 units/µL). Samples of cDNA were kept at 37 °C for 20 min with 1 µL of RNase H (Invitrogen) to remove remaining RNAs and subsequently stored at -20 °C until further analysis.

3.6.7.3 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to examine the expression of genes in liver tissue of test fish. The expression of target genes was normalized using β-actin as a housekeeping gene. The primers sequence used in this study is presented in Table 13. Duplicate qRT-PCR amplification was carried out using SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. The conditions for quantitative PCR were as follows: an initial denaturation step of 1 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The 40 cycles were then followed by the default dissociation step and one cycle of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s, as described by Zhao *et al.* (2015). The β-actin was used as an internal control for sample normalization of the target primers. The specificity of real-time PCR was confirmed by agarose gel electrophoresis for all samples. The relative gene expression model was applied to evaluate the “-fold” changes in mRNA expression between fish in different treatments using the ΔΔCT method (Livak and Schmittgen, 2001).

Table 13: Nucleotide sequences of primers

Genes	Primer sequences (5'→3')	Product size	References
TNF-α	F-GCTGGAGGCCAATAAAATCA	339 bp	Pirarat *et al.* (2011)
	R- CCTTCGTCAGTCTCCAGCTC		
IL-1β	F-AAGATGAATTGTGGAGCTGTGTT	175 bp	FF280564.1
	R-AAAAGCATCGACAGTATGTGAAAT		
TGF-β	F- TGCGGCACCCAATCACACAAC	105 bp	Harms *et al.* (2003)
	R- GTTAGCATAGTAACCCGTTGGC		
β-actin	F-ACAGGATGCAGAAGGAGATCACAG	155 bp	Thummabancha *et al.*
	R-GTACTCCTGCTTGCTGATCCACAT		(2016)

3.6.8 Data analysis

One-way ANOVA analysis was used to test for significant differences. Duncan's multiple range test was used to identify which means values were significantly different from each other. Differences were considered significant at $p<0.05$. All data will be statistically analysed using the SPSS and Excel 2013.

CHAPTER 4

RESULTS

4.1 Behaviors of Nile tilapia acquired experimental diets

After 10 weeks of feeding, Nile tilapia did not have abnormalities on swimming habit, feed acceptability, appearances, the colour of fish, fin and bone corrosion, and the wound on various organs under experimental diets supplementation.

4.2 Growth performance of Nile tilapia

4.2.1 Water quality

During feeding trial, water quality parameters such as dissolved oxygen (DO), pH and water temperature are summarized in Table 14. According to the measurement of DO values by using DO meter, the result revealed that DO values ranged from 6.00±0.59 mg. L^{-1} to 6.90±0.12 mg. L^{-1}. The highest DO value was observed in treatment 8 with the value of 6.90±0.12 mg. L^{-1}. It displayed no significant differences from other treatments comprising treatment 1, treatment 2, treatment 4, treatment 5, treatment 6, and treatment 7 with the values of 6.15±0.70, 6.64±0.17, 6.79±0.30, 6.50±0.57, 6.39±0.96, and 6.79±0.33 mg. L^{-1} respectively, but showed significant difference from Treatment 3 (6.00±0.59 mg. L^{-1}).

pH values were measured ranging from 6.67±0.14 to 6.97±0.09. The highest pH value was observed in treatment 7 which showed no significant differences from treatment 2 (6.82±0.04), treatment 4 (6.85±0.14), treatment 5 (6.77±0.13), treatment 6 (6.86±0.16), and treatment 8 (6.91±0.17) with exception to treatment 1 (6.67±0.14), and treatment 3 (6.73±0.10). The lowest pH value was found in treatment 1 (6.67±0.14).

The measurement of water temperature was performed throughout 10-weeks trial of Nile tilapia, the result showed that water temperature values ranged from 23.05±0.24 ˚C to 23.38±0.51 ˚C. There were no significant different of temperature values among all treatments.

Table 14: Water quality parameters of all treatments throughout 10 weeks trial.

Treatments	DO (mg. L^{-1})	pH	Temperature (°C)
1	6.15 ± 0.70^{ab}	6.67 ± 0.14^{c}	23.13±0.46
2	6.64 ± 0.17^{ab}	6.82 ± 0.04^{abc}	23.20±0.36
3	6.00 ± 0.59^{b}	6.73 ± 0.10^{bc}	23.28±0.43
4	6.79 ± 0.30^{ab}	6.85 ± 0.14^{abc}	23.33±0.33
5	6.50 ± 0.57^{ab}	6.77 ± 0.13^{abc}	23.05±0.24
6	6.39 ± 0.96^{ab}	6.86 ± 0.16^{abc}	23.25±0.39
7	6.79 ± 0.33^{ab}	6.97 ± 0.09^{a}	23.28±0.35
8	6.90 ± 0.12^{a}	6.91 ± 0.17^{ab}	23.38±0.51

Note: Data presented as means of four replicate samples. Values followed by different letters in each row are significantly different ($p < 0.05$).

4.2.2 Average weight of fish

The average weight of Nile tilapia under eight different experimental diets in different protein levels of SIS is shown in Table 15.

At the beginning of experiment, average initial weight of Nile tilapia in each treatment was recorded from 11.09±0.01 to 11.11±0.02 g/fish. The average final weights of Nile tilapia were significant increased ostensibly on week 2 ($p < 0.05$). Nile tilapias fed control diet 1 had the highest average final weight (17.14±0.78 g/fish) which showed no significant differences from fish fed other five experimental diets such as fish fed with control diet 2, SIS8 diet, SIS15 diet, SIS25 diet, and SIS75 diet at value of 16.36±0.85 g/fish, 16.36±0.80 g/fish, 16.84±0.57 g/fish, 16.51±0.89 g/fish, and 16.07±0.45 g/fish, respectively. However, Nile tilapia fed with SIS50 diet and SIS100 showed significant lower average final weight compared to control diet fish group. While, the lowest average weight of fish was observed in SIS100 diet (14.41±0.54 g/fish).

After 10-week trial, the result of average final weight Nile tilapia fed all experimental diets exhibited significant differences ($p < 0.05$). The average weight was revealed the values from 31.17±2.06 to 54.47±3.25 g/fish. Nile tilapia fed with SIS8 diet showed the highest average weight (54.47±3.25 g/fish), followed by SIS25 diet (53.66±2.67 g/fish) which displayed no significant differences from fish fed with

control diet 1 (51.61±2.57 g/fish), control diet 2 (51.90±3.23 g/fish) and SIS15 diet (51.38±1.10 g/fish), yet it revealed significant decreased from fish fed with SIS50 (44.39±2.54 g/fish), SIS75 (40.30±4.13 g/fish), and SIS100 diet (31.17±2.06 g/fish), explicitly. However, Nile tilapia fed SIS100 diet showed the significant lowest in final average weight compared to fish fed other diets.

Table 15: Average body weight of Nile tilapia fed with 8 different experimental diets during 10-weeks (g/fish).

Diets	Average body weight of Nile tilapia fed diet containing different levels of SIS					
	0	2	4	6	8	10
Control 1	11.09±0.02	17.14±0.78^{a}	24.73±1.6^{a}	32.62±3.13^{ab}	41.10±2.16^{a}	51.61±2.57^{a}
Control 2	11.10±0.02	16.36±0.85^{ab}	23.84±1.46^{ab}	31.98±1.89^{ab}	39.96±2.90^{ab}	51.90±3.23^{a}
SIS8	11.11±0.01	16.36±0.80^{ab}	24.44±1.26^{ab}	33.67±1.95^{a}	42.04±1.72^{a}	54.47±3.25^{a}
SIS15	11.10±0.02	16.84±0.57^{ab}	23.85±1.14^{ab}	32.61±1.97^{ab}	40.62±2.33^{ab}	51.38±1.10^{a}
SIS25	11.10±0.02	16.51±0.89^{ab}	24.10±1.00^{ab}	32.60±1.16^{ab}	42.31±2.26^{a}	53.66±2.67^{a}
SIS50	11.09±0.01	15.84±0.65^{b}	22.62±0.55^{bc}	29.86±0.77^{bc}	37.52±0.89^{b}	44.39±2.54^{b}
SIS75	11.11±0.02	16.07±0.45^{ab}	21.84±1.42^{c}	27.68±1.61^{c}	33.94±2.44^{c}	40.30±4.13^{b}
SIS100	11.09±0.02	14.41±0.54^{c}	17.77±0.92^{d}	22.07±1.40^{d}	27.38±1.37^{d}	31.17±2.06^{c}

Note: Data presented as means of four replicate samples. Values followed by different letters in each row are significantly different ($p < 0.05$).

4.2.3 Weight gain, specific growth rate, and survival rate

Weight gain, specific growth rate and survival rate of Nile tilapia receiving eight different experimental diets are represented in Table 16. Throughout 10-weeks trial, the result revealed that Nile tilapia fed SIS8 diet provided the highest weight gain 390.21±29.09%, while the lowest value of weight gain was observed in fish fed SIS100 diet 167.16±26.89%. However, fish fed SIS8 diet did not show significant differences from fish fed control diet 1 (365.34±22.94%), control diet 2 (367.50±28.40%), SIS15 diet (363.00±10.4%), and SIS25 (383.59±23.77%). Besides that, the weight gain of fish fed SIS50, SIS75 and SIS100 showed significant

decreases from 300.32±22.87%, 262.67±37.04%, and 167.16±26.89%, respectively. The lowest weight gain was observed in SIS100 diet (167.16±26.89%).

In term of specific growth rate, Nile tilapia fed SIS8 diet had the highest value (2.27±0.09%), while the lowest value was observed in SIS100 diet (1.47±0.09%). There were no significant differences ($p > 0.05$) between fish fed control diet 1 (2.20±0.07%), control diet 2 (2.20±0.09%), SIS8 diet (2.27±0.09%), SIS15 diet (2.19±0.03%), and SIS25 diet (2.25±0.07%). However, fish fed SIS50 (1.98±0.08%), SIS75 (1.83±0.15%), and SIS100 (1.47±0.09%) had significant lower compared to fish fed control diets.

Survival rate represented the percentage of fish that were still alive throughout 10-week of trialing. The results showed that survival rates of Nile tilapia ranged from 93.33±5.44 to 100±0.00%. The highest survival rate was observed in control diet 2, while the lowest survival rates were observed in SIS15 and SIS50 diet. However, there were no significant differences among fish fed all experimental diets.

Table 16: Weight gain, specific growth rate and survival rate of Nile tilapia receiving 8 experimental diets for 10 weeks trial.

Diets	**Growth performance of Nile tilapia fed diets containing different levels of SIS**		
	Weight gain (%)	Specific growth rate (%)	Survival rate (%)
Control 1	365.34±22.94[a]	2.20±0.07[a]	96.67±3.85
Control 2	367.50±28.40[a]	2.20±0.09[a]	100.00±0.00
SIS8	390.21±29.09[a]	2.27±0.09[a]	98.33±3.33
SIS15	363.00±10.40[a]	2.19±0.03[a]	93.33±5.44
SIS25	383.59±23.77[a]	2.25±0.07[a]	96.67±3.85
SIS50	300.32±22.87[b]	1.98±0.08[b]	93.33±5.44
SIS75	262.67±37.04[b]	1.83±0.15[c]	96.67±3.85
SIS100	167.16±26.89[c]	1.47±0.09[d]	95.00±6.38

Notes: Mean ± Standard error, in the same row with different superscript are significantly different ($p < 0.05$). Data presented as means of four replicate samples.

4.2.4 Feed intake, feed conversion ratio, protein efficiency ratio (PER), and productive protein value (PPV)

Feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), and productive protein value (PPV) of Nile tilapia fed with eight different experimental diets are represented in Table 17.

Throughout 10 weeks trial, the amount feed intake of Nile tilapia ranged from 2.33±0.09 to 3.19±0.21 g. $fish^{-1}.day^{-1}$. The highest feed intake value was observed in SIS100 diet, while the lowest value was observed in SIS25 diet. The feed intake of Nile tilapia showed significant difference between SIS50 and SIS75 diet. Moreover, there were no significant differences of feed intake of Nile tilapia fed control diet 1 (2.48±0.06 g. $fish^{-1}.day^{-1}$), control diet 2 (2.35±0.26 g. $fish^{-1}.day^{-1}$), SIS8 diet (2.35±0.25 g. $fish^{-1}.day^{-1}$), SIS15 diet (2.51±0.11 g. $fish^{-1}.day^{-1}$), and SIS25 diet (2.33±0.09 g. $fish^{-1}.day^{-1}$).

At the end of experiment, the lowest feed conversion ratio (FCR) of Nile tilapia was observed in SIS25 diet group (1.22±0.08) which showed no significant differences ($p > 0.05$) between control diet 1 (1.32±0.08), control diet 2 (1.27±0.17), SIS8 diet (1.24±0.16), and SIS15 diet (1.32±0.04). Nile tilapia receiving SIS50, SIS75, and SIS100 diet had significant increases in feed conversion ratio. The highest feed conversion ratio of Nile tilapia was observed in SIS100 diet (2.30±0.26).

The protein efficiency ratio (PER) of Nile tilapia receiving eight different diets had significant differences ($p < 0.05$) after 10 weeks of feeding trial. The result showed that the highest PER of Nile tilapia was observed in SIS8 diet with a value of 2.86±0.09% and the lowest PER was recorded in SIS100 diet with the amount of 1.56±0.01%. There were no significant differences between control diet 2 (2.80±0.04 percent), SIS8 diet (2.86±0.09%), SIS15 diet (1.37±0.04%), and SIS25 diet (1.35±0.08%). PER values of Nile tilapia receiving SIS50 diet, SIS75 diet, and SIS100 had significant decreases.

After Nile tilapia receiving 8 different experimental diet for 10 weeks, the result pf PPV of Nile tilapia showed significant differences ($p < 0.05$). The PPV of Nile tilapia ranged from 23.42±0.10 to 48.28±1.22%. The highest value was observed in SIS25 diet group, which the lowest value was observed in SIS100 diet group. The productive protein values of Nile tilapia fed with negative control diet did not show

significant difference from fish fed SIS8 diet and control diet with values of 46.34±0.62% and 45.54±1.45%, respectively. Besides, PPV of Nile tilapia receiving SIS50, SIS75, and SIS100 diets showed significant decreases compared to the other diets.

Table 17: Feed conversion ratio, protein efficiency ratio and productive protein value of Nile tilapia after 10 week-trial.

Diets	Growth performance of Nile tilapia fed diet containing different levels of SIS			
	FI (g. fish^{-1}.day^{-1})	FCR	PER (%)	PPV (%)
Control 1	2.48±0.06^{cd}	1.32±0.08^{c}	2.55±0.05^{d}	39.50±1.22^{d}
Control 2	2.35±0.26^{cd}	1.27±0.17^{c}	2.80±0.04^{ab}	46.34±0.62^{b}
SIS8	2.35±0.25^{cd}	1.24±0.16^{c}	2.86±0.09^{a}	45.54±1.45^{b}
SIS15	2.51±0.11^{cd}	1.32±0.04^{c}	2.67±0.07^{c}	41.68±1.03^{c}
SIS25	2.33±0.09^{d}	1.22±0.08^{c}	2.76±0.07^{b}	48.28±1.22^{a}
SIS50	2.81±0.18^{b}	1.59±0.13^{b}	2.24±0.06^{e}	35.27±0.89^{e}
SIS75	2.66±0.25^{bc}	1.63±0.27^{b}	2.11±0.05^{f}	33.01±0.82^{f}
SIS100	3.19±0.21^{a}	2.30±0.26^{a}	1.56±0.01^{g}	23.42±0.10^{g}

Note: Data presented as means of four replicate samples. Values followed by different letters in each row are significantly different ($P < 0.05$).

4.3 Whole-body composition

The whole-body composition of Nile tilapia is represented in Table 17.

In this present study, average moisture content value of initial Nile tilapia is 76.77±0.90%. After Nile tilapia fed all experimental diets for 10 weeks, moisture content values ranged from 72.97±0.78 to 73.72±0.69%. The result showed that highest moisture content value was observed in fish fed SIS15 diet, while the lowest moisture content value was observed fish fed SIS75 diet. However, there were no significant differences of moisture content among all fish fed all experimental diets.

Before starting the experiment, the whole-protein content of initial fish was 56.16±0.99%. After Nile tilapia fed eight different experimental diets for 10 weeks,

the whole-protein content values of fish fed the experimental diets showed significant differences ($p < 0.05$). The results revealed that the whole-protein content values ranged from 52.40±0.18 to 62.24±0.74%. The highest protein content of Nile tilapia was showed in SIS25 diet group (62.24±0.74%), followed by control diet 2 (59.18±0.28%), SIS15 diet group (57.31±0.81%), SIS8 diet group (57.10±0.39%), SIS50 diet group (56.27±0.57%), control diet 1 (56.01±0.82%), SIS75 diet group (54.27±0.77%); while the lowest protein content was observed in SIS100 diet. There were no significant differences between fish fed SIS8 and SIS15 diet; SIS50 diet and control diet 1; SIS8 diet and SIS50 diet.

The whole-fat content of initial Nile tilapia was recorded at 14.79±0.09%. After Nile tilapia fed 8 different diets for 10 weeks, the whole-fat content showed significant differences ($p < 0.05$). The result showed that Nile tilapia fed control diet 1 had the highest fat content (17.89±0.76%) and it did not show significant differences ($p > 0.05$) from fish fed SIS8 diet (17.77±0.25%), SIS15 diet (17.79±0.53%), and SIS25 diet (17.66±0.72%). Besides, Nile tilapia fed SIS50 diet, SIS75 diet and SIS100 diet showed significant decreases in whole-fat content ($p < 0.05$). The lowest fat content of fish was observed in SIS75 diet (5.82±0.16%).

The beginning of experiment, the whole-ash content value of Nile tilapia was recorded at 15.89±0.24%. After 10-week trial, the whole-ash content values of Nile tilapia receiving eight experimental diets were recorded from 9.92±0.11 to 16.41±0.30%. The highest ash content value was observed in fish fed control diet 1.

Table 18: Proximate composition of Nile tilapia fed eight experimental diets for 10 weeks.

Diet	Nutritional values of Nile tilapia fed eight experimental diets			
	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Initial fish	76.77±0.90	56.16±0.99	14.79±0.09	15.89±0.24
Control 1	73.30±0.85	56.01±0.82^{e}	17.89±0.76^{a}	16.41±0.30^{a}
Control 2	73.28±0.70	59.18±0.28^{b}	16.10±0.43^{b}	13.14±0.13^{c}
SIS8	73.15±0.18	57.10±0.39^{cd}	17.77±0.25^{a}	12.29±0.26^{d}
SIS15	73.72±0.69	57.31±0.81^{c}	17.79±0.53^{a}	13.19±0.11^{c}
SIS25	73.39±0.55	62.24±0.74^{a}	17.66±0.72^{a}	10.15±0.04^{f}
SIS50	73.25±0.56	56.27±0.57^{de}	5.91±0.31^{c}	14.97±0.17^{b}
SIS75	72.97±0.78	54.27±0.77^{f}	5.82±0.16^{c}	9.92±0.11^{f}
SIS100	73.08±0.49	52.40±0.18^{g}	5.93±0.74^{c}	10.89±0.70^{e}

Note: Data presented as means of four replicate samples. Values followed by different letters in each row are significantly different ($p < 0.05$).

4.4 Apparent digestibility coefficients

Apparent digestibility coefficient (ADC) of dry matter and protein of Nile tilapia fed the experimental diets is presented in Table 19. After 10-week trial, the apparent digestibility coefficient of dry matter values of Nile tilapia fed experimental diet showed significant differences ($p < 0.05$) and ranged from 62.77±1.97 to 69.44±0.54%. The highest value of ADC of dry matter was observed in fish fed SIS25 diet (69.44±0.54%), while the lowest value was observed in SIS8 diet (62.77±1.97%). Nile tilapia fed SIS25 diet showed no significant differences between control diet 1 (67.05±2.07%), control diet 2 (67.05±3.29%), SIS75 diet (66.89±0.84%), and SIS100 diet (67.14±0.46%). Moreover, ADC dry matter of fish fed SIS15 diet (64.52±0.15%) did not show significant differences ($p > 0.05$) from fish fed SIS8 diet and SIS50 diet (62.77±1.97% and 63.84±1.00%, respectively).

There were significant differences of ADC of protein ($p < 0.05$). The highest value of ADC of protein was observed in fish fed SIS25 diet and control diet 2 (87.98±0.01%) which did not show significant differences with control diet 1 (87.95±0.03%), but showed significant lower in fish fed SIS8, SIS15, SIS50, SIS75,

and SIS100 diets at 87.46±0.12%, 86.01±0.06%, 83.23±0.08%, 83.42±0.04%, and 81.42±0.06%, respectively.

Table 19: Apparent digestibility coefficients of Nile tilapia receiving different eight experimental diets.

Diet	Apparent digestibility coefficients	
	Dry matter (%)	Protein (%)
Control 1	67.05±2.07^{ab}	87.95±0.03^{a}
Control 2	67.05±3.29^{ab}	87.98±0.01^{a}
SIS8	62.77±1.97^{c}	87.46±0.12^{b}
SIS15	64.52±0.15^{bc}	86.01 ±0.06^{c}
SIS25	69.44±0.54^{a}	87.98±0.01^{a}
SIS50	63.84±1.00^{c}	83.23±0.08^{e}
SIS75	66.89±0.84^{ab}	83.42±0.04^{d}
SIS100	67.14±0.46^{ab}	81.42±0.06^{f}

Note: Data presented as means of four replicate samples. Values followed by different letters in each row are significantly different (P<0.05).

4.5 Blood biochemistry

The blood biochemistry parameters of Nile tilapia fed experimental diets are represented in Table 20. The result of this study showed that the haemoglobin concentration values of Nile tilapia ranged from 4.31±0.25 to 5.85±0.05 g/dL. The highest value of Nile tilapia haemoglobin concentration was observed in SIS15 diet (5.85±0.05 g/dL). It did not show significant differences between fish fed with control diet 1 (5.76±0.24 g/dL), SIS8 diet (5.23±0.40 g/dL), SIS25 diet (5.80±0.57 g/dL), SIS50 diet (5.47±0.17 g/dL), SIS75 diet (5.35±0.46 g/dL), and SIS100 diet (5.35±0.29 g/dL), respectively. Moreover, the haemoglobin concentration value had significantly lower ($p < 0.05$) in control diet 2 (4.31±0.25 g/dL) compared to other diets. Additionally, the haematocrit values (Packed cell volume) of Nile tilapia fed 8 different experimental diets showed significant differences ($p < 0.05$). Nile tilapia haematocrit values ranged from 21.57±0.89% to 48.76±0.87%. The highest haematocrit value of Nile tilapia was observed in SIS50 diet (48.76±0.87%), while the

lowest value was observed in SIS100 diet (21.57±0.89%). Compared to SIS50 diet, haematocrit values of Nile tilapia fed with other diet groups such as SIS25 diet (40.59±0.70%), SIS75 diet (39.24±0.80%), SIS15 diet (37.81±0.87%), SIS8 diet (36.53±2.58%), control diet 2 (34.27±3.41%), control diet 1 (31.29±1.41%), SIS100 diet (21.57±0.89%) showed significant lower. The highest red blood cell value of Nile tilapia was recorded in SIS25 diet group with the value of 2.20±3.74 $x10^6$ cells/mm^3, while the lowest value was found in control diet 1 with the value of 1.125±1.00 $x10^6$ cells/mm^3. Furthermore, white blood cells values of Nile tilapia receiving 8 experimental diets ranged from 79.94±4.61 to 160.69±22.03 $x10^3$ cells/mm^3. Nile tilapia fed with SIS25 diet had the highest value of white blood cells, while the lowest value was observed in SIS100 diet. Additionally, white blood cell values had significant increased after Nile tilapia receiving SIS up to 50% compared to fish fed with control diet; then, it started decreasing after Nile tilapia received SIS level in an excess of 50%.

Table 20: Blood biochemistry parameters of Nile tilapia receiving different experimental diets for 10 weeks.

Treatment	Haemoglobin	Haematocrit	RBC ($x10^6$ cells/mm^3)	WBC ($x10^3$ cells/mm^3)
Control 1	5.76±0.24^{ab}	31.29±1.41^{f}	1.125±1.00	95.06±4.11^{e}
Control 2	4.31±0.25^{b}	34.27±3.41^{e}	1.305±3.27	119.44±6.67^{cd}
SIS8	5.23±0.40^{ab}	36.53±2.58^{de}	1.285±3.05	123.44±12.71^{c}
SIS15	5.85±0.05^{a}	37.81±0.87^{cd}	1.59±2.30	150.63±7.76^{b}
SIS25	5.80±0.57^{a}	40.59±0.70^{b}	2.20±3.74	160.69±22.03^{a}
SIS50	5.47±0.17^{ab}	48.76±0.87^{a}	1.76±3.21	148.44±16.68^{b}
SIS75	5.35±0.46^{ab}	39.24±0.80^{bc}	1.61±1.95	114.98±9.81^{d}
SIS100	5.35±0.29^{ab}	21.57±0.89^{g}	1.285±3.65	79.94±4.61^{f}

Note: Different superscripts in the same row are significantly different (P<0.05).

The result of MCHC, MCH, and MCV of Nile tilapia fed eight different diets is presented in Table 21. The volume of mean corpuscular haemoglobin concentration (MCHC) values of Nile tilapia ranged from 11.22±0.21 to 24.85±2.01 g/dL (of erythrocytes). The highest volume of MCHC was recorded in Nile tilapia fed SIS100 diet (24.85±2.01 g/dL), followed by control diet 1 (18.41±0.33 g/dL), SIS15 diet (15.49±0.35 g/dL), SIS8 diet (14.38±1.47 g/dL), SIS25 diet (14.29±1.52 g/dL), SIS75 diet (13.64±0.94 g/dL), control diet 2 (12.64±0.85 g/dL), respectively; while fish fed SIS50 diet was reported as the lowest volume (11.22±0.21 g/dL). The MCHC of Nile tilapia fed Negative control diet had no significant differences from SIS8 diet, SIS50 diet, and SIS75 diet. In addition, there were no significant differences of MCHC of Nile tilapia receiving SIS8 diet, SIS15 diet, and SIS25 diet. Mean corpuscular haemoglobin (MCH) values of Nile tilapia receiving eight experimental diets showed significant differences ($p < 0.05$). MCH value ranged from 26.35±2.60 to 51.18±2.1 g/dL. The highest MCH was observed in Nile tilapia fed control diet 1 (51.18±2.1 g/dL), while the lowest value was observed in Nile tilapia fed SIS25 (26.35±2.60 g/dL). There were no significant differences of MCH of Nile tilapia fed SIS8 diet (40.72±3.08 g/dL) and SIS100 diet (41.63±2.28 g/dL). Moreover, MCH of Nile tilapia fed control diet 2 did not show significant difference with Nile tilapia fed SIS50 diet (31.09±0.99 g/dL) and SIS75 diet (33.26±2.86 g/dL). Moreover, the MCV of Nile tilapia receiving eight different diets showed significant differences ($p < 0.05$). The result showed that MCV of Nile tilapia ranged from 167.84±6.92 to 284.26±20.09 g/dL. The highest value MCV was observed in Nile tilapia fed SIS8 diet, which showed no significant difference between fish fed control diet 1 (278.10±12.53 g/dL) and SIS50 diet (277.06±4.94 g/dL). There were no significant differences between fish fed control diet 2 (262.59±26.09 g/dL) and SIS75 diet (243.70±4.98 g/dL); while fish fed SIS75 diet did not has significant difference with SIS15 diet (237.80±5.46 g/dL). The lowest MCV was observed in Nile tilapia fed SIS100 diet which showed no difference with Nile tilapia fed SIS25 diet

Table 21: Mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, and mean corpuscular volume of Nile tilapia receiving different experimental diets for 10 weeks.

Treatment	MCHC	MCH	MCV
Control 1	18.41 ± 0.33^{b}	51.18 ± 2.17^{a}	278.10 ± 12.53^{ab}
Control 2	12.64 ± 0.85^{de}	33.06 ± 1.95^{d}	262.59 ± 26.09^{bc}
SIS8	14.38 ± 1.47^{cd}	40.72 ± 3.08^{b}	284.26 ± 20.09^{a}
SIS15	15.49 ± 0.35^{c}	36.81 ± 0.33^{c}	237.80 ± 5.46^{d}
SIS25	14.29 ± 1.52^{cd}	26.35 ± 2.60^{e}	184.50 ± 3.19^{e}
SIS50	11.22 ± 0.21^{e}	31.09 ± 0.99^{d}	277.06 ± 4.94^{ab}
SIS75	13.64 ± 0.94^{d}	33.26 ± 2.86^{d}	243.70 ± 4.98^{cd}
SIS100	24.85 ± 2.01^{a}	41.63 ± 2.28^{b}	167.84 ± 6.92^{e}

Note: Different superscripts in the same row are significantly different ($P<0.05$).

4.6 Histological characteristics

4.6.1 Intestine tissue

The intestinal morphometric measurements are summarized in Table 22 and intestinal morphology of Nile tilapia receiving eight different experimental diets for 10 weeks are presented in Figure 9-16. The highest villus height was observed in fish fed SIS25 diet, while the lowest value was observed in fish fed SIS100 diet. There were no significant differences in villus height between fish fed control diet 1, SIS8 diet, SIS15 diet, and SIS50 diet. Moreover, there were no significant differences in villus width between fish fed SIS8 diet and SIS25 diet which showed significant differences from fish fed control diet 1, SIS15 diet, SIS50 diet, SIS75 diet, and SIS100 diet. Additionally, there were no significant difference fish fed SIS8 diet, SIS25 diet, SIS50 diet, SIS75 diet, and SIS100 diet which showed significantly differences from fish fed control diet 1 and SIS15 diet.

Table 22: Intestinal morphometric measurements of Nile tilapia fed diets for 10 weeks.

Diets	Villus height (µm)	Villus width (µm)	Serora
Control 1	360.71 ± 11.96^{b}	109.86 ± 5.00^{b}	53.37 ± 0.82^{b}
Control 2	N/A	N/A	N/A
SIS8	345.95 ± 19.61^{bc}	155.24 ± 34.51^{a}	59.70 ± 3.45^{a}
SIS15	357.35 ± 12.87^{b}	105.90 ± 12.17^{b}	44.67 ± 1.58^{c}
SIS25	397.24±8.62^{a}	135.82±3.75^{a}	60.00±5.95^{a}
SIS50	356.57±6.56^{b}	106.00±5.76^{b}	59.94±0.66^{a}
SIS75	335.57±6.45^{c}	107.43±3.60^{b}	58.89±1.06^{a}
SIS100	256.65±3.58^{d}	96.10±3.38^{b}	57.30±0.54^{ab}

Note: Different superscripts in the same row are significantly different (P<0.05).

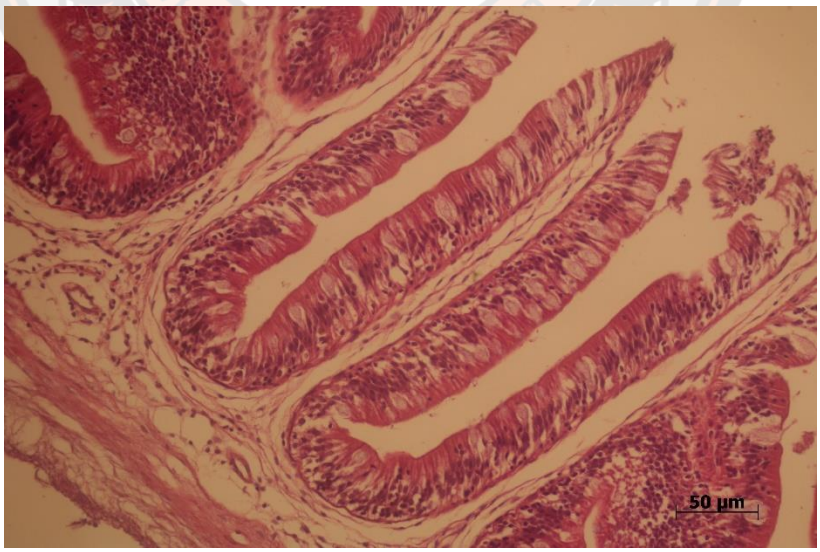

Figure 9: H&E stained intestinal tissue section of Nile tilapia fed control diet 1 throughout 10-week trial (H&E, 40).

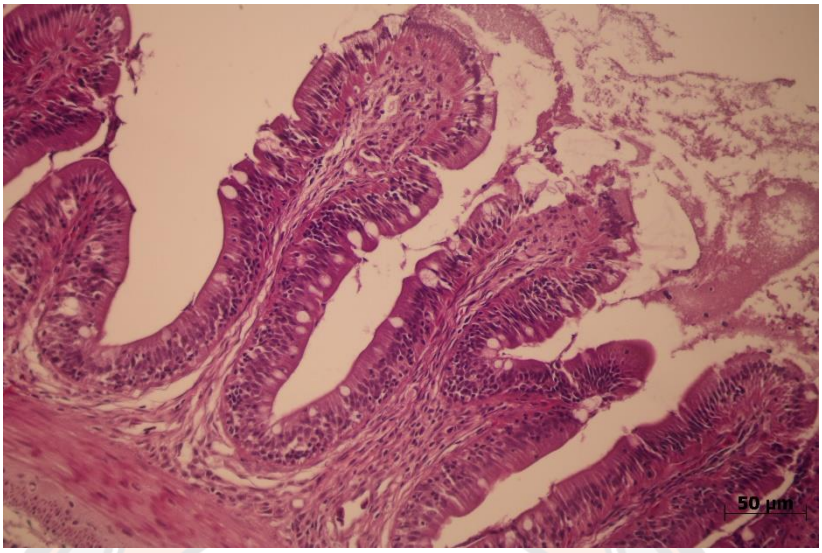

Figure 10: H&E stained intestinal tissue section of Nile tilapia fed control diet 2 throughout 10-week trial (H&E, 40).

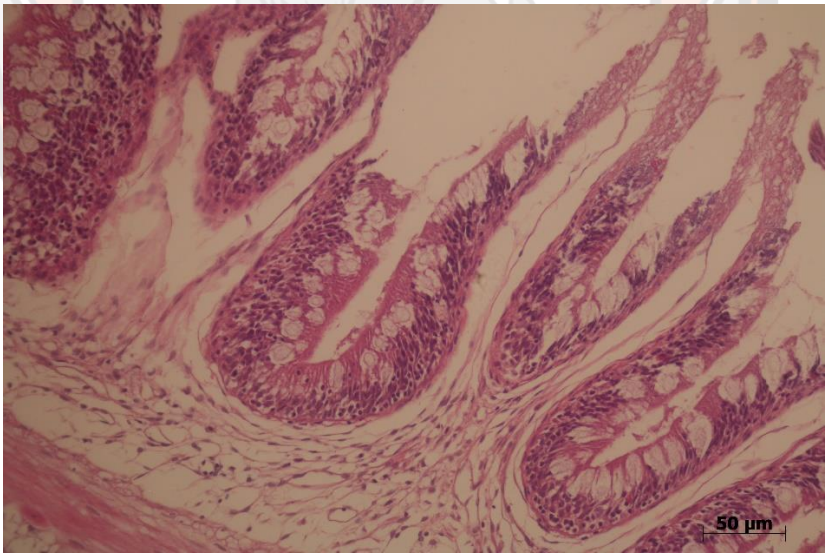

Figure 11: H&E stained intestinal tissue section of Nile tilapia fed SIS8 diet throughout 10-week trial (H&E, 40).

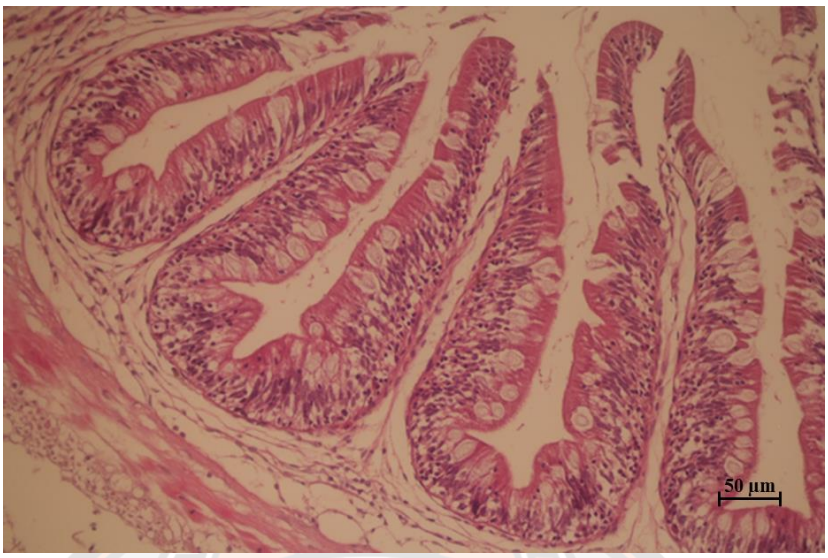

Figure 12: H&E stained intestinal tissue section of Nile tilapia fed SIS15 diet throughout 10-week trial (H&E, 40).

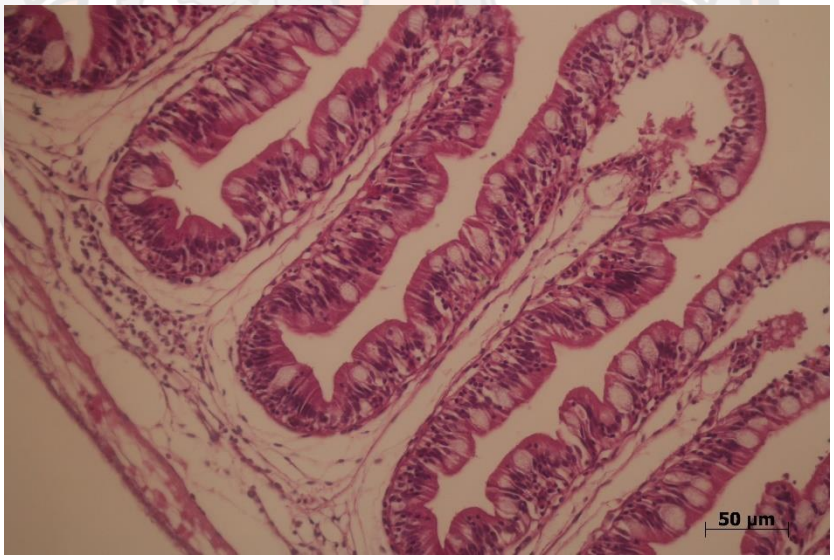

Figure 13: H&E stained intestinal tissue section of Nile tilapia fed SIS25 diet throughout 10-week trial (H&E, 40).

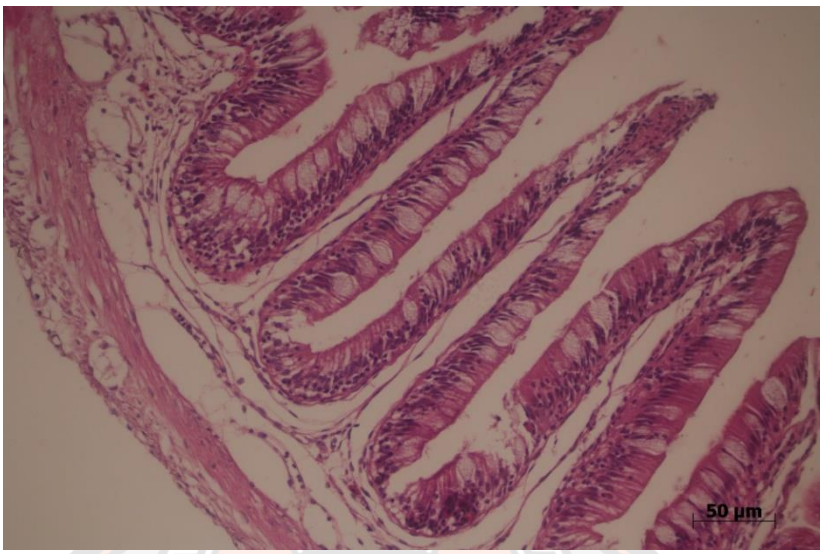

Figure 14: H&E stained intestinal tissue section of Nile tilapia fed SIS50 diet throughout 10-week trial (H&E, 40).

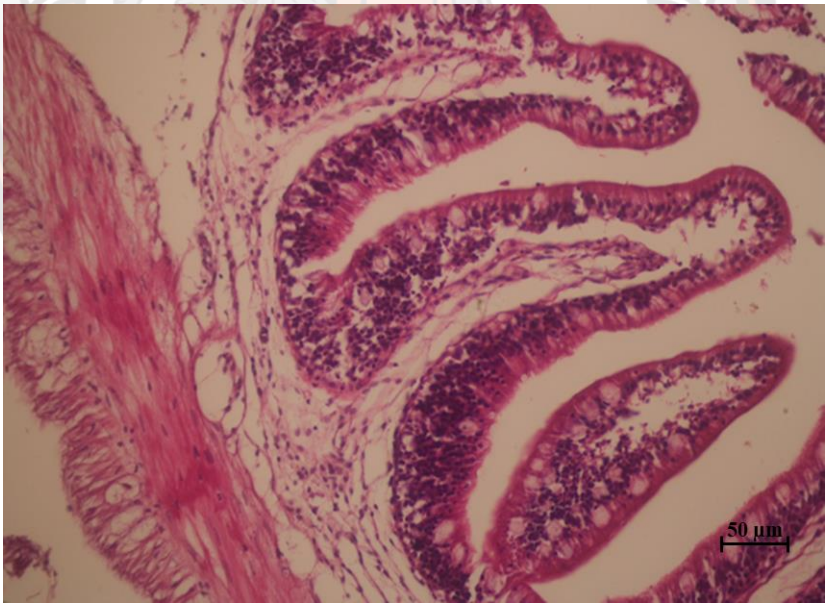

Figure 15: H&E stained intestinal tissue section of Nile tilapia fed SIS75 diet throughout 10-week trial (H&E, 40).

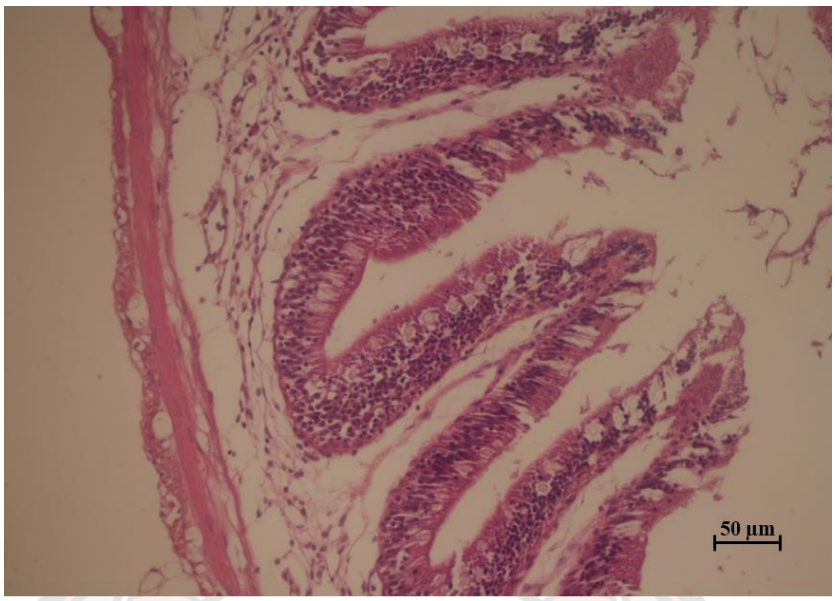

Figure 16: H&E stained intestinal tissue section of Nile tilapia fed SIS100 diet throughout 10-week trial (H&E, 40).

4.6.2 Liver tissues

The liver tissue sections of Nile tilapia fed eight different diets are presented in Figure 17-24. H&E staining revealed alterations in the histological appearance in liver tissues of Nile tilapia fed SIS substituting diets. The staining showed that liver cells of fish receiving SIS up to 50% showed no visible intracellular lipid deposition (Fig. 17-22), but nuclei of all treatment fish were damaged. Moreover, the liver tissues of Nile tilapia receiving SIS between 75%-100% protein levels showed intra cytoplasmic round circumscribed fat vacuoles. The relative size of hepatocytes increased as the proportion of the SIS in the diets increased, and this was associated with a much greater hepatic lipid deposition (Fig. 23-24).

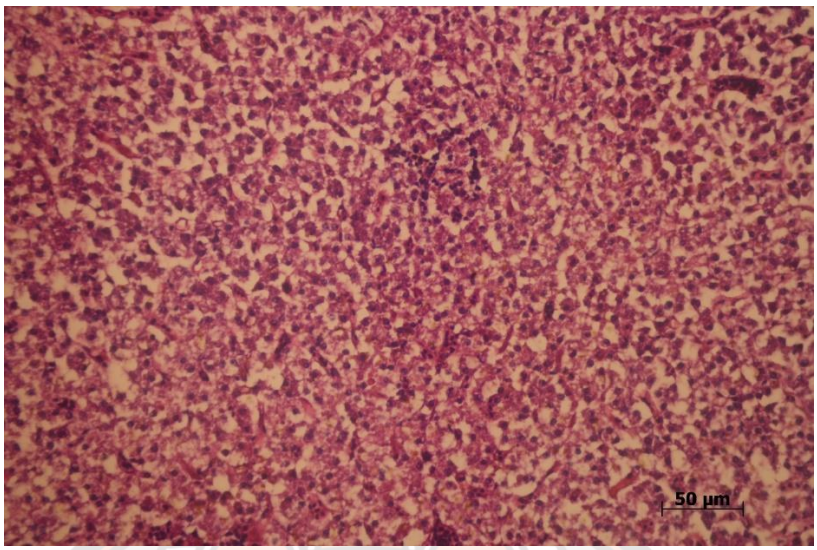

Figure 17: H&E stained liver tissue of Nile tilapia fed control diet 1 throughout 10-week trial (H&E, 40).

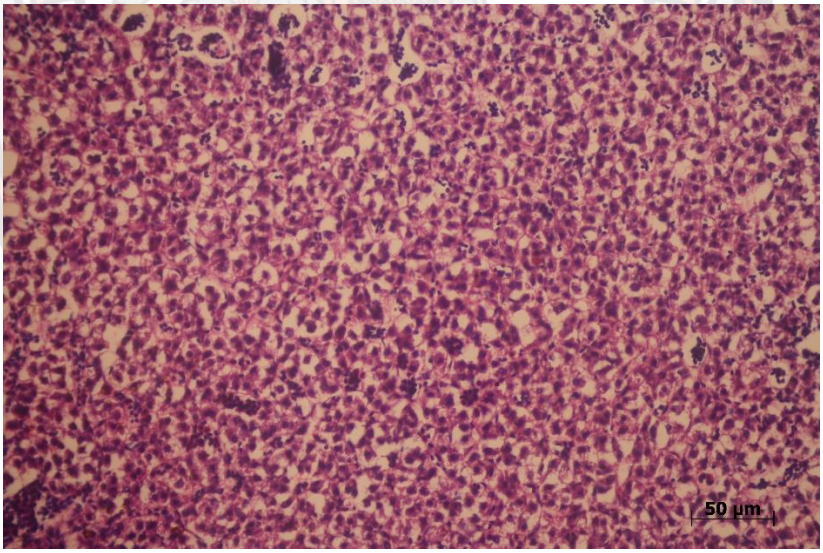

Figure 18: H&E stained liver tissue of Nile tilapia fed control diet 2 throughout 10-week trial (H&E, 40).

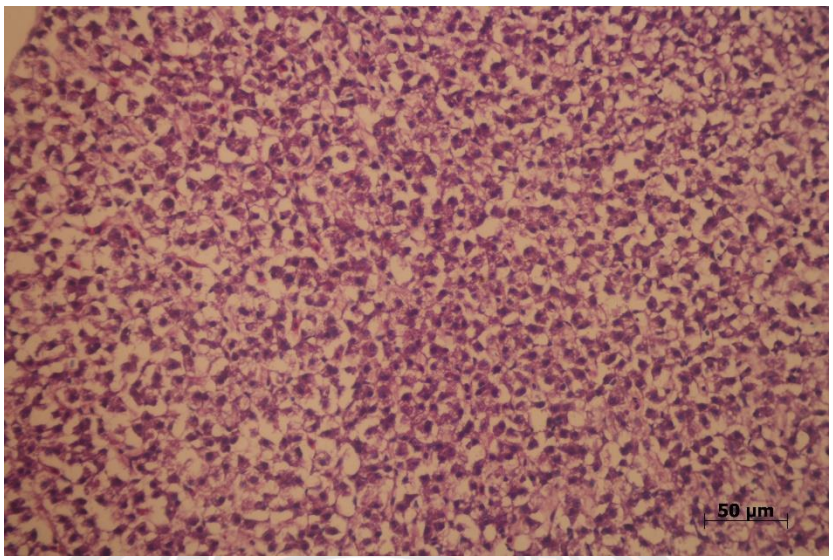

Figure 19: H&E stained liver tissue of Nile tilapia fed SIS8 diet throughout 10-week trial (H&E, 40).

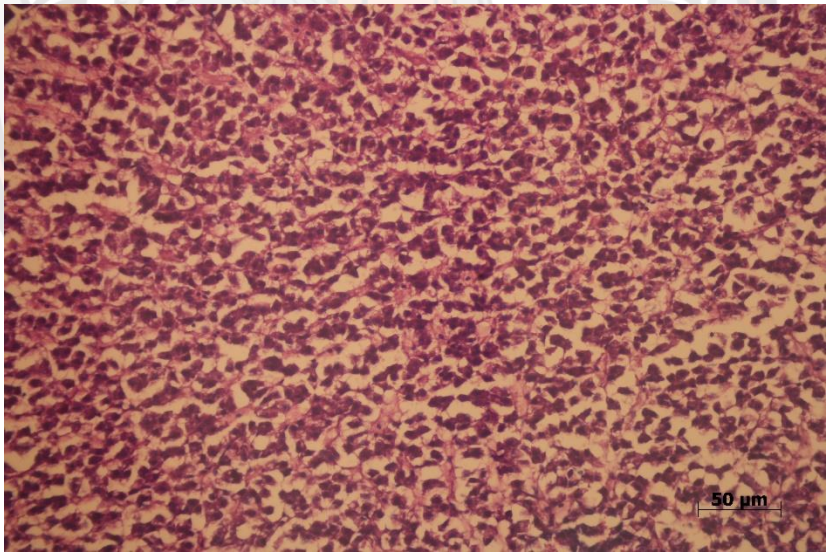

Figure 20: H&E stained liver tissue of Nile tilapia fed SIS15 diet throughout 10-week trial (H&E, 40).

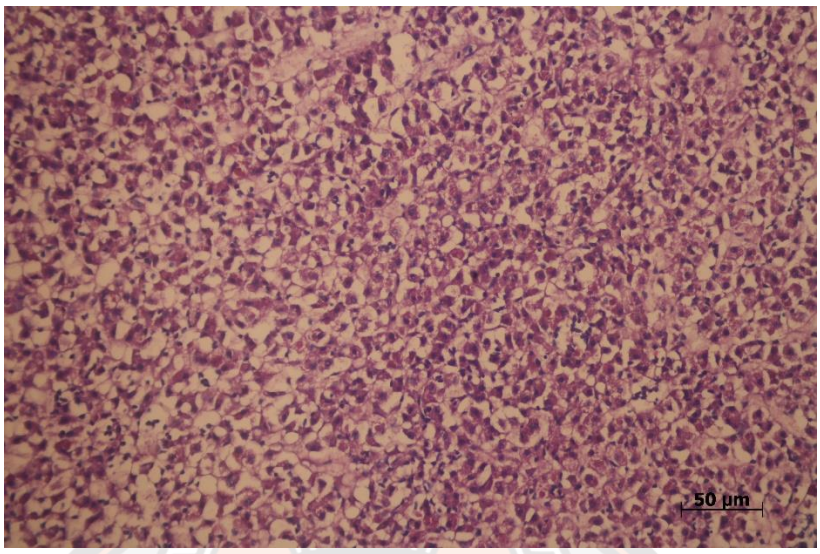

Figure 21: H&E stained liver tissue of Nile tilapia fed SIS25 diet throughout 10-week trial (H&E, 40).

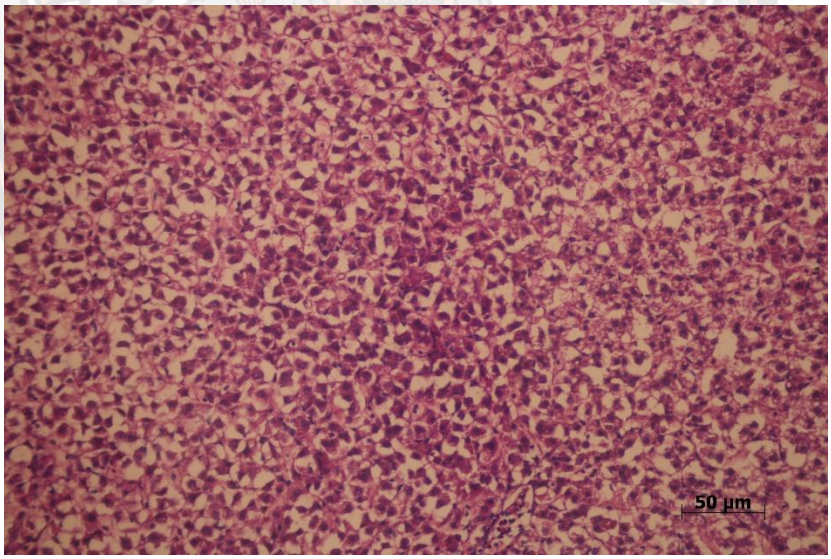

Figure 22: H&E stained liver tissue of Nile tilapia fed SIS50 diet throughout 10-week trial (H&E, 40).

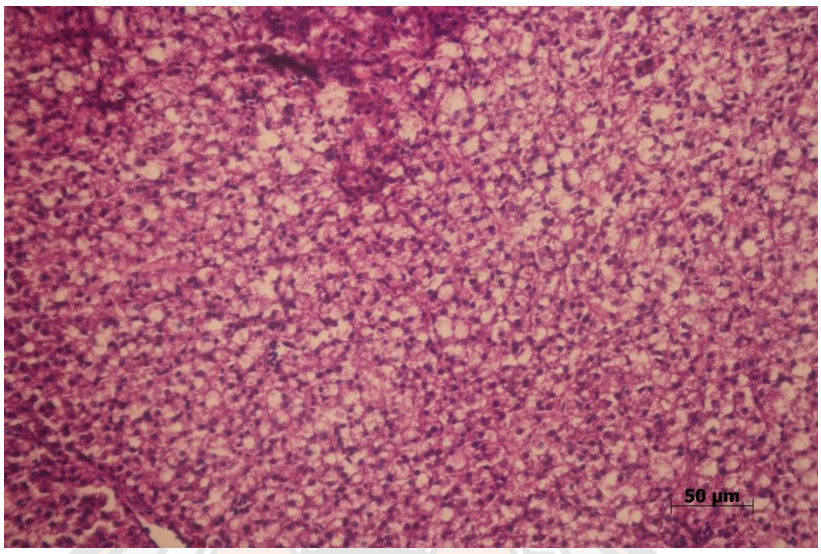

Figure 23: H&E stained liver tissue of Nile tilapia fed SIS75 diet throughout 10-week trial (H&E, 40).

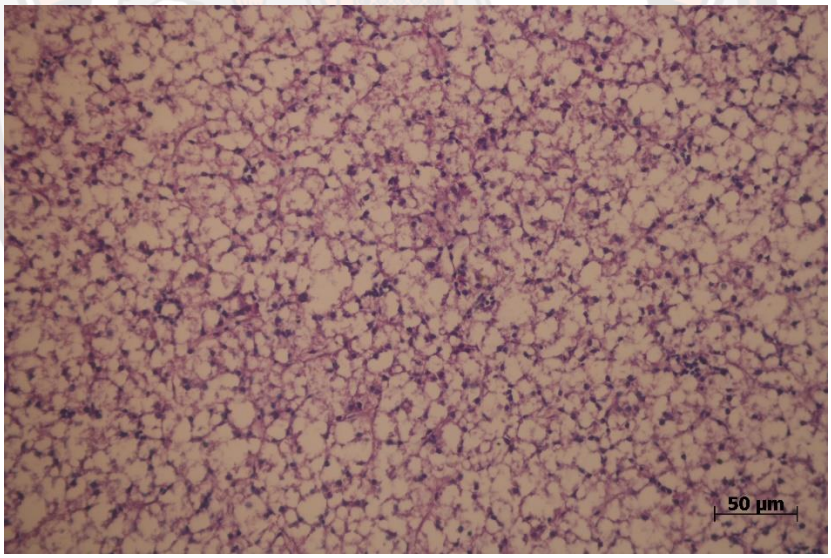

Figure 24: H&E stained liver tissue of Nile tilapia fed SIS100 diet throughout 10-week trial (H&E, 40).

4.7 Gene expression

After 10 weeks trial, expression profiles of TNF-α, IL-1β, and TGF-β gene in the liver of Nile tilapia fed the eight different experimental diets are presented in Fig. 25, 26, and 27, respectively. The results revealed that the expression levels of target genes (TNF-α, IL-1β, and TGF-β gene) did not show significant differences among all treatment groups.

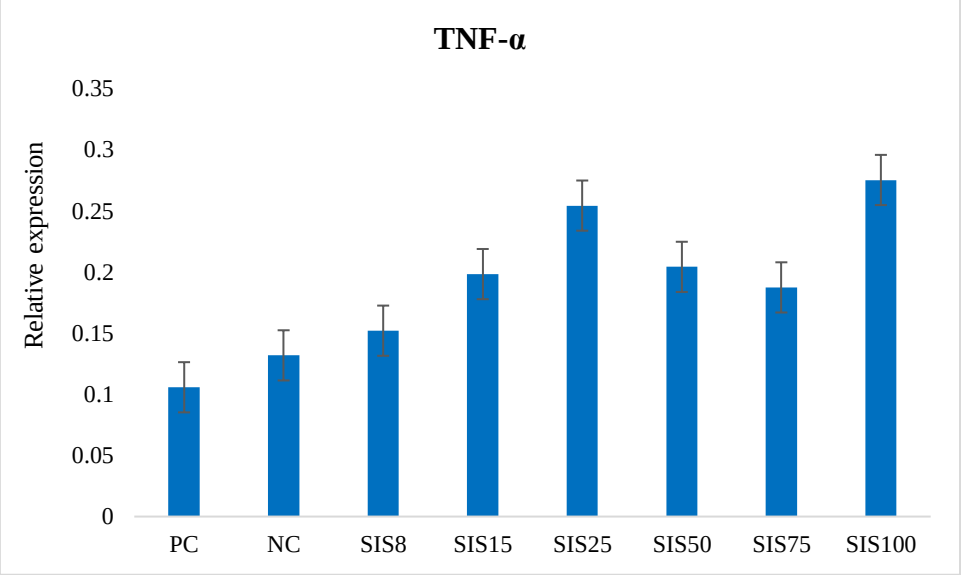

Figure 25: Relative expression of TNF-α gene of Nile tilapia fed the eight different experimental diets at the end of the feeding trial (t = 90 days). Results presented (mean ± SD) are normalized against β-actin.

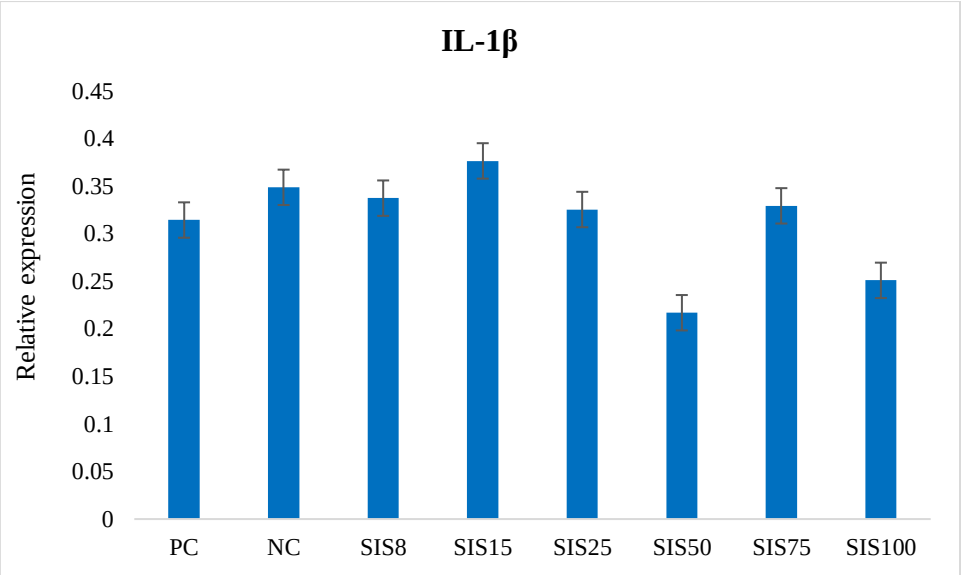

Figure 26: Relative expression of IL-1β of Nile tilapia fed the eight different experimental diets at the end of the feeding trial (t = 90 days). Results presented (mean ± SD) are normalized against β-actin.

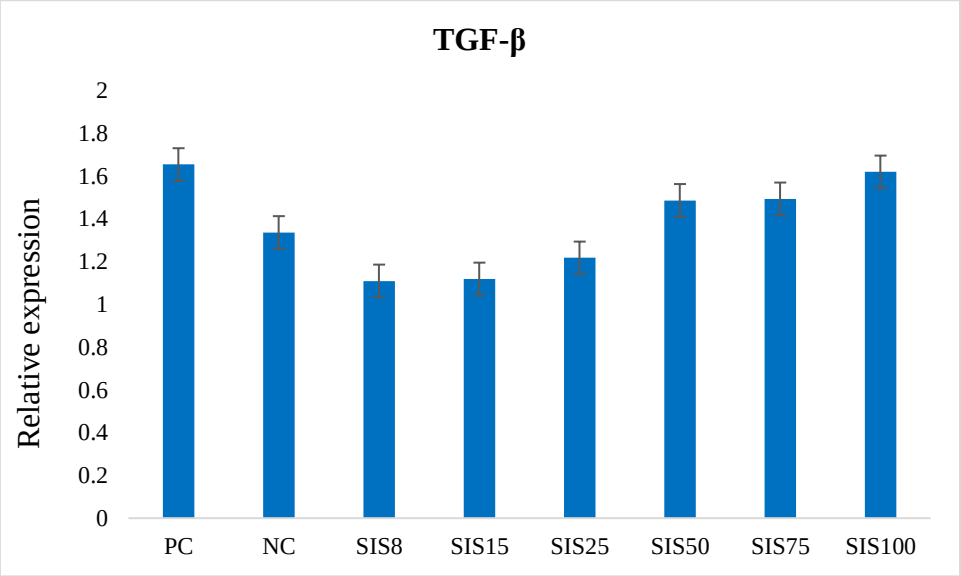

Figure 27: Relative expression of TGF-β of Nile tilapia fed the eight different experimental diets at the end of the feeding trial (t = 90 days). Results presented (mean ± SD) are normalized against β-actin.

4.8 Economic analyses of the diets fed to the Nile tilapia over the trial period

The dietary inclusion of SIS protein at all levels resulted in considerably lower feed cost compared to the control diet. The SIS8, SIS15, SIS25, SIS50, and SIS75 diets reduced feed costs relative to the cost of the control diet 1.

Table 23: Economic analyses of the diets fed to the Nile tilapia over the trial period

Diets	**Economic analyses of the diets fed to the Nile tilapia over the trial period**	
	Feed Cost (US$ kg^{-1})	Cost of feed used for trial (US$)
Control 1	1.09	0.78
Control 2	0.96	0.65
SIS8	0.95	0.65
SIS15	0.94	0.68
SIS25	0.93	0.62
SIS50	0.90	0.73
SIS75	0.88	0.68
SIS100	0.86	0.79

CHAPTER 5

DISCUSSION

5.1. Growth performance

According to the result of this present study, replacing protein sources of fishmeal with SIS at different levels (0%, 8%, 15%, 25%, 50%, 75%, and 100%) in Nile tilapia diets were performed for 10 weeks. The result showed that Nile tilapia fed SIS25 diet had the highest growth performance in term of weight gain, specific growth rate, and feed conversion ratio. Few studies reported the SIS were used as an ingredient for fish; for instance, Araujo-Dairiki *et al.* (2018) reported that SIS were used as diet ingredient for juvenile tambaqui and matrinxã with different proportion of protein (0, 15 and 30%) for 60 days. The result showed that no significant differences were observed among all diet treatments on growth parameters, except for FCR in tambaquis, which was significantly lower for the 30% sacha inchi feed in relation to control diet. The result showed that higher concentrations of SI might have some inhibitory effect, that may be due the elevated crude fiber content of sacha inchi (6.29%). The amount of feces siphoned from tanks housing tambaquis fed 30% sacha inchi diets was higher than in the other treatments. These previous results indicated that locally produced SIS have the potential to substitute more expensive non-regional sources of PUFA in tambaqui and matrinxã diets in Amazonian aquaculture. Lima *et al.* (2019) used sacha inchi oil to replace fish oil at different inclusion levels (0, 40, 60, and 100%) in the diet of rainbow trout juveniles. The results showed that the experimental diets did not affect fish growth, feed conversion, regardless of concentration level and experimental period, this finding may be due to the ability of rainbow trout to use dietary EFA to synthesize LC-PUFA. Meanwhile, replacing FO with SI oil increased protein deposition in the muscle. Fish fed exclusively with SI oil had increased total protein content in their muscle tissue after 90 days. The 40FO, 60FO, and 100FO groups had similar ARA and DHA percentages in their livers at 45 days, which is reflective of rainbow trout being able to synthesize LC-PUFA from dietary C18PUFAs. Although replacing FO with SI oil resulted in higher expression

levels of the desaturase and elongase genes at both time points, this increase was insufficient to maintain the high levels of muscular LCPUFA in the 40FO and 0FO groups. The percentages of C18PUFAs in the muscle of fish fed with the 0FO diet increased, while the percentage of n-3 LC-PUFA and the n-3: n-6 ratio decreased. Although the 0FO group exhibited higher transcription levels for the enzymes involved in LC-PUFA biosynthesis, the most promising results in terms of muscle FA profile were observed in the 60FO group. This finding indicates that the inclusion of 40% of SI into the diet of *O. mykiss* did not affect the percentage of ARA, EPA, and DHA. Taken together, these data suggest that SI represents a viable, promising replacement for FO in the feed for salmonids and potentially other aquacultured fish species as well. Al-Thobaiti *et al.* (2018) studied the replacement of 10% 20%, and 40% of fishmeal with alternative plant protein sources (corn gluten meal, wheat gluten meal, soybean meal and bagasse kenna mix) in Nile tilapia diets. The result showed that Nile tilapia can accept alternative plant protein sources up to 20% without any negative effect on growth performance (weight gain, specific growth rate, feed conversion ratio, and protein efficiency ratio). These study suggested that using alternative plant protein to replace fishmeal not only achieve better growth, but also help to reduce production costs. El-Saidy and Gaber (2003) stated that the replacement of fishmeal with soybean meal at high protein level caused the reduction fish growth, high feed conversion ratio, and low productive protein level compared to control diet group. Viola and Arieli (1983) stated that soybean meal could be used to substitute up to half of the fishmeal in Nile tilapia diets comprising 25% protein without any other nutritional additions. In the case of the report of Silva *et al.* (2017) which different protein levels of peanut meal (0, 25, 50, 75, and 100%) were used to substitute soybean meal in Nile tilapia diet. The result showed that many parameters were declined when increasing peanut meal in diet more than 25 percent such as protein efficiency ratio, protein retention and whole-body protein content. This study suggested that peanut meal can be used in feed considerable to 11.72 percent and coincide to 25 percent of soybean meal protein without causing fish growth. Similarly, Yang *et al.* (2009) studied the effects of fermented soybean meal (FSB) at difference levels of protein (0, 12, 24, 36, 48 and 60%) on growth performance body composition and hematological characteristics of Silver Perch (*Bidyanus bidyanus*).

The results showed that FSB up to 24% in diet did not have an adverse effect on growth of silver perch. Likewise, results in body proximate composition, biological measurements, and hematological characteristics indicated that the level of dietary FSB affected the lipid metabolism and deposition in the silver perch. This study indicating that the palatability of the diets might be considered as the possible causes for the poor performance in higher replacement levels of fish meal by FSB. Lacking suboptimal essential amino acid balance in diets containing plant protein is one of the important limiting factors and results in poor growth and feed utilization in fish. Also, it reduced the hematological parameter values. Based on our study, Nile tilapia fed SIS between 50%-100% had significant decrease in final body weight, weight gain, specific growth rate, FCR, PER, and PPV. In fish, reduced growth performance was detected when a large amount of the fishmeal was substituted by plant protein sources (Bilen and Müge Bilen, 2013). Although ingredients of plant origin are being increasingly used in fish feeds, the total replacement of fish meal by plant protein sources has rarely been successful, the high inclusion levels of plant protein sources usually resulting in reduced growth and less efficient feed utilization (Jobling *et al.*, 2001). These decreasing of growth performance may be due to the presence of antinutritional factors in SIS, especially saponins **(**Pariona Mendoza, 2008**)**. Dietary saponins depressed growth, feed consumption in gerbils and egg production in poultry (Jenkins and Atwal, 1994; Potter *et al.*, 1993; Sim *et al.*, 1984; Terapuntuwat and Tasaki, 1986). Many other factors reduced growth are low palatability (Mohsen and Lovell, 1990; Wang *et al.*, 2017), poor utilization of protein (Boonyaratpalin *et al.*, 1998), and amino acids imbalance (Hernández *et al.*, 2007). The palatability has an influence upon ingestion and digestibility (Al-Souti *et al.*, 2019). Moreover, animal growth depends on nutrient digestion and absorption (Cerezuela *et al.*, 2013). Likewise, lipid contents used in Nile tilapia diet were used from 8-15% after the supplementation of SIS between 50%-100%. The result showed that feed supplemented with SIS between 50%-100% had a significantly reduced growth performance indicating that high lipid content cause effect on fish growth performance. In relation to earlier studies, the most favorable value of lipid content in Nile tilapia range from 5-7.4 percent (Abdel-Tawwab *et al.*, 2010; Deng *et al.*, 2010; He *et al.*, 2015; Santiago and Reyes, 1993; Wang *et al.*, 2005; Xiong *et al.*, 2014).

Many fish species, limited dietary lipid caused in decreasing growth performance and a series of symptoms related to essential fatty acid efficiency (Takeuchi *et al.*, 1990; Watanabe, 1993). Additionally, some herbivorous and omnivorous fish species, such as grass carp and tilapia, have the ability to endure EFA deficiency for a relatively long period and synthesize lipid when dietary lipid is low (Chou and Shiau, 1996; Du *et al.*, 2005).

The feed conversion ratio (FCR) is an essential dimension for refereeing the commercial acceptability and suitability of artificial feed for aquatic animals. Because feed is expensive, feed conversion ratio or feed efficiency are important calculations for the grower. They can be used to determine if feed is being used as efficiently as possible (Craig *et al.*, 2017). In this present study, the result showed that FCR values of Nile tilapia fed SIS25 diet had the lowest value, but it did not show significant differences compared to fish fed control diets, while its value showed significant increases continuously with an increasing amount of SIS protein between 50%-100% in diets. In numerous studies claimed that low feed conversion ratio (FCR) value is an indicator of feed utilization efficiency of formulated feed (Koumi *et al.*, 2009). Relatively high FCR also reflects underfeeding of fish, i.e. the growth is less than expected due to lack of feed. This indicates that a relatively less energy is available for growth since energy for fundamental metabolism (ground metabolism) and motion is prioritized (Amoah *et al.*, 2011). This result suggests that substituting fishmeal with SIS in diets at 25% is the best value for Nile tilapia growth, while substituting of SIS between 50%-100% in Nile tilapia diets were affected to FCR value. It's likely because of the environmental parameters (temperature, dissolved oxygen, etc.) and the culture system, and the presences of anti-nutritional factors. It is related to Shiau *et al.* (1990), the FCR values tended to be higher due to the use of small aquaria and the relatively slow feeding habits of Nile tilapia. Further, the color of the diets (light brown) sometimes made them difficult to been seen in aquarium bottoms and may have resulted in overfeeding; however, feed supply must not be limiting in nutrition experiments and overfeeding is more desirable than under-feeding (Tacon and Cowey, 1985). Similarly, previous studies reported that feeding Nile tilapia with 30-36% protein shows FCR values around 1.3-2.0 which are more reasonable (Hafedh, 1999; Leal *et al.*, 2010). If the amount of plant proteins (soy proteins) in a given diet

increases, so will the amount of anti-nutritional factors like non-starch polysaccharides, phytin, and trypsin inhibitors. These factors also affect palatability for piscivorous fish (Enterria *et al.*, 2011; Francis *et al.*, 2001). Moreover, FCR is hinging on several different constituents consisting of feeding rate, nutritional contents of diet, species, sizes, and fish behaviors. An increase in the level of feeding can reduce feed conversion ratio (Craig *et al.*, 2017; Inayat and Salim, 2005).

Protein efficiency ratio (PER) is used as an indicator of protein retention of conversion ratio (Koumi *et al.*, 2009). After 10 weeks trial, the values of PER and PPV is the same trend to the weight gain of Nile tilapia which fish fed SIS25 did not show significant differences with fish fed control diets, but its values decreased significantly after fish received SIS between 50%-100% in diets. The values of PER (1.56±0.01 to 2.86±0.09%) in this present study is comparable to the previous studies (Hassaan *et al.*, 2019; Silva *et al.*, 2017). However, Abarra *et al.* (2017) reported that PER value of Nile tilapia fingerlings fed increasing knife fish meal replacement levels was recorded between 1.31±0.04 and 1.66±0.07% which was lower than this present study. Meanwhile, the PER values (2.61±0.02 to 3.10±0.01%) of Nile tilapia fed different levels of palm oil were a bit higher than our finding (Ayisi *et al.*, 2017) and It was expected that the highest PER would occur at 8% dietary PO rather than 6% PO as occurred because utilization of protein by most aquaculture fish can be improved by increasing dietary energy levels (Cho and Kaushik, 1985). The protein utilization (PER and PPV) herein decreased with increasing dietary protein levels and fish density. The maximum PER and PPV values were obtained when fish at lower density were fed on the 25% CP diet. These results may have occurred because weight gain was related to the deposition of protein, and protein accretion is a balance between protein anabolism and catabolism (Abdel-Tawwab, 2012).

Many studies reported the effects of plant protein used to replace fishmeal protein source on survival rate in Nile tilapia (Agbo *et al.*, 2011; Boussou *et al.*, 2017). After 10 weeks trial, Nile tilapia fed with all experimental diets was affected to survival rate with the exclusion of negative control diet indicating that substitution of SIS at different inclusion protein levels influenced to fish health. It is in relation to the study of Lightbourne (2011) which survival rate of Summer flounder ranged from 92-

100% in the various treatments of the feeding trial and was not significantly different among treatments when SBM was replaced by FM.

5.2. Whole-body composition

The proximate composition differs from species to species depending on various conditions such as age, sex, temperature, diets, stage of development, ration size and body weight, environment, and season (Huss, 1988; Papoutsoglou and Papaparaskeva-Papoutsoglou, 1978). Additionally, the variations in proximate composition of fish are closely related to the feed intake (Boran and T Karaçam, 2011). Agbede *et al.* (2007) described that plant protein sources have lower quality protein than the animal protein sources. In general, fish diet requires to be substituted with other protein sources comparatively abundant in the essential amino acids. In this present study, replacement of FM with SIS in fish diets was affected to the whole-body fish composition such as the whole protein, fat, and ash content with exception of the whole moisture content. The initial moisture content was 76.77±0.90%. After Nile tilapia receiving SIS in diets for 10 weeks, the final moisture content values of each treatment were found between 72.65±0.49% to 73.72±0.69% which did not show significant differences ($p > 0.05$) among all treatment fish groups. This is related to the study in Nile tilapia, *Oreochromis niloticus* (Carneiro *et al.*, 2017; Hafedh, 1999); African catfish, *Clarias gariepinus* (Abdel-Warith *et al.*, 2019) and juvenile parrot fish, *Oplegnathus fasciatus* (Kim *et al.*, 2016). Olopade *et al.* (2017) stated that the moisture content values in fish muscle range from 65.0-75.8% could be an acceptable value. The protein content in the whole-body fish ranged from 52.40±0.18-62.24±0.74% which showed significant differences ($p < 0.05$) among all treatment groups. These values can be compared to Ayisi *et al.* (2017) which the whole-body protein composition ranged from 57.26-61.58%. Based on the whole-body composition, substituting SIS protein level up to 25% in diets showed significant increase in whole-body fat content, and it showed significant decreases with the increasing SIS between 50%-100% in diets. Several studies have similarly found that body lipid content decreased in fish fed diets with high inclusion level of plant protein sources (Opstvedt *et al.*, 2003; Tibaldi *et al.*, 2006). In fish, lipid is stored mainly in perivisceral adipose tissue, liver and muscle or even subcutaneous

tissue, with specific difference in the localization of lipid deposits (Corraze, 2001). It is difficult to give a simple but comprehensive explanation for the effect of dietary plant protein on lipid deposition of fish (Yang *et al.*, 2009). Differences among these studies in species and fish size (Corraze, 2001), levels of dietary intake (Gómez-Requeni *et al.*, 2004), plant protein sources selected and specific botanical component (Dias *et al.*, 2005), and amino acid pattern (Kaushik *et al.*, 1995) as well as rearing conditions and the duration of feeding experiments (Noble *et al.*, 1998) may in part account for the discrepancies in lipid deposition. These results indicate that the protein and fat content were affected by SIS protein levels in diets. Ash content of Nile tilapia were found from 9.92±0.11 to 16.41±0.30% which showed significant differences between treatments. The quantities of ash content among the dry fishes from 9-30% on the source of moisture and dry matter content values (Flowra *et al.*, 2012). Moreover, Ash contains different kinds of minerals which play an important role in body structure such as calcium, iron, magnesium, zinc and so on (Islam, 2017).

5.3. Apparent digestibility coefficients

ADC dry matter of numerous protein feedstuffs offers an estimate of overall digestibility, and a low value usually indicates that a high level of indigestible material is present in the feedstuff (Li *et al.*, 2013). Thus, dry matter ADCs have been considered to provide a better estimate of the amount of indigestible material present in feedstuffs in comparison with digestibility coefficients for individual nutrients (Luo *et al.*, 2008). The protein quality of the dietary ingredients is usually the leading factor affecting fish performance and protein digestibility and is the first measure of its availability for fish (Yu *et al.*, 2013). In this present study, the ADC of dry matter and protein in SIS25 diet had the highest value compared to control diet. After substituting SIS between 50%-100% in diet, the ADC showed significant decreases. This study is in an agreement with (Gaber, 2006) and reported that the low protein digestibility (BBM) recorded here is likely due to the quality of raw material. The presence of higher concentrations of total phenolic substance reduced protein digestibility and amino acid availability through phenolics-protein and/or phenolics-protein enzyme complexes (Grabner and Hofer, 1985; Siddhuraju and Becker, 2001). ADC values depend on many factors such as the feed composition (fiber content of the feed) as

well as the marker used (Austreng *et al.*, 2000; Goddard and McLean, 2001; Silva *et al.*, 2017), total fecal collection or the use of an indicator are expensive, time consuming and require relatively large amounts of feed (Schneider and Flatt, 1975). Moreover, Saponins reduce protein digestibility probably by the formation of sparingly digestible saponin–protein complexes (Potter *et al.*, 1993). Diets that contain a high level of animal protein and those composed principally of plant-based ingredients are all highly digestible. Improvement of protein digestibility could be attributable to the reduction or elimination of different antinutrients during the pretreatment process, especially phytic acid and tannins which are known to interact with protein to form complexes. This can be also related to higher efficiency of the thermal treatment, reducing trypsin and chymotrypsin inhibitory activities (Alonso *et al.*, 2000; Musa *et al.*, 2012).

5.4. Blood biochemistry

In fish, the haematological characteristics have been studied to founding common blood values and ranges with respect to sex, stage of development, size, and environmental and physical factors (Fazio *et al.*, 2013; Gabriel *et al.*, 2007; Kori-Slakpere, 1985; Sowunmi, 2003). Many aquaculturists and fishery biologists are extremely anxiety of global warming which caused to increasing of water temperature. The water temperature variations possibly will significantly motivate the regular physiological processes, growth and survival of fish, thus decreasing the fish abundance (Pörtner and Peck, 2010). However, fish maintained in different production systems may present haematological changes that are reflective of their health status or an imbalance in their physiology (Ghiraldelli *et al.*, 2006). According to many researchers reported about aquatic haematology, explanation of standard value ranges are reasonably hard and complex (Vácha, 1980). Comparing the present records to earlier researches, there is evident agreement with the previously published range in term of haematological indices values. This present study, hemoglobin, red blood cell, and white blood cell values in Nile tilapia fed all experimental diets were significant differences ($p < 0.05$). As the results, the hemoglobin concentration values range from 4.31±0.25 to 5.85±0.05 $g.dL^{-1}$, the amount of red blood cells from 1.125±1.00 to 2.20±3.74 $x10^{6}$ $cells/mm^{3}$, the amount of white blood cells 79.94±4.61

to 160.69±22.03 $x10^3$ cells/mm^3, and haematocrit values from 21.57±0.89 to 48.76±0.87 percent, respectively. The parameter values of this founding can be compared to the study of Steckert *et al.* (2019) which were found the numbers of red blood cells range from 1.4×10^6 and 2.4×10^6 μL^{-1} as its minimum and maximum value respectively. The higher red blood cells levels can increase the oxygen carrying capacity of blood, thus supplying oxygen to major organs in response to higher metabolic demand, which is a manifestation of stress (Ruane *et al.*, 1999). Together with the hematocrit values, the lowest and highest value were recorded as 25.1 and 36.5%. Comparing the present data of hematological values in Nile tilapia to previous studies, there is evident agreement with Dal'Bó *et al.* (2015) which haemoglobin concentration values were extended from 4.75 g/dL to 6.48 g/dL, total number of red blood cells between 1.03 to 1.70 $x10^6$/ml. The assortment of haemoglobin within species suggests an association with blood oxygen transport as an index of the aerobic metabolic capabilities of fish (Graham *et al.*, 1985). Similarly, Soltan *et al.* (2008) investigated the effects of using cottonseed, sunflower, canola, sesame and linseed meals as the plant protein mixture diet (PPM diet) to replace fishmeal with different protein levels (15, 30, 45, 60, 75, 90, and 100%) in Nile tilapia diets. The result revealed the lowest to the highest value of hemoglobin as 4.23 g/dL and 7.10 g/dL respectively, which the highest value observed in control diet (fishmeal-based diet). These results suggested that, the lower hemoglobin values in plant protein mixture diet was affected by the binding of Phytic acid and gossypol molecules and the other toxic presences in plant protein mixture to iron and amino acids affecting their low availabilities in the body and increased red blood cell flimsiness. Weber (1996) reported a decrease in the concentration of triphosphate nucleotides in some fish species after they were submitted to hypoxia. While the temperature increases, water oxygen concentration falls. Additionally, sex, stage of development, seasons, and the environmental characteristics can affect to hemoglobin concentrations. Other similar reference values were established in researches of Azevedo *et al.* (2006) and Ghiraldelli *et al.* (2006). The related study was found in report of Navarro *et al.* (2018) which studied the hematological parameters by using different oil sources (linseed oil, soy oil, fish oil, and corn oil) into 4 different diets in Nile tilapia diets. The result showed that the hematocrit values ranged from 35-44%. This study

recommended that the higher concentration of omega-3 polyunsaturated fatty acid may have caused a better resistance in the cell wall of erythrocytes and consequently a higher percentage of hematocrit. Based on the characterization, hematocrit is an uncomplicated, indirect scale of hemoglobin and $CaO_{2.}$ In fish, the measurements of hematocrit value have been investigated (Gallaugher, 1994). Several studies reported that lively aquatic species display higher hematocrit, hemoglobin concentration and red blood cells values, compared to less active species (Wilhelm Filho *et al.*, 1992). For example, hematocrit values of fish were recorded up to 53% of tuna (*Thunnus thynnus*), mackerel (*Auxis rochei*), compared with values for more sedentary species such as 8.5% hagfish (*Eptatretus cirrhatus*) (Gallaugher, 1994). In case of splenic contraction and red blood cell swelling, these can cause the increasing of hematocrit values (Hrubec and Smith, 2010). Hematocrit can vary in fish in relation to their swimming performance. This present study, white blood cells values of Nile tilapia increased to the highest value after receiving SIS25 diet and it started decreasing when supplementation of SIS in diets between 50%-100%. These suggesting that SIS used in Nile tilapia diets between 50%-100% were affected to the changes of WBCs. The changes in increasing or declining numbers of white blood cells are a normal reaction to the exposure of toxicants (Kori-Siakpere *et al.*, 2006). Moreover, white blood cells of fish respond to different stress conditions comprising infections and chemical vexations (Christensen *et al.*, 1978). In animals, the differentiations of white blood cell profiles are sensitive to variety of physiological and environmental changes, and health status (Ritchie et al., 1993). The responses normally found when fish are subjected to an array of toxicants are lower percent lymphocytes and higher percent neutrophils (Witeska, 2005). Many researchers studied the mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), and mean corpuscular volume (MCV) in Nile tilapia (Mahmoud *et al.*, 2020; Navarro *et al.*, 2018; Sebastião *et al.*, 2011). To the best of our knowledge, this is the first report which perform the effects of dietary SIS supplementation on haematological parameters of fish. In this present study, MCHC, MCH, and MCV of Nile tilapia fed all experimental diets showed significant differences ($p<0.05$). Wepener *et al.* (1992) indicated that the MCHC is a good indicator of red blood cell swelling, the ratio of blood haemoglobin concentration as resisted to the haematocrit value. Normally,

MCHC is not influenced by the blood volume nor by the number of cells in the blood but it can be interpreted incorrectly when new cells, with a different haemoglobin concentration, are released into blood circulation (Soivio and Nikinmaa, 1981). Additionally, MCHC is reduced (hypochromic) in some types of the microcytic anemia, in which the degree of loss of hemoglobin is more than loss of cell volume, but normal (normochromic) in macrocytic anemia, in which hemoglobin is increased, but the cell size is also proportionally increased (Zheng *et al.*, 2015). However, the decrease of (MCHC) and (MCH) may pave the way for hypochromic anaemia which means a decrease in the fish blood colour, as a result of decrease in erythrocyte haemoglobin content (Zuckerman, 2007). These responses suggest that haematological parameters of Nile tilapia receiving all different experimental diets were influenced by supplementation of SIS in diets.

5.5. Histological characteristics

5.5.1 Intestinal tissue

In this present study, Nile tilapia fed with SIS up to 25% protein level was not affected to intestinal morphology. After substituting SIS between 50%-100% in diet, the villi of intestines were decreased. These results indicate that the presence of anti-nutritional factors of SIS are the main factor of intestinal morphology changes. In fish, various studies have been reported the morphor-physiological characteristics of intestines and used them as an important criterion for classification of feeding habits (Albrecht *et al.*, 2001; de Moraes *et al.*, 2004; Fagundes *et al.*, 2016). Among all the findings, changing in intestinal tissue depends on many factors including the uses of plant protein sources in diet (Sáenz de Rodrigáñez *et al.*, 2009), probiotic administration (Caballero *et al.*, 2003), the presences of anti-nutritional factors in plant ingredients. The anti-nutritional factors which were found in SIS are total phenol, condensed tannin, saponins, trypsin inhibitor, DPPH radical scavenging, and FRAP, these factors have made negative impacts to the intestinal tissue structure and lead to an intestinal inflammation in some species (Francis *et al.*, 2001; Rawdkuen *et al.*, 2016; Venou *et al.*, 2006). In this study, SIS were roasted at high temperature to destroy anti-nutritional factors. Similarly, Bueno-Borges *et al.* (2018) reported that oven-roasting SIS at 160 ˚C for 15 min has ability to reduce the anti-nutritional factor

and enhanced antioxidant activity, except for saponins, which cannot be damaged by high temperature (Reddy and Pierson, 1994). Saponins are widely distributed plant natural products with vast structural and functional diversity (Moses *et al.*, 2014). In fish, saponins have been reported to be highly toxic to fish because of their damaging effect on the respiratory epithelia (Roy *et al.*, 1990). They are also considered to be the active components of many traditionally used fish poisons, like mahua oil cake (Francis *et al.*, 2001). Fish have also been shown to exhibit stress reactions to the presence of saponins in water. Roy and Munshi (1989) reported that the O_2 uptake of perch (*Anabas testudineus*) increased with a concomitant increase in the erythrocyte, hemoglobin and packed cell volume levels, after the fish had been in water containing 5 mg Quillaja saponin/l for 24 h. *Penaeus japonicus* that had been previously exposed to concentrations of 20 mg saponin/l for 24 h increased both respiration rate and metabolism (measured as increase in O_2 uptake and NH_4 excretion) during a 6 h detoxification process (Chen and Chen, 1997). There are also numerous reports of negative effects of dietary saponins. These negative effects have been ascribed to several properties of saponins such as reduced feed intake caused by the astringent and irritating taste of saponins (Oleszek *et al.*, 1994), reduction in intestinal motility (Klita *et al.*, 1996), reduction in protein digestibility (Shimoyamada *et al.*, 1998) and damage to the intestinal membrane and inhibition of nutrient transport described earlier in the present review. Thus, the structure of the intestinal cell rapidly and reversibly responds to dietary changes. For instance, alterations in the intestinal mucous of fishes fed diets with unbalanced fatty acid composition have been described for Artic charr (Olsen *et al.*, 1999; Olsen *et al.*, 2000). In fish, the length of intestine varies and depends on the diet, but mostly between 0.4 and 38 times longer than the body length. The amount of plant constituents in diet is the major determination factor for intestinal length. Generally, herbivorous fish have longer intestine compared to carnivorous fish (Clements and Raubenheimer, 2006). In this present study, the length of intestines of Nile tilapia fed positive and negative control diet (Fishmeal-based control diet), SIS8 diet, SIS15 diet, SIS25 diet showed significant increases while the length of intestine significantly decreases after increasing amount of SIS in excess of 25% in diet. The result indicated that Nile tilapia can accept SIS at level of 25%, while substituting SIS between 50%-100% the

villi of Nile tilapia can be damaged. Devoid of exclusion, all of the plant protein sources additions are quite lower in methionine and lysine than animal protein sources, and many are also lower in total sulfur containing amino acids (O'Keefe, 2003; van Vliet *et al.*, 2015). In mammals, numerous studies reported that methionine deficiency suppressed epithelial cell growth, reduced intestinal antioxidant capacity and affected intestinal cell fate (Li *et al.*, 2014; Pan *et al.*, 2016). The methionine deficiency decreased the height of intestinal villus and microvilli, as well as the number of goblet cells in turbot (Gao *et al.*, 2019).

5.5.2 Liver tissue

According to the liver tissue straining, the liver cells of fish receiving SIS up to 50% did not show significant changes in hepatic cells, but the nuclei of all treatment fish were damaged. Its damages were probably caused by enzyme which was used in technical processes. Moreover, the liver tissues of Nile tilapia receiving SIS between 75%-100% protein levels showed intra cytoplasmic round circumscribed fat vacuoles. These responses might be affected by the dietary diets with high fat content up to 15%. This present study is in an agreement with the study of Obirikorang *et al.* (2018) which dietary inclusions of swine fat at 7% and 10% (12% and 15% of fat content in diet) affected hepatic lipid metabolism of Nile tilapia, and the histological examinations showed varying degrees of degenerative features such as lipid deposition and nuclear migration. Also, histological section of liver from *Clarias gariepinus* fed with high maize gluten meal (75%) showed severe intracellular lipid deposition and abnormal nuclei (Abdel-Warith *et al.*, 2014). This could be attributed to high level of carbohydrate in MGM 75% diet compared with other two diets (substituting MGM at 25% and 50% protein levels). Interestingly, in the present experiment, isolated areas of necrosis in hepatocytes were located in the livers of fish fed higher levels of MG, indicating possible irreversible effects on fish health due to nutritional imbalances (Mosconi-Bac, 1990).

Furthermore, hepatic histological alterations due to nutritional imbalances provide insights into cell and tissue conditions and can complement somatic growth in evaluating the nutritional requirements and status of fish (Kumar *et al.*, 2005; Wang *et al.*, 2014). A recent trend in fish feeds is to use higher levels of lipids in the diet.

Although increasing dietary lipids can help reduce the high costs of diets by partially sparing protein in the feed, problems such as excessive fat deposition in the liver can decrease the health and market quality of fish (Craig *et al.*, 2017).

Similarly, plant ingredients used in the aquatic diet can affect the process of digestion because of the existence of anti-nutritional factors (Francis *et al.*, 2001). Besides, proteases activities in the intestine and hepatopancreas may be affected by the plant protein source (Lin *et al.*, 2010), potentially inhibiting the growth and feed utilization of Nile tilapia.

5.6. Gene expression

Gene expression studies are known as powerful methods in toxicology and environmental biomonitoring (Abo-Al-Ela *et al.*, 2017a; Iguchi *et al.*, 2006; Knapen *et al.*, 2007). Identification of genes that related to immunity in fish and determination of their expression patterns receive a great attention (Abo-Al-Ela *et al.*, 2017b). Many researchers have been studied the expression of genes in Nile tilapia (Amer *et al.*, 2019; Chen *et al.*, 2016; Hassaan *et al.*, 2019; Zhi *et al.*, 2018) such as TNF-α and IL-1β (Dawood *et al.*, 2020; Kayansamruaj *et al.*, 2014), TGF-β (Elabd *et al.*, 2019). TNF-α is a central regulatory cytokine in antimicrobial and inflammatory responses (Grayfer *et al.*, 2008). It is also largely expressed in non-specific cytotoxic cells of tilapia (Praveen *et al.*, 2006). Correspondingly, it is involved in viral (Purcell *et al.*, 2004) and ectoparasitic (Saurabh *et al.*, 2011) invasions. Moreover, the expression of TNF could be considered for the assessment of fry-rearing environment (Wai-San Lam *et al.*, 2011). It appears to be the key regulator and effector in immune responses by regulating cell death and survival (Locksley *et al.*, 2001). Zhang *et al.* (2018) suggested that IL-1β and TNF-α are both involved in the enteritis process as their expression level in SM-fed fish was higher than FM fed fish. It is a key player in the defence against microorganism invasion and tissue injury and is able to induce immune responses by stimulating lymphocytes or by enhancing the release of other cytokines that can activate NK cells, macrophages and lymphocytes (Low *et al.*, 2003). In fish, IL-1β gene is constitutively expressed in numerous tissues such as spleen, head kidney, as well as liver, while higher expression has been found out in the spleen (Lu *et al.*, 2008; Tafalla *et al.*, 2005). It is a highly inducible gene that is

upregulated shortly after stimulation (Hong *et al.*, 2001). Even a change in a dietary components in hybrid tilapia (Zhang *et al.*, 2014) or the addition of oxytetracycline, formic and propionic acid/salt mixture to the feed of Nile tilapia (Reda *et al.*, 2016) changed the expression of IL-1β and disease resistance. Furthermore, in chronically stressed fish (Bermejo-Nogales *et al.*, 2007), during the smolting of Atlantic salmon (Ingerslev *et al.*, 2006), as well as in acute stress in common carp, *Cyprinus carpio* L. (Metz *et al.*, 2006) there was an increase in the expression of pro-inflammatory cytokines (IL-1β, TNF-α). The regulation of IL-1β and TNF-a is accomplished by a network of counter-acting cytokines such as transforming growth factor β (TGF-β) (Li *et al.*, 2006). TGF-β is a pleiotropic cytokine that controls the initiation and resolution of inflammatory responses (Xu *et al.*, 2018). TGF-β 1–3 are unique multi-functional growth factors that are only expressed in mammals, and mainly secreted and stored as a latent complex in the extracellular matrix (Xu *et al.*, 2018). In this present study, the expression of TNF-α, IL-1β, and TGF-β gene levels did not affect to fish fed all the experimental diets. Similar report was observed in Japanese seabass which the expression of IL-1β in midgut did not show significant differences among all diets when fishmeal was replaced with soybean meal at different inclusion protein levels (0 ,50, 75%) (Zhang *et al.*, 2018). Although TNF-α and TGF-β expression levels did not show significant differences among all treatments, but its values tend to rise with an increasing protein levels of SIS up to 25%. Up-regulated expression of pro-inflammatory genes by SM administration has been reported in turbot (Gu *et al.*, 2016), common carp (Urán *et al.*, 2008). Similarly, Immunoregulatory TGF-β expression was enhanced by Moringa incorporation throughout the entire experiment (Elabd *et al.*, 2019). TGF-β functions in the non-specific immune response and the expression of TGF-β can also be stimulated by plant extracts (Panprommin *et al.*, 2011). These responses suggest that substituting FM with inclusion levels up to 25% of SIS can be a consequence of improved immune response.

CHAPTER 6

CONCLUSION

This present study was performed the substitution of fishmeal with sacha inchi seed in Nile tilapia diets at different inclusion protein levels from 0, 8, 15, 25, 50, 75, and 100%. Nile tilapia fed with SIS at 25% protein level was the best in growth performance, the whole-body composition, apparent digestibility coefficients of dry matter and protein. Meanwhile SIS used in diets have influenced to hematological parameters and histological characteristics. Moreover, no significant differences were detected in expression levels of TNF-α, IL-1β, and TGF-β genes. However, the expression levels of TNF-α, IL-1β, and TGF-β genes tend to enhance when substituting FM with SIS up to 25%. The increased levels of target genes were the same trend with the WBCs value. According to economic analyses of experimental diets showed that the SIS25 diet offers the best economic viability, taking into consideration the cost of feed fed over the trial period. These responses suggest that substituting FM with inclusion levels up to 25% of SIS can be a consequence of improved immune response and showed the best growth performance in Nile tilapia.

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APPENDIX

1. Determination of moisture content (AOAC, 2000)

Method

1. Dry the crucible tube in the oven at 105 ^{o}C for 3 hours and transfer to desiccator to cool. Weigh the crucible tube.
2. Weigh about 2 g of sample to the crucible tube. Spread the sample to the uniformity.
3. Place the crucible tube to the hot air oven. Dry for 3 hours at 105 ^{o}C.
4. After drying, transfer crucible tube to the desiccator to cool. Reweigh the crucible tube and its dried sample.

Calculation

$$\textbf{Moisture (\%)} = \frac{(\mathbf{W1}-\mathbf{W2}) \times \mathbf{100}}{\mathbf{W1}}$$

Where: W1 = weight (g) of sample before 1drying
W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 2000)

Reagents

1. Kjedehl catalyst: Mix 100 g of potassium sulphate (K_2SO_4) with 7 g of copper sulphate ($CuSO_4$)
2. 93-98% of Sulfuric acid (H_2SO_4)
3. 45% of Sodium hydroxide (NaOH) solution
4. 0.1 N of Hydrochloric acid (HCl) solution
5. 4% of Boric acid, H_3BO_3
6. Indicator solution: Mix 100 ml of 0.2 g methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Method

1. Place sample (0.5 g) in digestion tube.

2. Add 2 g Kjedehl catalyst and 15 ml of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

$$\textbf{Protein (\%)} = \frac{(\mathbf{A}-\mathbf{B})\times \mathbf{N} \times \mathbf{1.4007} \times \mathbf{6.25}}{\mathbf{W}}$$

Where A = volume (ml) of 0.2 N HCl used sample titration
B = volume (ml) of 0.2 N HCl used in blank titration
N = Normality of HCL
W = weight (g) of sample
1.4007 = atomic weight of nitrogen
6.25 = the protein-nitrogen conversation factor for fish and its by-products

3. Determination of ash content (AOAC, 2000)

Method

1. Place the crucible and lid in the furnace at 550 ^{o}C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.

4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.
5. Heat at 550 ^{o}C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to grey. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

$$\textbf{Ash} = \frac{\textbf{Weight of ash} \times \textbf{100}}{\textbf{Weight of sample}}$$

4. Determination of fat content (AOAC, 2000)

Reagents

- Petroleum ether

Method

1. Place the bottle and lid in the incubator at 105 ^{o}C overnight to ensure the weight of bottle is stable.
2. Weigh about 3-5 g of sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into Soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the Soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min)
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90 ^{o}C until solvent is completely evaporate and bottle is completely dry.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

Calculation

$$\textbf{Fat (\%)} = \frac{\textbf{Weight of fat} \times \textbf{100}}{\textbf{Weight of sample}}$$

BIOGRAPHY

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