EXOPOLYSACCHARIDE FROM THE LIQUID CULTURE OF *Ganoderma lucidum* AS FEED ADDITIVE FOR RED HYBRID TILAPIA (*Oreochromis* sp.)

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FACULTY OF SCIENCE
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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)

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EXOPOLYSACCHARIDE FROM THE LIQUID CULTURE OF *Ganoderma lucidum* AS FEED ADDITIVE FOR RED HYBRID TILAPIA (*Oreochromis* sp.)

ABSTRACT

Feed additive plays a significant role in sustaining fish production. Exopolysaccharide (EPS) obtained from the fermented mycelium of *Ganoderma lucidum* produced by using submerged-liquid fermentation (SLF) was proposed as a feed additive in this study for red hybrid tilapia (*Oreochromis* sp.). Body composition, organosomatic indices, and haematological parameters were observed in fish fed with three different diets supplemented with EPS (1 g/kg, 2 g/kg, and 3 g/kg). Although higher concentration EPS feed was observed to produce minor effect on body composition and organosomatic indices, significant effect on both parameters was observed in fish fed with 1 g/kg diet. All diets showed condition factor (CF) values of above 1.4, indicating good fish health and isometric growth. Furthermore, the blood analysis of EPS-treated red hybrid tilapia showed that haemoglobin (HGB: 7.43 g/dl), haematocrit (HCT: 37.50 %), and red blood cell (RBC: 2.69 10^6/mm^3) were significantly increased (P<0.05) at 3 g/kg while the white blood cell (WBC: 176.65 10^3/mm^3) and mean corpuscular haemoglobin concentration (MCHC: 198.50 g/L) were numerically high at 3 g/kg. These findings showed that high EPS supplemented diet could enhance the health status of fish by boosting its immunity. However, the diet supplemented with 1 g/kg is sufficient in contributing to the nutritional value of fillet and improving the health status of red hybrid tilapia, which is suitable for fish farming.

Keywords: *Ganoderma lucidum*, exopolysaccharide, red hybrid tilapia, body composition, organosomatic index, haematological.

EXOPOLYSACCHARIDE DARI FERMENTASI CECAIR *Ganoderma lucidum* SEBAGAI TAMBAHAN MAKANAN UNTUK TILAPIA HIBRID MERAH (*Oreochromis* sp.)

ABSTRAK

Aditif makanan memainkan peranan penting dalam mengekalkan pengeluaran ikan. *Exopolysaccharide* (EPS) daripada miselium fermentasi *Ganoderma lucidum* yang dihasilkan menggunakan fermentasi cecair terendam (SLF) dicadangkan sebagai aditif makanan dalam kajian ini untuk tilapia hibrid merah (*Oreochromis* sp.). Komposisi badan, indeks organosomatik, dan parameter hematologi telah diperhatikan pada ikan yang diberi makan dengan tiga jenis makanan yang mengandungi EPS (1 g/kg, 2 g/kg, dan 3 g/kg). Walaupun makanan yang mengandungi EPS yang tinggi menghasilkan kesan kecil pada komposisi badan dan indeks organosomatik, tetapi kesan yang ketara pada kedua-dua parameter boleh diperhatikan dalam ikan yang diberi makanan 1g/kg EPS. Semua diet menunjukkan nilai faktor keadaan (CF) di atas 1.4, di mana ia menunjukkan kesihatan ikan yang baik dan pertumbuhan isometrik. Tambahan pula, analisis darah tilapia hibrid merah yang dirawat EPS menunjukkan bahawa hemoglobin (HGB: 7.43 g/dl), hematokrit (HCT: 37.50 %), dan sel darah merah (RBC: 2.69 10^6/mm^3) meningkat dengan ketara (P<0.05) pada 3 g/kg manakala sel darah putih (WBC: 176.65 10^3/mm^3) dan purata kepekatan hemoglobin korpuskular (MCHC: 198.50 g/L) menunjukkkan nilai yang tinggi pada 3 g/kg dari segi nilai purata. Penemuan ini menunjukkan bahawa diet EPS yang tinggi boleh meningkatkan imuniti. Walau bagaimanapun, makanan yang ditambah dengan 1 g/kg mencukupi untuk menyumbang kepada nilai pemakanan fillet dan meningkatkan status kesihatan tilapia hibrid merah, yang sesuai untuk penternakan ikan.

Kata kunci: *Ganoderma lucidum*, *exopolysaccharide*, tilapia hibrid merah, komposisi badan, indeks organosomatik, hematologi.

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ºC	:	Degree Celsius
cm	:	Centimetre
g	:	Gram
h	:	Hour
kJ/g	:	Kilojoule per gram
mg L^{-1}	:	Milligrams per litre
min	:	Minute
mL	:	Millilitre
%	:	Percentage
rpm	:	Revolutions per minute
CA	:	Crude ash
CBC	:	Complete blood counts
CF	:	Crude fibre
CF	:	Condition factor
CL	:	Crude lipid
CP	:	Crude protein
DCP	:	Dicalcium phosphate
d.m.	:	Dry matter
DO	:	Dissolved oxygen
DW	:	Dry weight
GE	:	Gross energy
HGB	:	Haemoglobin
HSI	:	Hepatosomatic index
Ht	:	Haematocrit
MCH	:	Mean corpuscular haemoglobin

MCHC	:	Mean corpuscular haemoglobin concentration
MCV	:	Mean corpuscular volume
NFE	:	Nitrogen free-extract
RBC	:	Red blood cell
VSI	:	Viscerosomatic index
WBC	:	White blood cell
wt	:	Weight

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Tilapia is one of the freshwater fish widely produced in the world as a protein source for food, including in Malaysia. Red hybrid tilapia is in high demand due to its high versatility, rapid growth and nutritional value (Haque et al., 2016; Islam et al., 2006). Based on the report by FAO (2019a), Malaysians prefer to consume red hybrid tilapia (*Oreochromis* sp.) because of its attractive colour. FAO (2019a) also stated that the production of freshwater fish is predicted to increase up to 725, 119 fishes in 2020 from 190, 000 fishes in 2014. As the demand for this fish is very high, there is a need to search for an alternative way to increase the growth rate and the quality of fish by using feed additive. Besides, fish diseases may influence the production of red hybrid tilapia. Therefore, a potential feed additive can be used to reduce the problems and to improve its quality of production.

Disease is one of the vital issues in fish production. Currently, the newly concern disease is Tilapia Lake Virus (TiLV), which mainly affects tilapines in Africa, South America and Asia (Jansen et al., 2018). A case reported by Amal et al. (2018) stated high mortality of red hybrid tilapia experienced with TiLV disease in Malaysia farm. Other researchers also stated that TiLV affected other tilapines such as *Sarotheroden* (Tilapia) *galilaeus*, commercial hybrid tilapia (*O. niloticus* × *O. aureus*) and farmed tilapia *O. niloticus* (Bacharach et al., 2016; Eyngor et al., 2014; Ferguson et al., 2014). Thus, to overcome this problem, more researchers conduct studies by including feed additive containing immunostimulant properties to replace vaccines due to its limited spectrum of activity. However, this study is a preliminary study prior to the immune study to measure the response of EPS on fish body composition, organosomatic indices and haematological effect.

A feed additive is an ingredient supplemented in feed without replacing any primary components. The supplementation helps to increase the aquaculture production efficiently, to enhance fillet quality together thus reducing the waste discharges, and lastly to minimize the usage of other medication (Goncalves & Santos, 2016).

G. lucidum is well known as mushroom (fungi) which contains many valuable properties useful in medication for curing and preventing human disease. It is well-known as Chinese traditional medicine and has the ability to enhance the body resistance to disease like cancer (Cao et al., 2018; Meng et al., 2014). Roy et al. (2015) stated that *G. lucidum* has high nutritional potentials including protein. It also contains some functional metabolites which can be a source of antioxidant property (Cohen et al., 2002). The specialities of this *Ganoderma* sp. encouraged it to be introduced as a feed additive in aquaculture. *G. lucidum* has been proven to be a good feed additive in aquaculture by Mohan et al. (2016) on giant freshwater prawn (*Macrobranchium rosenbergii*) and by Chithra et al. (2016) on the grass carp (*Ctenopharyngodon idella*). *G. lucidum* contains two types of polysaccharides called exopolysaccharide and intracellular-polysaccharide. To obtain the polysaccharide from *G. lucidum*, Supramani et al. (2019) suggested to use submerged-liquid fermentation (SLF) and go for a low scale production by fed-batch fermentation for experimental purpose. The potential feed additive in this study for the red hybrid tilapia is the exopolysaccharide from the fermented mycelium of *Ganoderma lucidum*.

Body composition and organosomatic indices are essential to determine the feed quality, the fish consumption desire and the growth (Breck, 2014). The body composition, the hepatosomatic index, the viscerosomatic index and the condition factor are used as indicators to assess the food quality value (Ighwela et al., 2014; Jin et al., 2015; Li et al., 2012; Nalawade & Bhilave, 2011).

Haematology parameters are indicators to determine the physical condition of cultured fish after feeding with feed additive according to the allocated diet values (Fagbenro et al., 2013). Based on Alsaid et al. (2015) study, haematology parameters act as health indicators in which contribute to dealing with the diagnosis of fish disease. Hence, it is essential to take into consideration the body composition, the organosomatic indices and the haematological indices to determine the feed efficiency as well as the health condition of the fish.

To the best of author's knowledge, this study is the first report on exopolysaccharide of the fermented mycelium of *G. lucidum* to be used as a feed additive for red hybrid tilapia (*Oreochromis* sp.). Therefore, this study could help to fulfil the gaps of knowledge as a preliminary study.

1.1 Hypothesis

Exopolysaccharide from the fermented mycelium of *Ganoderma lucidum* can be an alternative feed additive without any negative effect on red hybrid tilapia body composition, organosomatic indices and haematological indices.

1.2 Objectives

1. To analyse the body composition of red hybrid tilapia (*Oreochromis* sp.) fed with diets containing exopolysaccharides from the fermented mycelium of *Ganoderma lucidum*.
2. To determine the organosomatic indices of red hybrid tilapia (*Oreochromis* sp.) fed with diets containing exopolysaccharides from the fermented mycelium of *Ganoderma lucidum*.

3. To measure the haematological indices of red hybrid tilapia (*Oreochromis* sp.) fed with diets containing exopolysaccharides from the fermented mycelium of *Ganoderma lucidum*.

CHAPTER 2: LITERATURE REVIEW

2.1 Malaysian Aquaculture

Aquaculture is widely practiced in Malaysia due to its demand that can contribute to the growth of Malaysian economy besides acting as a protein source. According to Yusoff (2015), by comparing all types of the aquaculture industry, freshwater aquaculture contributes to the second highest production of fishes (163, 757 tonnes of fishes) which valued at RM 992 million in 2012. The fishes include catfish (*Clarias* sp.), black and red tilapia (*Oreochromis* sp.), riverine catfish (*Pangasus* sp.) and freshwater giant prawn (*Macrobranchium rosenbergii*). Amongst all the species cultured, one of the top Malaysian commercially important freshwater fish is tilapia. Therefore, any problem related to the production of tilapia fish needs to be overcome in order to enhance the production of tilapia in Malaysia.

2.1.1 Tilapia Fish

Tilapia is a term used to classify the three main aquaculture genera such as *Oreochromis*, *Sarotherodon* and *Tilapia* (Popma & Masser, 1999). The reproductive behaviour shows the best way to distinguish between these three genera. Based on Popma & Masser (1999) study, all *Tilapia* hatch their babies in the nest while *Sarotherodon* and *Oreochromis* species fertilize eggs and hatch babies in the mouth. Therefore, they are called as mouthbrooders. The difference between *Sarotherodon* and *Oreochromis* is *Sarotherodon* species practices mouthbrooding in male or both male and female. However, *Oreochromis* species practice mouth brooding only in female species. Tilapia is an omnivorous fish from the Cichlidae family. It is one of the famous fish species in Malaysia as a protein source for human consumption.

The most common red hybrid tilapia (*Oreochromis* sp.) used for research purpose are the hybrid of *O. niloticus* and *O. mossambicus* (Islam et al., 2006; Kohinoor et al., 1999;

Lim et al., 2016). According to Pongthana et al. (2010), the widely farmed tilapia in Malaysia are red hybrid tilapia (*Oreochromis* sp.) and Nile tilapia (*O. niloticus*). Ramli et al. (2016) stated that in Malaysia, there are four red hybrid tilapia which are hybrid of *Oreochromis niloticus*, hybrid of *Oreochromis mossambicus*, hybrid of *O. mossambicus* × *O. niloticus*, and hybrid of *O. niloticus* × *O. mossambicus*. The reason for more red hybrid tilapia production from different strains is because of unreported certain breeding practices and also inappropriate management (Karuppannan et al., 2013). Red hybrid tilapia has been known to have high growth rates and adaptive to environmental changes (Islam et al., 2006).

Figure 2.1: The red hybrid tilapia (*Oreochromis* sp.) used in this study.

Basically, for commercial aquaculture, the most important environmental considerations in preventing disease outbreaks are water quality, food quality and stocking density (Faruk & Anka, 2017; Zamri-Saad et al., 2014). Although tilapia can tolerate high stocking density as mentioned by El-Sayed (2006), the high stocking density is susceptible for outbreaks of exotic diseases (Amal & Zamri-Saad, 2011; Zamri-Saad et al., 2014). The disease outbreak is because of high stocking density aquaculture will result in changes of water quality which is ideal condition for the living fish pathogens and other infectious organisms such as virus, bacteria, fungi, protozoa, crustaceans and helminths (Faruk & Anka, 2017; Li et al., 2016). A combination of proper aquaculture farm

practices with the introduction of immunostimulants instead of the vaccine will reduce the disease outbreaks (Zamri-Saad et al., 2014).

2.1.2 Tilapines Diseases

The latest disease outbreak affecting the tilapines is tilapia lake virus (TiLV) disease or also known as syncytial hepatitis of tilapia (SHT) (Ferguson et al., 2014). TiLV infection can spread very fast at the early stage of tilapia, such as during eggs, fingerlings and juveniles stages (Jansen et al., 2018). The immune system of tilapia at early stage is not as efficient as bigger size fish, due to under developing immune system. Therefore, Dong (2019) composed some viral disease prevention steps which is by improving the immunity of the fish host. Another way to improve immunity is by avoiding the introduction of infected fish in aquaculture and enhancing the water environment. Dong et al. (2017) stated that the main countries that experiencing TiLV disease on tilapia are Ecuador, Egypt, Thailand, Colombia and Israel. Then, the virus is introduced to other countries, including Malaysia, by translocation of these fingerlings, either directly or indirectly.

In ASEAN countries, tilapia lake virus (TiLV), nervous necrosis virus (NNV), and infectious spleen and kidney necrosis virus (ISKNV) are potential threats of farmed tilapia (Dong, 2019). Apart from TiLV outbreak, another type of infection that can affect the tilapia production in the world is Streptococcosis disease or bacterial infection from *Streptococcus* sp.. This disease also takes advantage of tilapia when its immunity is disturbed by environmental stress, where it is susceptible to disease outbreak (Amal & Zamri-Saad, 2011). Therefore, it can be concluded that immunostimulants are needed to boost the immunity of host tilapia to counter react to this problem in aquaculture.

Based on the study by Zamri-Saad et al. (2014), they reported that vaccination is one of the treatments use to prevent Streptococcosis disease. It has the ability to activate

specific immune response so the chances for disease and the mortality can be avoided, but not to eliminate fully, the bacteria from the surviving fish. Therefore, several vaccines have been developed according to the current situation to control the disease outbreak.

Vaccination is not a proper way for controlling intracellular pathogens because the vaccines only control a particular disease, although other disease found at the same place. Therefore, vaccination is not a feasible method to manage all aquatic animal-related diseases (Wang et al., 2016). So, instead of vaccine, another way to control the disease outbreak is the introduction of immunostimulants, which can boost the non-specific immune response in fishes. This may reduce time, cost and medication work.

2.2 Fish Feed

Fish feed is an essential source of protein in the aquaculture industry for producing marketable size fish in a short period of time. Other nutrients involve are carbohydrates, lipids, vitamins and minerals. These nutrients become a source of energy and need for maintaining growth, health and carrying out normal body function (Prabu et al., 2017). According to Bhilave (2018), the role of fish feed is very important in the economy of aquaculture because of the high investments required for importing ingredients and also for formulating feed thus contributing to the hiking of feed price. One of the ways to improve the fish disease management is by adding immunostimulants in fish feed. By doing this method, it will reduce the usage of medication and cost in the aquaculture industry (Bassleer, 2017).

2.2.1 Feed Additive

Nowadays, feed additive is added during the fish feed preparation which mainly to improve the feed quality, the feed intake and the health status of fishes. Besides, feed

additives are non-nutritious and can be used as immunostimulants, antioxidants, antibiotics and probiotics in the aquaculture industry (Bharathi et al., 2019).

Immunostimulant is a feed additive that can enhance the non-specific immune response by communicating directly to the immune system cells and boosting their activities (Mastan, 2015). The most promising immunostimulants in aquaculture are polysaccharides, herbs, microorganisms, nutrients, prebiotics, oligosaccharides, and different biological factors (Wang et al., 2016). According to Assefa & Abunna (2018), immunostimulant is one of the best approaches to manage the disease outbreaks in aquaculture.

In the present study, to overcome the tilapia getting affected by infectious disease, the immunostimulant diet was chosen. Immunostimulants are mostly produced from the natural compound which stimulates the immune system, reduces susceptibility to infections, and also protects fish from the high-stress environment (during translocation, sorting, breeding and vaccination) and diseases in aquaculture (Bricknell & Dalmo, 2005; John et al., 2007). Feed additive containing immunostimulant properties could improve the immune system and ultimately, the fish production.

The study from Katya et al. (2014) deduced that mushroom could be a source of protein to replace fishmeal and it also contains bioactive compounds that could stimulate the fish immunity thus reducing susceptibility to disease. One of the successful studies on mushroom as alternative of fishmeal is *Ganoderma lucidum* polysaccharide (GLP) meals as fishmeal replacement for giant freshwater prawn (*Macrobranchium rosenbergii*) (Mohan et al., 2016). Studies made by Supramani et al. (2019) stated that *G. lucidum* contains valuable polysaccharide which are exopolysaccharide (EPS), intracellular polysaccharide (IPS) and also its biomass. However, to date, no research has been done by utilizing this particular polysaccharide as a feed additive for fish.

2.3 *Ganoderma lucidum*

Ganoderma lucidum is a fungus which originated from family Polyporaceae/ Ganodermataceae, order Aphyllophorales and class Basidiomycetes (Donk, 1964; Hapuarachchi et al., 2018). *G. lucidum* is basically a phyto-pathogenic fungus where it can decay wood (Loyd et al., 2018). Although many *Ganoderma* species have this pathogenic characteristic, in Asia, it is also believed that *G. lucidum* have medicinal attributes and used as Asian traditional medicine (Bishop et al., 2015; Jargalmaa et al., 2017).

According to Wasser (2002) studies, higher Basidiomycetes mushrooms like *G. lucidum* can generate bioactive polysaccharides in its mycelium, fruiting body and broth culture. *G. lucidum* has been used for prevention or treatment of various diseases. The *G. lucidum* polysaccharides establish a good antitumor, antifungal, anticancer and immunomodulation properties (Wan-Mohtar et al., 2017). The metabolites from *G. lucidum* like polysaccharide can help in improving the biological activity and the therapeutic use (Cor et al., 2018). The efficiency of the productions of *G. lucidum* polysaccharides can be achieved by using its mycelium in submerged-liquid fermentation (Supramani et al., 2019).

According to the study by Chithra et al. (2016), *G. lucidum* polysaccharides can become a potential feed additive for the grass carp, *Ctenopharyngodon idella* due to the high growth performance and the low mortality rate. Other mushrooms such as *Lentinula edodes* can enhance the immune response of rainbow trout, *Oncorhynchus mykiss* and resistance to the infections from *Lactococcus garvieae* (Baba et al., 2015). Apart from that, other mushrooms that can be used as feed additives or supplements are *Agaricus blazei*, *Agaricus bisporus*, *Cordyceps inensis*, *Cordyceps militaris*, *Fomitella fraxinea*, *Flammulina velutipes*, *Hericium coput-medusae*, *Lentinula edodes*, *Pleurotus ostreatus*

and *Pleurotus eryngii* (Bederska-Łojewska et al., 2017). Besides fish, the bioactive substances from *G. lucidum* also provide positive effect on the poultry performance and the health condition (Bederska-Łojewska et al., 2017; Khan et al., 2019; Ogbe et al., 2009).

2.3.1 Exopolysaccharide

Fungal polysaccharides are known as prebiotic substances which become one of the nutritional ingredients that contribute to aquaculture by maintaining fish body condition and promoting fish growth. Review study made by Mohan et al. (2019) discussed that fungal polysaccharide not only improves the growth of the aquatic organisms, it also helps in boosting immunity and resistance against diseases. The beneficial effects of fungal polysaccharides on aquatic organisms are supported by researches made by Rodriguez et al. (2007), Murthy et al. (2009) and Sivagnanavelmurugan et al. (2014).

Exopolysaccharides (EPS) are composed of sugar residues and usually secreted by plants, algae, fungi and bacteria (Dilna et al., 2015). The optimum synthesis of exopolysaccharide in fungi and the secretion of it to the environment can only be achieved when the EPS meet the optimum medium and condition for the specific strains of fungi (Mahapatra & Banerjee, 2013). In this study, the EPS from the fermented mycelium of *G. lucidum* was observed as in Figure 2.2 and Figure 2.3, which was prepared in a liquid environment before proceeding it to dry stage.

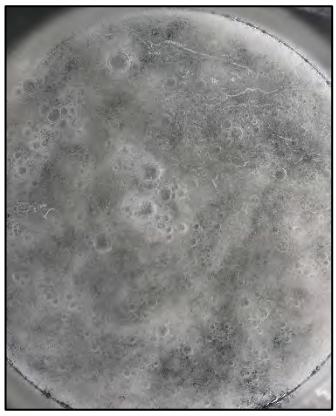

Figure 2.2: Top view of EPS from the fermented mycelium of *G. lucidum* in a shake flask.

Figure 2.3: Side view of EPS from the fermented mycelium of *G. lucidum* in a shake flask.

Exopolysaccharides from the fermented mycelium of *G. lucidum* contain antioxidant properties which can act as antioxidants (Mahendran et al., 2012). Katya et al. (2014) stated that beta-glucans from exopolysaccharide can activate innate immunity and act as a defence mechanism in fish. β-D-glucans possess antitumor effects and the polysaccharides act as a shield against free radicals from mutagens, and this can prevent the cell from being harmed (Cherian et al., 2011). Complex β-1,3-glucans polysaccharides (its peptides known as peptidoglycan) is one of the *G. lucidum* bioactive polysaccharides which has the ability to interact and to boost the immunity (Cor et al., 2018). According to Barman et al. (2013), glucans can be obtained from the cell walls of fungi, plants and bacteria, providing the most promising effect in all tested fish and

shrimp. While doing their research, they found oral application is the route to get a good result.

Mohan et al. (2019) summarised studies from other researchers that fungal polysaccharides provide a good impact in body composition, growth performance, haematological and biochemical studies of fishes from Cichlidae family. However, the limitation of the study is it observed the fungal polysaccharides as immunostimulants only in Cichlidae family.

2.4 Nutrient Requirement

Proteins, lipids, carbohydrates, vitamins, and minerals are the important nutrient requirement for fish to sustain their life. Continuous supply of protein is needed for the metabolic process of animal, including tilapia fish (Lim & Webster, 2006). Tissue production prior to growth of aquatic animals requires high amount of protein supply (Prabu et al., 2017). According to Lim & Webster (2006), lipid plays a major role in food taste, feed texture, steroid hormones, prostaglandins and fatty acid contents. Besides, it also functions as pellet binder, serves as a precursor for the formation of various metabolic intermediates, essential for growth and has sparing effect on the utilization of dietary protein (Lim & Webster, 2006). According to Polakof et al. (2012) and Sangiao-Alvarellos et al. (2005) studies, fish can keep carbohydrates in their tissues in the form of glycogen and use them during hypoxic conditions, food scarcity and high stocking density. The fish culture in a closed system cannot get enough natural food. Therefore, vitamins must be provided to the fish in other way by adding supplemental vitamins in their diets. Based on Lim & Webster (2006), tilapia needs minerals for tissue formation and metabolic processes such as osmoregulation, acid-base balance and well function of muscle and nerves. Typical essential minerals found in tilapia fish are calcium, sodium, potassium, copper, phosphorus and iron (Jim et al., 2017).

2.5 Organosomatic Indices

2.5.1 Hepatosomatic Index

The hepatosomatic index can be calculated by using liver weight and body weight. The fish liver is the metabolic organ which is vital during the metabolic process (Sadekarpawar & Parikh, 2013). The role of the liver is well explained by Pope et al. (2010), whereby the excess energy produced in the fish body can be stored in the liver as glycogen. The bigger the size of the liver as compared to body weight, the higher the hepatosomatic index of fish, indicating the liver contains high energy. Thus, this situation deduced that the fish is well fed (Pope et al., 2010). Besides, Sagada et al. (2017) explained that the higher the insertion of dietary lipid and protein in fish feed, the higher the lipid deposition in the fish liver. Therefore, it is a route of influencing the HSI level.

Lenhardt et al. (2009) proposed that the changes in hepatosomatic index show the sign of water pollution by chemicals as the fish liver helps in detoxification (Araujo et al., 2018). Actually, the liver has the second most ability in accumulation of metals after skin (Yılmaz et al., 2010). One of the possible reasons of the increasing HSI level in fish is due to the occurrence of the detoxification process as a response from toxic compounds in surrounding (Liebel et al., 2013; Pereira & Kuch, 2005). On the other hand, the decreasing of HSI level can be caused by high chemical stress exerted from the fish populations (Kopecka et al., 2006).

2.5.2 Viscerosomatic Index

Viscerosomatic index can be calculated by using viscera weight and body weight. Viscera weight represents the wet weight of visceral organs and associated fat tissues (Abbas et al., 2015). According to Sogbesan et al. (2017), increase in the value of the viscerosomatic index is due to the presence of bioactive chemicals in the additive diets.

Besides, increase in the value of VSI also indicates that fish consume fish feed that contains high dietary lipid (Yildiz, 2004) which will then affect the body lipid deposition in the viscera (Sagada et al., 2017). Based on Ahmad et al. (2012) study, carp fish commonly has the ability to utilize carbohydrate fully from feed up to 15%. However, when there is an excess of dietary carbohydrate above 15%, the carp has difficulty in using it due to omnivorous feeding habits. Lastly, the carbohydrate is converted into viscera lipid and this situation is unfavourable for economical fish production.

2.5.3 Condition Factor

Fulton's condition factor (K) is an essential parameters to determine the growth and also the physiological pattern of fish when compared with two populations living in specific environment (Suleiman et al., 2018). Ridanovic et al. (2015) explained that the condition factor is also a tool in mastering fish pathology and biology. Conversely, Ahmed et al. (2011) deduced that condition factor is also used in determining the condition, the fatness and the welfare of fishes.

K value is calculated by using the body weight and the total length of body fish. Jin et al. (2015) stated that this K value can predict the changes in nutritional conditions in fish body. Barnham & Baxter (1998) proposed that the fish which has weak growth, long and thin size, the K value should be at 1.00. However, when the fish condition is stable and for angler fish, the K value should be at 1.20. Apparently, the K value for the excellent growth and well-formed fish should be approximated to 1.40.

Based on Ayoade (2011) studies, the recommendable K value of fish in fish farming should be more than 1.0, which shows a good health status and isometric growth of fish. The monosex and the mixed-sex culture of tilapia produce different K value trends. According to Olurin & Aderibigbe (2006), the mixed-sex culture of tilapia shows a variation in K value due to the sex of fish where utilization of feed to sustain life is

different. However, this is not applicable in Anani & Nunoo (2016) study because monosex male culture of tilapia in the dietary experiment does not influence the variation of K values.

2.6 Blood Haematological

Haematological indices are the parameters that used to detect physiological and pathological of animals (Etim et al., 2014). The involved parameters are red blood cell, white blood cell, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and serum total protein. According to Kefas et al. (2015), haematological values are influenced by physiological stress, disease, and environmental factors, including toxic pollutant. Besides, the stocking density and the feeding habits of fish also contribute to the changes in blood parameters (Hrubec et al., 2000; Naderi et al., 2017; Parrino et al., 2018).

According to Rehulka (2003), there are three necessary elements that need to be considered in order to enhance fish production, including feed, feed composition and metabolic adaptation. At the same time, these three elements are responsible for changes in haematological parameters of fish. The most abundant cells in the blood circulatory system for vertebrates are red blood cells and white blood cells. Generally, red blood cell involves in gas exchange while white blood cell involves in the immune response. Study made by Shen et al. (2018) deduced that fish red blood cell does not only play a role in respiratory, but it also expresses immune-related genes and responses. Serum total protein studies revealed that it helps in identifying the organs affected by toxicity, general health status and also promotes early warning of damages that can occur in stressed organisms (Folmar, 1993).

CHAPTER 3: METHODOLOGY

3.1 Exopolysaccharide Production

3.1.1 Fungal Source

Ganoderma lucidum was obtained from Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Universiti Malaya (UM). The strain of the *Ganoderma lucidum* used was QRS 5120 (Supramani et al., 2019).

3.1.2 *Ganoderma lucidum* Mycelium Cultivation

G. lucidum was dried in a laminar flow after it undergoes aseptic preparation by washing it with 99.9% ethanol (Sigma-Aldrich, Dorset, UK) for 10 seconds. The dried *G. lucidum* was scratched using a scalpel, and its content was removed by twisting its body and with the help of forceps. Mycelium growth took place by placing the content which was called a tissue, on malt extract agar (MEA) (Sigma-Aldrich, Dorset, UK) under controlled temperature (room temperature). The generated mycelium was undergone the same culture in newly fresh MEA. The freshly obtained pure mycelium then cultured in a potato dextrose agar (PDA) (Sigma-Aldrich, Dorset, UK) slant at 4°C for inoculum production.

3.1.3 Exopolysaccharide Cultivation

Submerged-liquid fermentation (SLF) was used in this study. The mycelium of *G. lucidum* undergone two seed fermentation. Basically, 1st seed for inoculum production took ten (10) days. After that, it proceeded to 2nd seed for EPS production for eleven (11) days. According to Supramani et al. (2019), 11th day can generate high EPS production, so it was harvested on that day. 1st seed carried out by using 250-ml Erlenmeyer flask and start working medium was at 100 ml where 50% of media, 30% of glucose, and 20% of

distilled water. The mycelium from prepared agar was used in the 1st seed culture at size 5 mm × 5 mm.

The medium composition of seed culture in the shake flask was constant at gram per litre: 1.0 g/L of yeast extract (Oxoid no. LP0021, Dardilly, France), 0.5 g/L of potassium dihydrogen phosphate anhydrous (KH_2PO_4) (Bendosen Laboratory Chemicals no. C0637, Bendosen, Norway), 0.5 g/L of di-potassium hydrogen orthophosphate anhydrous (K_2HPO_4) (Bendosen Laboratory Chemicals no. C0680-2296192, Bendosen, Norway), 0.5 g/L of magnesium sulfate ($MgSO_4$) (Bendosen Laboratory Chemicals no. C0481, Bendosen, Norway), 4.0 g/L of ammonium chloride (NH_4Cl_2) (Bendosen Laboratory Chemicals no. c0055, Bendosen, Norway), and 30.0 g/L of glucose (D (+) – glucose monohydrate (dextrose)). The other constant conditions were initial pH at 4, agitation at 100 rpm, and temperature at room temperature (27 - 30°C) (Mohtar et al., 2016; Supramani et al., 2019).

Then the 1st seed culture was kept on a shaker (SK 300) for agitation. On the 10th day, they were upscaled to 200 ml medium for 2nd seed culture by using 500-ml Erlenmeyer flasks. The percentage of the medium was in accordance to the 1st seed, but the inoculum was replaced with distilled water. Before it was proceeded to 2nd seed, the 1st seed product on the 10th day was ground by using electron grinder for the 20s to obtained homogenised inoculum. The media composition and conditions was in accordance to the 1st seed culture. Later, they were kept on the shaker. Then, the production of exopolysaccharide was improved by using the 2L or 4L stirred tank bioreactor. It followed the media composition and conditions as in 2nd seed and the inoculum from 2nd seed at 10th day was used during the upscaling process to the bioreactor (Mohtar et al., 2016).

Figure 3.1: 2nd seed culture by using shake flask.

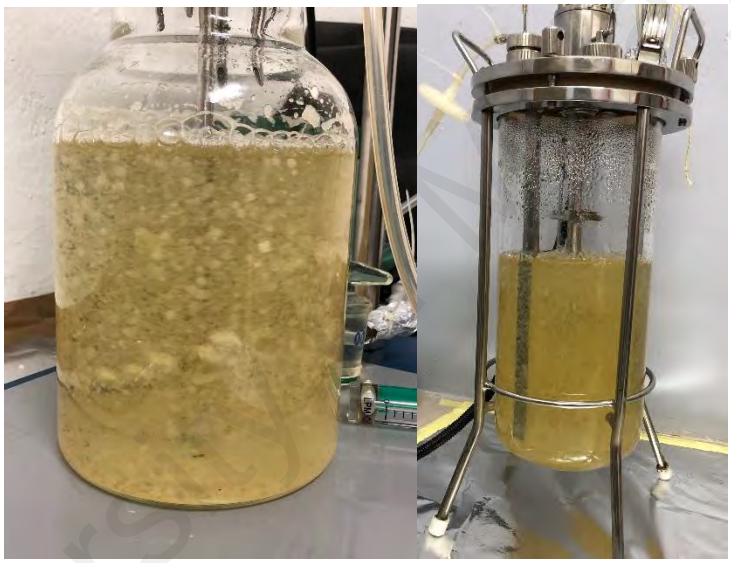

Figure 3.2: Upscaling culture into 2L (on the left of the picture) and 4L (on the right of the picture) stirred tank bioreactor.

3.1.4 Exopolysaccharide Extraction

On the 11th day of 2nd seed and the bioreactor culture, the samples were then filtrated with Buchner funnel to obtain the supernatant and then washed it thrice (3) with distilled water to secure the remained supernatant. The obtained supernatant was mixed with ethanol (95%: 4 volumes), well stirred, and it left overnight at controlled temperature (4^0C). The mixture was centrifuged at 10,000 rpm for 15 min after transferred to 50 mL Falcon tubes. The precipitate then filtered and dried to obtain exopolysaccharide (EPS). The EPS yield was collected prior to the proximate analysis.

3.2 Experimental Diets

Raw materials for the experimental diets including fishmeal, rice bran, soybean meal, corn meal, dicalcium phosphate (DCP), minerals and vitamins were purchased from local livestock fish centre. The chemical composition of fish feed ingredients was tabulated in Table 3.1. The formulation and chemical composition of fish feed diets were tabulated in Table 3.2.

Table 3.1: Chemical composition of fish feed ingredients.

Nutrients	**Corn meal (%)**	**Fishmeal (%)**	**Rice bran (%)**	**Soybean meal (%)**	**EPS (%)**
Protein	6.64	54.29	11.23	43.01	17.67
Lipid	2.60	2.41	8.76	2.14	0.28
Fibre	9.81	14.54	19.40	9.64	10.38
Dry matter	91.01	89.36	93.52	92.51	91.95
Ash	1.73	23.16	5.30	5.16	2.08
Carbohydrate	79.23	5.60	55.30	40.04	69.59

Table 3.2: Formulation and proximate composition of experimental feed.

Ingredients (g/kg)	**Control**	**1 g/kg EPS**	**2 g/kg EPS**	**3 g/kg EPS**
EPS	0.00	1.00	2.00	3.00
Fishmeal	300.00	300.00	300.00	300.00
Soybean meal	236.80	236.60	236.30	236.10
Corn meal	193.90	193.50	193.10	192.70
Rice bran	199.30	198.90	198.60	198.20
Vitamin premix[1]	2.00	2.00	2.00	2.00
Mineral premix [2]	3.00	3.00	3.00	3.00
Dicalcium phosphate (DCP)	10.00	10.00	10.00	10.00
Fish oil	40.00	40.00	40.00	40.00
Lysine	10.00	10.00	10.00	10.00
Methionine	5.00	5.00	5.00	5.00
Total (g)	1000.00	1000.00	1000.00	1000.00

Table 3.2, continued.

	Control	1 g/kg EPS	2 g/kg EPS	3 g/kg EPS
Protein (% d.m.)	37.86	36.30	37.02	35.19
Lipid (% d.m.)	6.29	6.29	4.84	6.67
Fibre (% d.m.)	1.63	1.70	1.92	1.76
Dry matter (%)	87.52	66.63	71.47	72.05
Ash (% d.m.)	12.00	12.86	12.40	12.45
NFE/carbohydrate (%)[3]	42.22	42.85	43.82	43.93
Gross energy (kJ/g)[4]	18.98	18.72	12.96	18.80

[1]The vitamin premix supplied the following per 100g diet: vitamin A, 500IU; vitamin D3, 100IU; vitamin E, 0.75 mg; vitamin K, 0.02 mg; vitamin B1, 1.0 mg; vitamin B2, 0.5 mg; vitamin B3, 0.3 mg; vitamin B6, 0.02 mg; folic acid, 0.1 mg; biotin, 0.235 mg; pantothenic acid, 1.0 mg; inositol, 2.5 mg.

[2]The mineral premix supplied the following per kg diet: selenium, 0.2 mg; iron, 8.0 mg; manganase, 1.0 mg; zinc, 8.0 mg; copper, 0.15 mg; potassium chloride, 0.4 mg; magnesium oxide, 0.6 mg; sodium bicarbonate, 1.5 mg; iodine, 1.0 mg; cobalt, 0.25 mg.

NFE = nitrogen-free extract

d.m. = dry matter basis

[3]NFE (%) = 100 – (% *CP* + % *CL* + % *CF* + % *CA*) (Schulz et al., 2005).

[4]Gross energy was calculated as 23.9, 39.8, and 17.6 kJ/g for protein, lipid, and NFE, respectively (Schulz et al., 2005).

Four (4) formulated diets were used in the feeding trials. The diets contained (1 g/kg, 2 g/kg, and 3 g/kg) of EPS and 0 g/kg of EPS was treated as control. Formulation of the diets was carried out by using Winfeed version 2.8 software. The dry ingredients were ground by using a dry blender (Pensonic no. PB-3203, Malaysia). Then, mixed well with vitamins, minerals, and DCP with the addition of some water to form pellets in 0.3 cm diameters size by using a mini pelleting machine (KCM, Y132M-A). The pellets were dried in an oven for 24 h at 70°C and then stored in a cold room (4°C).

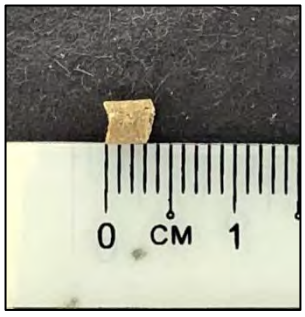

Figure 3.3: Fish feed in pellet form (± 0.3 cm).

3.3 Experimental Fish and Set-up

Red hybrid tilapia (*Oreochromis* sp.) fingerlings were purchased from a local fish farm in Sungai Buloh. The experiment facilities were set up in Freshwater Aquarium Laboratory at Institute of Biological Sciences, Faculty of Science, Universiti Malaya. Two hundred (200) fishes were purchased at size ranged from 10 – 20g (fingerlings). These fishes were acclimatised for two (2) weeks in natural condition and fed with commercial diet twice (2) per day at 0900 h and 1500 h. The water quality was maintained regularly and any mortalities were recorded. A closed recirculation system was used with eight (8) tanks (3' × 2' × 1') which contain up to 100 litres of water. Each tank was provided with a filter pump for oxygen supply and aeration. Dechlorinated water was used and 20 – 30% of the water in each tank was replaced once in two (2) days.

After acclimatisation period, the experimental diets containing EPS supplemented at 0 g/kg (control), 1 g/kg, 2 g/kg, and 3 g/kg were fed to duplicate groups of fishes. Each tank contains fifteen (15) acclimatised fishes (16.19 ± 0.24 g). The feeding trial was carried out for forty-two (42) days. The feeding trial began by feeding the fish at 3% of their body weight (BW) ratio for the first two weeks, reduced to 2.8 % at week 3 & 4 and finally to 2.6 % at week 5 & 6. At the end of the trial, all fish were weighed, sacrificed for body composition and frozen at -20°C for further analysis.

Water quality parameters were monitored and maintained daily which included pH at 6.0 – 6.8 by using pH meter (YSI, Pro DO), temperature at 28 - 29°C and dissolved oxygen (DO) above 5.0 mg/L by using DO meter (YSI, Pro DO), and ammonia less than 0.8 mg/L and nitrate less than 1.9 mg/L by using a portable photometer (HANNA instruments, Romania), weekly (Taufek et al., 2017).

3.4 Proximate and Chemical Analysis

The experimental ingredients, diets, and body composition for proximate analysis were analysed according to the Association of Official Analytical Chemist method (AOAC, 2003).

3.4.1 Crude Protein

The method for crude protein proximate composition was based on the Kjeldahl method. The sample was prepared by weighing it at 0.15 g and transferred it into a Kjeldahl digestion tube. A tablet of Selenium Kjeltabs Catalyst (0.10 g) and 6 ml of concentrated sulphuric acid were added into the tube accordingly. The tubes were digested in FOSS Tecator Digestor Auto at 420^{0}C for 1 hour. Then, the sample was left in the machine for 15 minutes for the cooling process. While waiting, proceeded to the preparation for distillation process by pouring 25 ml of 4% boric acid into a 250 ml conical flask and 7 to 8 drops of bromocresol green + methyl red indicator was added in the flask, accordingly. The titration indicator was prepared by firstly dissolved 0.10 g of bromocresol green in 100 ml methanol and then only added with 70 ml methyl red solution in 100 ml methanol. The Kjeltec semi auto-analyser was set up with 80 ml of deionized water and 50 ml of sodium hydroxide for each digestion tube. The deionized water and sodium hydroxide was prepared sufficiently for the whole process and connected them to the Kjeltec semi auto-analyser. After 15 minutes of the cooling process, the digestion tube with sample and the prepared conical flask were undergone distillation process by using Kjeltec semi auto-analyser. Finally, the product from the distillation process was titrated by using hydrochloric acid and the titration value was recorded.

All samples and blanks were analysed in duplicates.

The protein content was calculated by using the samples and blanks titration value as below.

$$\text{Nitrogen (\%)} = \frac{(S - B)\,(N)\,(14.007) \times 100}{\text{g of sample} \times 1000} \times 100 \qquad (3.1)$$

Protein (%) = (% Nitrogen) (6.25)

Based on the standardized protein factor, 6.25 was used as a multiplication factor to convert the titre volume to % protein.

S = titre HCl for sample (ml)
B = titre HCI for blank (ml)
N = normality for HCl

3.4.2 Crude Lipid

Crude lipid contents of feed and fish body composition were measured by using the Soxhlet method with petroleum ether extraction (FOSS Soxtec 2055). The extraction cups were dried in an oven and then weighed. The samples were weighed approximately for 2g and added into the cleaned cellulose thimbles. The extraction cups were filled with 80 ml of petroleum ether. The prepared thimbles and extraction cups were placed in the FOSS Tecator Extraction Unit for the extraction process for 1 hour. Then, the extraction cups were dried in the oven for 2 hours at 120°C. After that, the extraction cups were cooled off in a desiccator and subsequently weighed. The samples were kept in thimbles for crude fibre analysis. All samples were analysed in duplicates.

The lipid content of the samples were calculated as follows:

$$\text{Lipid (\%)} = \frac{(W3 - W2)}{W1} \times 100 \qquad (3.2)$$

Where *W1* = weight of sample (g)
W2 = weight of extraction cup initial (g)
W3 = weight of extraction cup final (g)

3.4.3 Dry Matter

The dried empty crucibles were weighed to obtain the dry weight. Next, the samples were weighed for 2g and placed in the weighed empty dried crucibles. The weight of the samples with crucibles were recorded. They were then dried in the oven at 105°C for 24 hours to obtain the final weight.

The dry matter content of the samples were calculated as follows:

$$\text{Dry matter (\%)} = \frac{(W3 - W1)}{(W2 - W1)} \times 100 \quad (3.3)$$

Where *W1* = Weight of empty crucible (g)
W2 = Weight of crucible + sample (g)
W3 = Weight of crucible + sample after drying in 105°C (g)

3.4.4 Crude Ash

The ash content analysis was the continuous process from the dry matter analysis to determine the ash content of the diets and body composition by using the dried samples obtained from dry matter analysis. The samples with the crucibles were continued to dry in the muffle furnace (Naberthem) at 600°C overnight. Then, the samples were transferred in a desiccator to cool. Lastly, they were weighed to determine the ash content.

The ash content was calculated as follows:

$$\text{Ash (\%)} = \frac{(W4 - W1)}{(W3 - W1)} \times 100 \quad (3.4)$$

When *W1* = Weight of empty crucible (g)
W3 = Weight of crucible + sample after drying in 105°C (g)
W4 = Weight of crucible + sample after drying in 600°C (g)

3.4.5 Crude Fibre

The residues from the crude lipid extraction analysis which known as de-fatted samples were used in crude fibre analysis. The samples were undergone in alkali and acid digestion to obtain the crude fibre. The dried empty fibre capsules together with their lids were weighed. Approximately, 0.5 g of sample was weighed and transferred into the weighed fibre capsule with its lid. The extraction vessel was placed with 350 ml of 1.25% sulphuric acid (H_2SO_4) on the hot plate and heated to boil. When the reagent (H_2SO_4) started to boil, the heating volume was reduced to approximately 5 – 7 (stable condition). The capsule tray with fibre capsules containing samples was placed in the carousel and put on the stopper to lock the capsules in place.

Then, the carousel with capsules was lowered partially (to make sufficient immersed of the samples) into the extraction vessel containing the boiling reagent. It continued with placing the condenser on the top of the extraction vessel. The tap water was opened at the speed of 0.41/min for the reflux system at the condenser. The samples were boiled for 30 minutes and then washed with hot water three (3) times (each time used fresh hot water for 2 min).

The extraction vessel was replaced with 350 ml of 1.25% sodium hydroxide (NaOH) on the hot plate and heated to boil. The same procedures were repeated with sulphuric acid with four (4) times of washing process; first two (2) times with hot water, one time with 1% hydrochloric acid (HCl), and finally one time with hot water.

The capsules tray with the capsules were moved to the tray holder for final de-fatting of the fibre residue. The de-fatting process was done by washing once with acetone. The capsules were dried in an oven at 105°C for 5 hours. They were cooled off to room temperatures in a desiccator (~ 30 min) and weighed with the precision of ± 0.1 mg (*W3*). The capsules were placed in pre-dried and pre-weighed ashing crucibles (45 × 60 mm)

(*W4*). The samples were ashed in the ashing crucibles at least 4 hours at 600°C. They were then cooled off in desiccator until reached room temperature and then weighed (*W5*) to determine the crude fibre content.

The crude fibre content was calculated as follows:

$$\text{Fibre (\%)} = \frac{W3 - (W1 \times C) - (W5 - W4 - D)}{W2} \times 100 \quad (3.5)$$

Where *W1* = Initial capsule weight (g)

W2 = Sample weight (g)

W3 = Capsule + residue weight (g)

W4 = Empty ashing crucible (g)

W5 = Total ash (g)

$$C = \frac{W3 \text{ (Blank)}}{W1 \text{ (Blank)}}$$

D = *W5* (Blank) – *W4* (Blank)

3.4.6 Nitrogen Free Extract

An equation from Schulz et al. (2005) was used to calculated nitrogen free extract (NFE) or carbohydrate (%) as follows:

$$\text{NFE (\%)} = 100 - (\%\ CP + \%\ CL + \%\ CF + \%\ CA) \quad (3.6)$$

CP = Crude protein

CL = Crude lipid

CF = Crude fibre

CA = Crude ash

3.4.7 Gross Energy

An equation from Schulz et al. (2005) was used to determine the gross energy (GE) for each diet and body composition as follows:

$$GE\ (kJ/g) = CP\ (\times 23.9\ kJ/g) + CL\ (\times 39.8\ kJ/g) + NFE\ (\times 17.6\ kJ/g) \qquad (3.7)$$

CP = Crude protein (%)

CL = Crude lipid (%)

NFE = Nitrogen free-extract (%)

The value 23.9 kJ/g, 39.8 kJ/g, and 17.6 kJ/g were the factors for crude protein, crude lipid, and NFE, respectively.

3.5 Sample Preparation

At the end of the feeding trial, three (3) fishes for liver and five (5) fishes for blood sample were selected randomly from each tank. The fishes were sacrificed and marked individually. Each fish was weighed and its total body length also recorded. The liver and viscera were weighed to determine the hepatosomatic index (HSI %), viscerosomatic index (VSI %) and condition factor (CF). The fillets of the same fish have undergone the same proximate analysis as stated in the topic 3.4 (proximate and chemical analysis) to measure the lipid, protein, fibre, ash, dry matter, carbohydrate and energy content found in the fish fillet.

Figure 3.4: The fillets of red hybrid tilapia for body composition.

Blood samples were collected randomly from five (5) fishes in each tank. Approximately 1 ml of the blood sample was collected by using a 1-ml syringe with a needle size of 22G 1 ½ inch and pooled into treatment groups for blood analysis.

Figure 3.5: Blood collection from red hybrid tilapia by using a 1-ml syringe with a needle size of 22G 1 ½ inch.

3.6 Haematological Studies

Blood samples were collected from five randomly selected fishes in each tank. The blood was drawn from caudal vein and transferred into heparinized vacutainer tube for the determination of haematological analysis. Part of the samples was used for determination of haematological parameters (complete blood counts). After that, for the serum, blood was allowed to clot for 2h and later centrifuged at 5 000 × g at 4^0C for 10 min. The isolated serum was then stored in -20^0C for further total protein analysis. Complete blood counts (CBC) which consisted of red blood cells (RBC), white blood cells (WBC), haemoglobin (HGB), haematocrit (HCT), and RBC indices namely: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were measured using automatic haematology analyzer (Sysmex XN, Germany). Serum total protein was measured by using Advia 2400 Chemistry System (Siemens Healthineers, Germany).

3.7 Analysis of Experimental Data

Hepatosomatic index (HSI), viscerosomatic index (VSI), and condition factor (CF) were calculated after the data generated from the experiment. The following are the calculations for the parameters mentioned before:

$$\text{HSI (\%)} = (\text{wet weight of liver (g)}/ \text{final weight of fish (g)}) \times 100 \quad (3.8)$$

$$\text{VSI (\%)} = (\text{viscera weight (g)}/ \text{whole body weight (g)}) \times 100 \quad (3.9)$$

$$\text{CF} = [\text{body weight (g)}/ (\text{body length (cm)})^3] \times 100 \quad (3.10)$$

3.8 Statistical Analysis

One way analysis of variance (ANOVA) test was used to analyse the data in this study by using SPSS version 23.0 (SPSS Inc., Chicago IL. USA). Duncan's post hoc test was used to compare the difference between means at a 95% confidence interval ($P<0.05$). Data were presented as the mean ± standard error of the mean (SEM).

CHAPTER 4: RESULTS

This study presents the exopolysaccharides from the fermented mycelium of *Ganoderma lucidum* was used as feed additive in the diets of red hybrid tilapia (*Oreochromis* sp.) at different levels which are at 1 g/kg, 2 g/kg and 3 g/kg of EPS and without EPS as control (0 g/kg EPS). The production of exopolysaccharide from fermented mycelium of *G. lucidum* and the usage of it in this study are shown in Figure 4.1.

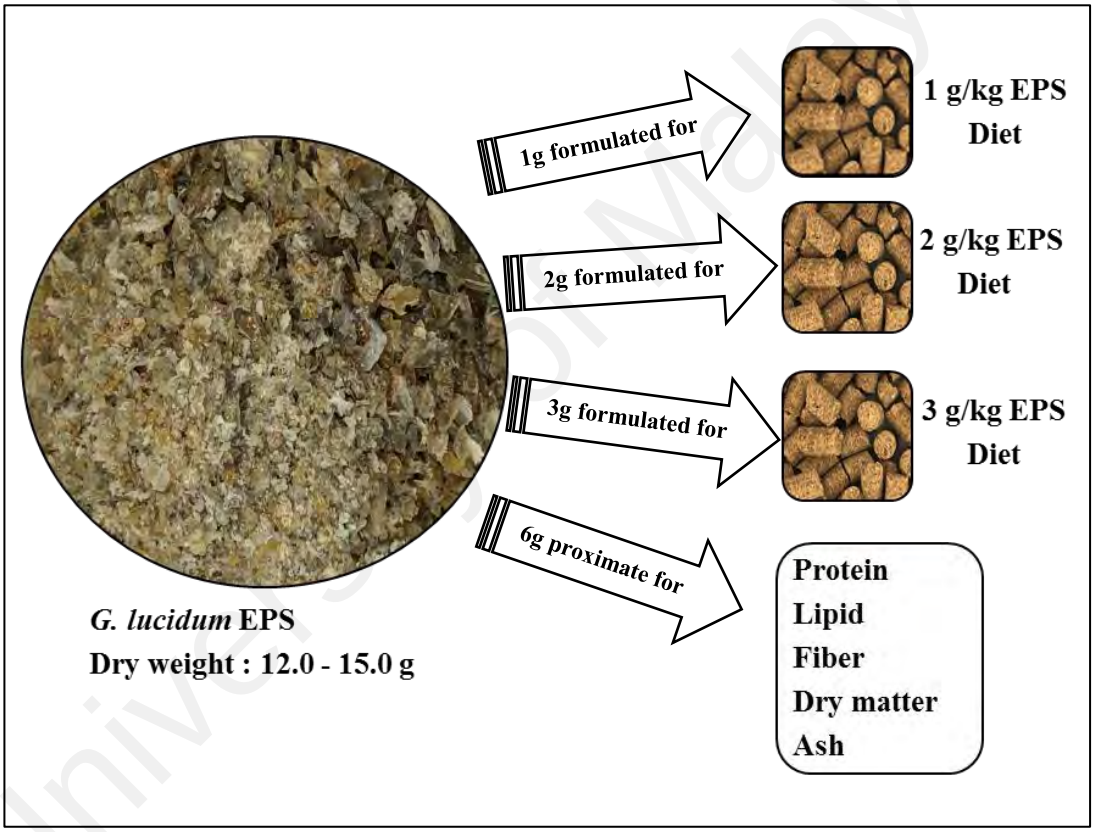

Figure 4.1: Total production of *G. lucidum* EPS and its usage in this study.

The body composition, organosomatic indices and haematological effect of the exopolysaccharides from the fermented mycelium of *G. lucidum* on red hybrid tilapia are presented in this chapter.

Figure 4.2 shows the growth of red hybrid tilapia (*Oreochromis* sp.) fed with different experimental diets which containing 0 g/kg EPS (control), 1 g/kg EPS (1E), 2 g/kg EPS

(2E), and 3 g/kg EPS (3E). In general, red hybrid tilapia in the group fed with 3E showed significantly higher growth, followed by 2E, 1E, and control. Similarly, the length of the fish increases with the increasing levels of EPS supplementation in fish feed. During the feeding trial, only two mortalities were recorded during the 5^{th} and the 6^{th} weeks, both involving fish in 1E and 3E groups.

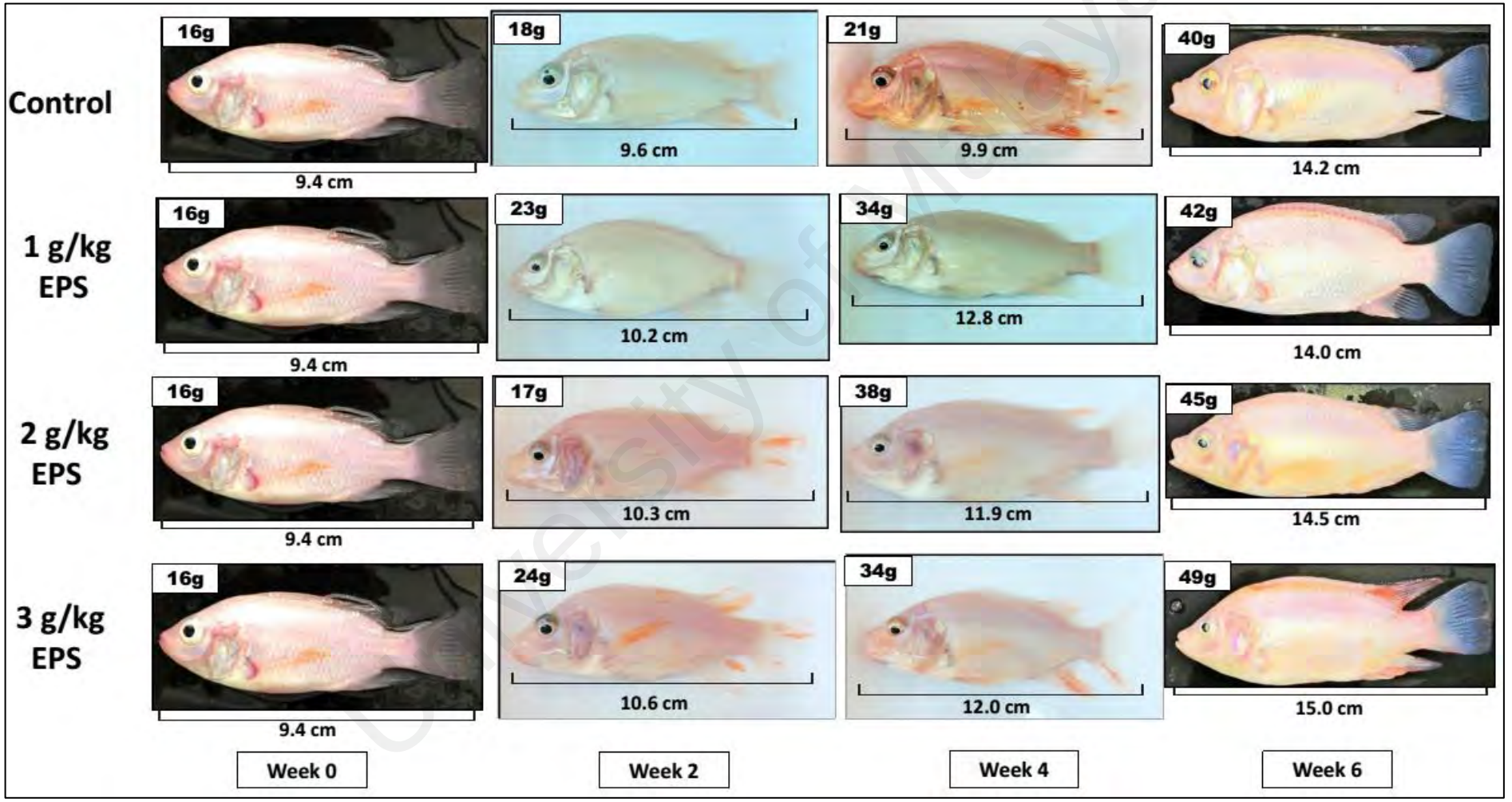

Figure 4.2: Growth of red hybrid tilapia fed with different experimental diets.

4.1 Body composition

4.1.1 Fish Fillet Nutritional Composition

Table 4.1: Fillet composition of fish fed with the experimental diets.

Components (%)	Control	1 g/kg EPS	2 g/kg EPS	3 g/kg EPS
Protein	81.87 ± 1.23	88.70 ± 1.42	83.80 ± 2.21	83.21 ± 1.04
Lipid	1.50 ± 0.06	1.68 ± 0.08	1.37 ± 0.17	1.45 ± 0.23
Fibre	0.03 ± 0.00^{a}	0.20 ± 0.00^{bc}	0.22 ± 0.06^{c}	0.06 ± 0.04^{ab}
Dry matter	95.27 ± 0.37	95.22 ± 0.09	94.86 ± 0.11	94.76 ± 0.07
Ash	6.60 ± 0.21	6.93 ± 0.32	5.90 ± 0.45	6.55 ± 0.53
NFE/carbohydrate[1]	10.00 ± 0.94	2.50 ± 1.69	8.70 ± 2.59	8.73 ± 2.04
Gross energy (kJ/g)[2]	21.93 ± 0.15	22.52 ± 0.18	22.11 ± 0.14	22.00 ± 0.02

*The result represents the mean ± SEM of 3 fishes per tank with a total of 6 fishes per diet.

Mean values in the same row with different superscripts are significantly different ($P<0.05$).

[1]NFE % = 100 – (% *CP* + % *CL* + % *CA* + % *CF*) (Schulz et al., 2005).

[2]Gross energy was calculated as 23.9 kJ/g, 39.8 kJ/g, and 17.6 kJ/g for protein, lipid, and NFE, respectively (Schulz et al., 2005).

The fish fillet composition (% of dry weight) of crude protein, crude lipid, crude fibre, ash, dry matter, carbohydrate, and gross energy after the feeding trial are shown in Table 4.1. Generally, no significant differences were found amongst treatments for protein, lipid, dry matter, ash, gross energy and carbohydrate contents in fish fillet ($P>0.05$). However, only fibre content in fish fillet was significantly affected by the supplementation of EPS ($P<0.05$). The fibre value of the fish fillet fed with 2E (0.22 ± 0.06 %) was comparatively higher than other treatments, while the lowest was recorded in control (0.03 ± 0.00 %) group.

The highest protein content in fish fillet was observed in diet 1E (88.70 ± 1.42 %) followed by 2E (83.80 ± 2.21 %), 3E (83.21 ± 1.04 %) and control (81.87 ± 1.23 %). The 1E fillet recorded the highest lipid value (1.68 ± 0.08 %) while 2E fillet recorded the lowest lipid value (1.37 ± 0.17 %). The dry matter was the highest in control fish (95.27 ± 0.37 %) by a slight difference in its value as compared to other value from the fish fed

EPS contained diets. However, for ash content in fish fillet, 1E (6.93 ± 0.32 %) showed the highest value followed by control (6.60 ± 0.21 %), 3E (6.55 ± 0.53 %) and 2E (5.90 ± 0.45 %). The carbohydrate content in fish fillet was highly detected at control (10.00 ± 0.94 %), and the lowest was in 1E (2.50 ± 1.69 %). Lastly, fillet in 1E showed the highest gross energy (22.52 ± 0.18 kJ/g) compared to other treatments due to numerically higher protein and lipid content in this fillet.

4.2 Organosomatic Indices

Table 4.2: Organosomatic parameters for fish fed with experimental diets.

Variables	Experimental diets			
	Control	**1 g/kg EPS**	**2 g/kg EPS**	**3 g/kg EPS**
HSI (%)	1.97 ± 0.06^{b}	1.42 ± 0.21^{a}	2.27 ± 0.16^{b}	2.22 ± 0.18^{b}
VSI (%)	11.02 ± 0.40^{b}	9.75 ± 0.43^{ab}	10.71 ± 0.48^{ab}	9.42 ± 0.36^{a}
CF	1.60 ± 0.08	1.51 ± 0.03	1.48 ± 0.02	1.46 ± 0.02

*The result represents the mean ± SEM of 3 fishes per tank with a total of 6 fishes per diet.

Mean values in the same row with different superscripts are significantly different (P<0.05).

HSI = Hepatosomatic index; VSI = Viscerosomatic index; CF = Condition factor

Based on the organosomatic indices of red hybrid tilapia observed after feeding with the experimental diets, no significant differences were recorded amongst the treatments for CF (P>0.05) (Table 4.2). However, the HSI and the VSI were significantly affected by the supplementation of EPS in the feed (P<0.05).

There was no significant different amongst control, 2E and 3E groups for HSI value, but the lowest HSI value was significantly shown in 1E. Numerically, the highest HSI value was observed in 2E (2.27 ± 0.16 %) followed by 3E (2.22 ± 0.18 %), control (1.97 ± 0.06 %), and 1E (1.42 ± 0.21 %). A small supplementation of EPS in the fish feed at the amount of 1 g/kg produced less HSI value as compared to control (0 g/kg). However, exceeding the supplementation of EPS more than 2 g/kg elevated the HSI value compared to 1 g/kg.

The VSI values for 2E (10.71 ± 0.48 %) and 1E (9.75 ± 0.43 %) were not significantly different compared to the control (11.02 ± 0.40 %) and 3E (9.42 ± 0.36 %) groups. At the same time, there was a significant difference noticed amongst the control and 3E. The control group showed numerically higher values compared to other diets, while the lowest was observed at 3E. Thus, the results showed a variation of VSI trend during supplementation of EPS in fish feed. CF was not significantly different amongst diet, but the control showed higher values compared to the treated diets.

4.3 Haematological Parameters Analysis

Table 4.3: Haematological parameters of red hybrid tilapia after feeding with various experimental diets.

Parameters	Control	1 g/kg EPS	2 g/kg EPS	3 g/kg EPS
HGB (g/dl)	5.75 ± 0.35^{a}	6.98 ± 0.10^{b}	7.08 ± 0.26^{b}	7.43 ± 0.11^{b}
HCT (%)	30.00 ± 1.73^{a}	36.00 ± 0.71^{b}	39.00 ± 1.16^{b}	37.50 ± 0.29^{b}
RBC (10^6/mm^3)	2.04 ± 0.14^{a}	2.47 ± 0.07^{b}	2.57 ± 0.12^{b}	2.69 ± 0.01^{b}
WBC (10^3/mm^3)	133.88 ± 13.92	155.20 ± 3.32	161.63 ± 18.17	176.65 ± 11.00
MCV (fl)	148.00 ± 1.73^{bc}	145.75 ± 2.18^{b}	152.50 ± 2.02^{c}	139.50 ± 0.65^{a}
MCH (pg)	28.20 ± 0.34	28.28 ± 0.47	27.83 ± 2.26	27.65 ± 0.31
MCHC (g/L)	190.75 ± 1.57	193.75 ± 2.18	182.25 ± 12.42	198.50 ± 1.44
Serum total protein (g/dl)	3.20 ± 0.06	2.75 ± 0.20	3.23 ± 0.05	3.03 ± 0.13

*The result represents mean ± SEM of 5 fishes per tank with a total of 10 fishes per diet was pooled together and ran in duplicate for each diet.

Mean values in the same row with different superscripts are significantly different (P<0.05).

HGB = Haemoglobin; HCT = haematocrit; RBC = red blood cell; WBC = white blood cell; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration.

The haematological parameters of red hybrid tilapia fed with various experimental diets revealed that there were no significant differences observed amongst all treatments for WBC, MCH, and MCHC (P>0.05). However, the HGB, HCT, RBC and MCV were significantly affected by the supplementation of EPS (P<0.05) (Table 4.3).

Although no significant difference for HGB was observed amongst 1E, 2E and 3E groups, there was a significant difference when comparing control with other EPS

supplemented groups. A similar trend was observed in HGB, HCT and RBC of fish fed with the experimental diets.

Both HGB and RBC revealed the highest value at 3E with the values of (7.43 ± 0.11 g/dl) and (2.69 ± 0.01 10^6/mm^3), respectively. However, the lowest HGB and RBC values were observed in control with the values of (5.75 ± 0.35 g/dl) and (2.04 ± 0.14 10^6/mm^3), respectively. Besides, similar trends were observed in HGB and RBC with increasing values from control up to 3E. The 2E group showed the highest HCT value (39.00 ± 1.16 %), while the lowest was at control (30.00 ± 1.73 %).

The MCV for control did not show any significant difference when comparing with 1E and 2E, but the control showed significantly increased MCV value when comparing with 3E. However, the highest MCV value was recorded at 2E (152.50 ± 0.02 fl), and the lowest was at 3E (139.50 ± 0.65 fl).

The WBC, the MCH, and the MCHC values did not show significantly effects among the groups. However, numerically higher values were recorded for WBC in 3E (176.65 ± 11.00 10^3/mm^3), MCH in 1E (28.28 ± 0.47 pg) and MCHC in 3E (198.50 ± 1.44 g/L). The lowest values were recorded for WBC in control (133.88 ± 13.92 10^3/mm^3), MCH in 3E (27.65 ± 0.31 pg), and MCHC in 2E (182.25 ± 12.42 g/L).

There were no significant differences found amongst the group for serum total protein. By comparing the group with the numerical value of serum total protein (Table 4.5), 2E (3.23 ± 0.05 g/dl) showed slightly higher value whereas 1E (2.75 ± 0.20 g/dl) showed the lowest value amongst the other diets.

CHAPTER 5: DISCUSSION

The obtained results from the current study showed that the body composition, the organosomatic indices and the haematological parameters of red hybrid tilapia (*Oreochromis* sp.) were affected by the supplementation of exopolysaccharide (EPS) from fermented mycelium of *Ganoderma lucidum* in the experimental diets at a different level.

The raw ingredients including fishmeal, rice bran, corn meal, soybean and *G. lucidum* exopolysaccharides were analysed for their proximate composition prior to feed formulation. All the feed were then formulated isonitrogenous (35 – 37 %). The protein content of the feed ranged from 35 – 37 %. This range might be due to the overestimation of the ingredient's protein content during the proximate composition. However, the values formulated for these feeds were within the red hybrid tilapia nutrient requirement (Mjoun et al., 2010). Although FAO (2019b) suggested 30 – 35 % of crude protein needed for tilapia fish size ranged from 10 – 25 g, the reviewed study by Mjoun et al. (2010) deduced that 30 – 56 % of crude protein is sufficient for growth performance of fry sized tilapia and for juvenile is in the range of 30 – 40 %. Therefore, the percentage of crude protein in fish feed in the current study was at acceptable level for red hybrid tilapia fingerlings (10 – 20 g). The other studies that used isonitrogenous diets within the range 35 – 37 % are Kirimi et al. (2016) which utilised 35 % of crude protein for Nile tilapia fingerlings, Din et al. (2012) used 35 % of crude protein for red tilapia fingerlings, and Leal et al. (2009) formulated 37 % of crude protein for Nile tilapia juvenile. Their fish obtained good growth performance after consuming isonitrogenous diet within 35 – 37 % of crude protein.

The lipid and the carbohydrate of the feed ranges in the current study were 4 – 6 % and 42 – 44 %, respectively. Although FAO (2019b), stated that the dietary lipid of 5 %

was the minimum requirement to improve the tilapia growth and the protein utilization efficiency, Ighwela et al. (2014) proved that 4 % was sufficient to sustain the growth of tilapia. According to FAO (2019b), there was no exact carbohydrate percentage required for tilapia species. However, the digestible carbohydrate that can be utilized fully by tilapia was at 35 – 40 %. In this study, the lipid and the carbohydrate levels in all diet feeds were at acceptable levels.

During this study, the mortality rate of red hybrid tilapia was under control. Ahmed et al. (2017) study supported that beta-glucan contained feed was not toxic at 3 g/kg supplemented diet. However, the mortality might be due to the environmental or physical factor, and it was supported by Muin et al. (2015) study. Besides, the raw EPS of *G. lucidum* has been tested previously as feed additive on zebrafish embryo (zebrafish carries most of the higher vertebrates characteristics). The result showed that *G. lucidum* EPS is a non-toxic product that can be safely used as feed additive in the aquaculture industry (Taufek et al., 2020).

5.1 Body Composition of Fish Fillet

Tilapia is one of the fish that can contribute as a protein source for human consumption. The edible part of the fish and expected to contain high nutritional value is the fillet of the fish. Therefore, the body composition was evaluated on red hybrid tilapia fillet in this study to see the nutrient contents of the fillet instead of the carcasses. The fillet can be said as pure meat because it did not involve the head, the scales, the fins and the viscera (Ali et al., 2004).

The high protein content in fish fillet indicates that red hybrid tilapia utilizes well the feed to grow its muscle and body (Breck, 2014). The feed containing *G. lucidum* EPS produced high protein content of red hybrid tilapia fillet as compared to control. It was proven when comparing with other studies which maintained feed protein content within

35 – 37 %. For example, Din et al. (2012) study used isonitrogenous diets (35 %) and the higher supplementation of mushroom stalk meal (*Pleurotus sajor-caju*) at 10 % experienced high protein content (70.41 %) in the whole red tilapia's body which was lower than the reading obtained by feed containing EPS in the present study. This shows that *G. lucidum* EPS could act as an alternative protein source in aquaculture. Besides, the utilization of the feed to boost the red hybrid tilapia growth might be due to the present of beta-glucan in EPS. It was well supported by Ai et al. (2007) study which reported beta-glucan in fungus could act as an efficient growth promoter for fish. In the current study, *G. lucidum* exopolysaccharide proved it by producing high growth and also high protein in fillet of red hybrid tilapia.

Based on the lipid content, the fillet of red hybrid tilapia fed with EPS from *G. lucidum* was slightly higher (1.37 – 1.68 %) as compared to those given red algae (*Gracilaria arcuata*) as a dietary supplement for Nile tilapia (0.40 – 0.46 %) (Younis et al., 2018). However, according to Sheikhzadeh et al. (2019) study, the lipid content in the fillet of *Oncorhynchus mykiss* was higher than the present study, which was at 5.41 – 5.80 %. The lipid content in the fish feed also was different from the current study, which was three times higher (19.65 – 19.83 %). Therefore, it can be concluded that when the uptake of crude lipid by fish decreases, the lipid content in the fillet also decreases and vice-versa. This situation is relevant to the current study, where the lowest lipid content in red hybrid tilapia fillet was observed in 2E, which had the lowest lipid content in the feed. The influence of dietary lipids on fish fillet is supported by Chaiyapechara et al. (2003) study.

The dry matter content of the fillet for all diets were approximately 95 %, as this study used dried samples. The reason for choosing the dried sample over the wet sample is to reduce the contamination of the sample during long-time period storage for proximate analysis. Besides, the dried samples were required for nutrient analysis such as crude

protein, lipid, fibre, and ash instead of the wet sample (AOAC, 2003). The wet sample was only used to calculate the body water-level (El-Sayed et al., 2004), which in this study was not done. Although not many studies used the dry sample to obtain the dry matter contents in the fillet, but the range obtained by Taufek et al. (2017) study (92 – 98 %) validated the dry matter level in the current fish fillet study.

As far as the author's knowledge, there was very little study by other researchers on fibre and carbohydrate contents in fish fillet after feeding the tilapia with the supplemented feed. However, there is a study related to the current study where they used *G. lucidum* polysaccharide as feed supplement for *Macrobranchium rosenbergii*. The study showed significant improvement in carbohydrate content (22 – 36 g/kg wet wt) in its muscle at different level (1 g/kg, 1.5 g/kg, 2.0 g/kg, and 2.5 g/kg) as compared to their control's carbohydrate content (22 g/kg wet wt) (Mohan et al., 2016). Based on the fibre content in red hybrid tilapia fillet of the present study, it revealed that the values were not much different amongst the experimental diets although there were small changes occurred between them. The carbohydrate level was also not consistent in red hybrid tilapia fillet (dry weight) because it depended on the crude protein, the lipid, the ash and the fibre contents in the fish fillet. By referring to Schulz et al. (2005) equation on NFE/carbohydrate, it could be concluded that as the crude protein, the lipid, the ash and the fibre increases, the amount of the carbohydrate decreases in the fish fillet. After the observation of the lipid content in fish fillet and also the HSI and the VSI trends in the present study, it could be concluded that the decrease in carbohydrate content in fish fillet might be due to the usage of carbohydrate as energy in fish metabolism and some were stored in muscle as lipid deposition (Ighwela et al., 2014; Li et al., 2018).

Gross energy is the energy present in fish fillet in the form of protein, lipid and carbohydrate. There was a variation trend of gross energy observed amongst all groups

fish fillet because it might be slightly influenced by the protein, the lipid and the carbohydrate levels in fish fillet (Schulz et al., 2005). Nevertheless, the overall value of gross energy revealed that all experimental fish fillet was approximately at 22 – 23 kJ/g. Thus, no difference was observed at the energy content in fish fillet amongst all diets. These results were in accordance with Ballestrazzi & Lanari (1996) studies where their result showed that the gross energy of body composition of sea bass showed no difference in all diets and maintained between 24 – 25 kJ/g DW.

5.2 Effect of *G. lucidum* EPS on Organosomatic Indices

Hepatosomatic index (HSI) basically gives information about the condition of the liver and the body, water pollution effect and also to determine energy reserved in fish (Ighwela et al., 2014; Liebel et al., 2013; Sadekarpawar & Parikh, 2013; Taddese et al., 2014). Based on the current findings, supplementation of *G. lucidum* in red hybrid tilapia feed provided a good effect on HSI, which started from 2 g/kg of EPS as compared to control. It might be due to the EPS structure, which made up of sugar residue that may contribute to the increase of carbohydrate value in EPS feed as compared to control. By referring to the normal tilapia's utilizable carbohydrate level (35 – 40 %), the findings conveyed that 2E and 3E groups had excess dietary carbohydrate because both groups had high carbohydrate content in the feed which were 43.83 % and 43.93 %, respectively (FAO, 2019b). So, as the number of EPS in fish diets increases, the excess dietary carbohydrate also increases. Eventually, it increases the liver size by storing carbohydrate in the form of glycogen which may lead to an increase of HSI value (Ahmed et al., 2015). The studies that related to the current study also stated that the high supplementation of raw polysaccharide (beta-glucan) in Nile tilapia feed increased the HSI value as compared to the other diets (Ahmed et al., 2017). The contribution of carbohydrates in reserving energy in the liver of fish was well supported by studies made by Ahmad et al. (2012)

and Ighwela et al. (2014). The increasing trend of HSI indicated the fish was well fed during the feeding trial (Pope et al., 2010).

Generally, VSI determines the condition of viscera and body. Besides, VSI also plays a role in fish metabolism and identifies the fish health status, which correlated with HSI (Ighwela et al., 2014; Sogbesan et al., 2017). The similarities that correlated the HSI and the VSI are the excess dietary carbohydrate which influences the viscera and the liver weight. As the VSI value increases, it reveals that there is a lipid deposition at viscera by using the excess dietary carbohydrate (Ahmad et al., 2012). However, in this study, the VSI value of red hybrid tilapia was high in control as compared to the EPS groups. The diet 3E found the lowest VSI value. This study revealed that the supplementation of EPS in red hybrid tilapia feed provided good effect on VSI. This result is also supported by Chaiyapechara et al. (2003) study where viscera lipid should be decreased to avoid discarded by-product, health problem, and low flavours and nutrition in fish fillets. The VSI value of Nile tilapia (*O. niloticus*) fed with carbohydrate and lipid-based diet from Xie et al. (2017) study showed high VSI value with the range of 12 – 15 % as compared to the current study value (9 – 11 %). So, it shows that the excess dietary carbohydrate and the lipid deposited in viscera of tilapia as fat and eventually increased the VSI value.

Condition factor (CF) is the ratio of body weight over cubic body length. It determines the condition, the fatness, and the health of fish (Ahmed et al., 2011). There was a variation of CF trend on the experimental diets with the increasing amount of supplementation of EPS. It is because of the influence of fish sex (Anani & Nunoo, 2016). Apart from fish sex, Barnham & Baxter (1998) deduced that it might be a disturbance of maturation stage, gut fullness, season, age of fish sex, food intake, growth of muscle and lipid reserved. They also stated that gonads could contribute to equal or more than 15 % of the whole body weight. However, female fish will experience a rapid drop in CF value

when its eggs are shed. Therefore, Barnham & Baxter (1998) concluded that the greatest impact on CF value was observed during the developmental stage of reproductive organs of fish. The mentioned factors that disturbed the CF value was supported by studies from Olurin & Aderibigbe (2006) and Hamid et al. (2015).

According to Anani & Nunoo (2016), CF also shows the well use of feeding sources by other internal organs such as fish gonad. Therefore, it influences the fish body parameters that involve total body weight and visceral weight, like CF and VSI. Mozsar et al. (2014) study also revealed that the decreases in dietary lipid and carbohydrate might contribute to low CF value because it influences the liver size and reserves the lipid in fish organs that might lead to the increase of fish body weight while vice-versa reason was noted for higher CF. Although a lot of factors could influence the trend of CF, the CF values for all diets in the current study were about 1.4 which indicated good fish health and isometric growth as well as desirable in fish farming. This was proven by study made by Ighwela et al. (2011), where the mean of CF was more than 1.0 for *O. niloticus*, and it showed an isometric growth. Based on the CF trend in all diets, it can be concluded that increased EPS supplementation in fish feed produced lean fish instead of fatty fish. This is well supported by Mozsar et al. (2014) study where low CF value, determined by the length-weight relationship showed less fat reserved in fish muscle and other parts of the body that lead to low fatness of fish.

5.3 Effect of *G. lucidum* EPS on Haematological Indices

Haematological parameters are essential tools in understanding the physiological status of the experimental fishes. Blood conditions has the ability to indicate the general condition of the animal in a very convenient way (Kefas et al., 2015). By looking at haematological results, the toxicity level, the feed quality and the surrounding condition of the fish can be predicted (Ayoola et al., 2014; Svobodová et al., 2005). The changes in

red blood cells count, white blood cells count, and haematocrit could be the sign of toxicity level of feed and the environmental effect on fish health status (Ozovehe, 2012). However, haematocrit, red blood cell and mean corpuscular volume values can predict the anemias, the hydration status and the red blood cell production. On the other hand, the total protein, albumin and globulin values are essential indicators for immune status, liver function, hydration and osmoregulation (Hrubec & Smith, 2000).

The current study discovered that the increasing of RBC was correlated with the increasing trend of HGB and HCT. This is in accordance with the synergistic linkage of the blood cells of haematocrit and haemoglobin which corresponding to red blood cell counts (Audu et al., 2014). By comparing with the normal range of HCT value for red hybrid tilapia (27 – 37 %) (Hrubec et al., 2000), the HCT value of fish fed with experimental diets increased from control to 3E. Nevertheless, slightly elevated levels were observed in 2 g/kg and 3 g/kg compared to the normal range. The increase in HCT value shows good absorption of nutrients and transportation of oxygen in the fish blood (Isaac et al., 2013). Kefas et al. (2015) stated most of the fishes rarely contain above 50 % of HCT value. If too high HCT value, it might generate polycythemia (Etim et al., 2014). If it decrease from the normal range, it may be damaged at the fish gill and/or weakening of osmoregulation which may lead to anaemia and haemodilution (Mohammed & Sambo, 2008).

Although no significant differences were found among treatments for WBC, when comparing the mean value with normal range, there was slightly higher effect of EPS on the immunity of red hybrid tilapia as compared to control. The WBC value was higher than the usual red hybrid tilapia range (21.56 – 154.69 $10^3/mm^3$) (Hrubec et al., 2000), but the control was within the range. The RBC value increased as going up from control

to 3E and all the values were within the normal range for red hybrid tilapia (1.91 – 2.83 $10^6/mm^3$) (Hrubec et al., 2000).

The two main components in the blood circulatory system are RBC which is responsible for gaseous exchange and WBC solely for immunity (Shen et al., 2018). However, other recent studies discovered that RBC also plays a role in expressing immunity genes and its responses to infections (Jung et al., 2019; Puente-Marin et al., 2019; Shen et al., 2018). In this study, feed containing EPS produced high RBC and WBC as compared to control, which could promote EPS as a good immunostimulant that can be used as a feed additive in boosting fish immunity. An increase in RBC also improved fish well-being by facilitating oxygen transportation capacity.

Based on Ighwela et al. (2012) study, maltose from barley was used as a feed additive in energy-based feed for Nile tilapia (*O. niloticus*) where by the highest WBC value (29.75 $10^3/mm^3$) was observed at 30 % malt supplementation. In the present study, the *G. lucidum* EPS feed also showed high energy contained feed. However, a small supplementation of EPS in the feed also experienced the highest WBC value (176.65 $10^3/mm^3$) at 3 g/kg EPS, which equivalent to 0.3 % EPS supplemented feed. Therefore, it showed that the presence of EPS, which might contain beta-glucan in fish diet could help in activating the innate immune system by increasing the number of white blood cells in the red hybrid tilapia body in this study (Sanchez-Martinez et al., 2017). As compared to Hrubec et al. (2000) study, the current research showed WBC value was higher than the normal range of red hybrid tilapia (21.56 – 154.69 $10^3/mm^3$). It might be due to the strain of hybrid tilapia, the rearing system used and also external factors such as the fish feed, the water quality, the condition of culture and the age of fish (Hrubec et al., 2000; Hrubec & Smith, 2000).

There is a lack of study about the impact of exceeding normal haematology range for red hybrid tilapia, especially on white blood cell counts. However, there is a study done on medicinal herbs as a feed additive for growth promoter in *O. niloticus* feed (Dada, 2012). Dada (2012) reported that the best growth response was at 5 g/kg of superlive powder (medicinal herbs) supplemented feed which brought to high white blood cell counts ($9800 \times 10^3/\mu l$ equivalents to $9800 \times 10^3/mm^3$) which was higher than the value obtained in the present study. Dada (2012) also reported that all diet fish were in a healthy state. Therefore, the present study could be claimed that the obtained WBC values for all *G. lucidum* EPS fed fish were safe for red hybrid tilapia.

Red blood cell indices are mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) which used to diagnose the level of anaemia (Sahan & Duman, 2010). In this study, MCH values for all diets were close to the normal range (28.3 – 42.3 pg), but MCHC values were below the normal range (220 – 290 g/L) (Hrubec et al., 2000). Haemoglobin values for all diets were within the normal range (7.0 – 9.8 g/dl) except for control where it dropped to 5.8 g/dl. The decreased in MCHC and unstable in MCH values in all diets, together with low HGB (control) value indicated that there might be a slight disturbance in hematopoietic organs of fish (Alsaid et al., 2015). Besides, it might be the influence of fish sex and size on blood characteristics (Gabriel et al., 2011). MCV values were within the normal range (115 – 183 fl) (Hrubec et al., 2000), although the unstable trend was observed amongst the diets. According to the previous study by Hardig & Hoglund (1983), the maturation of red blood cells determined the values of MCV and MCH. Hence, immature red blood cells which have larger cell volume and lower haemoglobin (Carvalho & Fernandes, 2006), might be the reason for the unstable trend of MCV values.

Overall, the HGB, the HCT and the RBC had significant effects on all EPS diet fish as compared to control, and the highest influenced was at 3E. Although no significant effect observed for WBC in all fish, an increasing trend was observed from 1E to 3E, accordingly, which corresponded to the trend of RBC which played a role in immunity as compared to control. A similar trend was observed in the study done by Chitsaz et al. (2018) during supplementation of *Lentinula edodes*, a medicinal mushrooms extract in great sturgeon juvenile (*Huso huso*) as compared to control diet. By referring to the reviewed studies made by Mohan et al. (2019), the increased in RBC and WBC values might be due to the presence of beta-glucan in exopolysaccharide.

All fish in the current study showed approximately the same serum total protein value, although a slightly higher value was observed in 2E fish. The total protein was within the normal red hybrid tilapia value (2.3 – 3.6 g/dl) (Hrubec et al., 2000). According to Svetina et al. (2002), blood and serum total protein concentrations are normally used as a primary indices of general biological well-being. The increase of serum total protein might be related to the mechanism of beta-glucan action in the fish body (Ringo et al., 2012; Vetvicka et al., 2013).

CHAPTER 6: CONCLUSION

Based on the presented results, it can be concluded that 3 g/kg EPS supplemented feed provided the best effect on haematological studies which became the early symptom prior to immune studies. Generally, the inclusion of at least 1 g/kg of EPS is sufficiently projected positive response in terms of body composition and organosomatic indices. This indicated that the small supplementation of EPS was sufficient to improve the nutritional value of its fillet, to enhance organosomatic indices and to boost the haematological value. Therefore, 1 g/kg of exopolysaccharide from the fermented mycelium of *G. lucidum* can act as a feed additive in aquaculture industry for the tilapia feed.

Nevertheless, there were some limitations observed throughout the research in this preliminary study. This included the duration of research, the sample size, the lack of available or/and reliable data, and the lack of prior research studies on the topic. So, this might be the obstacles for future works which should be taken into consideration.

The future works concerning the immune response, which exerted some challenges on the EPS fed red hybrid tilapia could be further studies. This could have a significant impact for the aquaculture industry where *G. lucidum* EPS will be accepted as immunostimulants in enhancing the innate immune system of red hybrid tilapia against diseases and maintained red hybrid tilapia as the important protein source in Malaysia.

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