FERMENTED MYCELIAL BIOMASS OF *Ganoderma lucidum* AS A POTENTIAL ADDITIVE FOR JUVENILE RED HYBRID TILAPIA (*Oreochromis* sp.)

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FERMENTED MYCELIAL BIOMASS OF *Ganoderma lucidum* AS A POTENTIAL ADDITIVE FOR JUVENILE RED HYBRID TILAPIA (*Oreochromis sp.)*

ABSTRACT

The presence of antibiotic compounds in aquaculture is gaining higher interest worldwide due to the emergence as dangerous pollutants of the environment and to human health. However, feed additive play an important role in agriculture to reduce the usage of antibiotics and aquaculture productive performance. Therefore, the polysaccharide produced by *Ganoderma lucidum* is recognised due to its essential biological activities. Growing biomass from mycelium of *Ganoderma lucidum* (MGL) through submerged liquid fermentation (SLF) has been a fast and straightforward alternative compared to fruiting bodies. The study aims to observe the inclusion of biomass from MGL as a dietary supplement on growth performance, antioxidant activity and fatty acid profile of red hybrid tilapia (*Oreochromis sp*) (RHTO). The experimental diets were 5g/kg, 10g/kg and 15g/kg of MB from MGL.Growth performance was measured using growth indicator parameters, antioxidant activity was measured for Glutathione S-Transferase (GST) and Catalase (CAT) while fatty acid profile was determine using the gas chromatography method. From the finding, the highest value for final weight and specific growth rate (SGR) was 35.4g and 2.14 in diet 15g/kg respectively. For feed conversion ratio (FCR) and feed intake (FI), the best result showed by 15g/kg diet. For antioxidants, the 10g/kg shows better results as GST (52.05nmol/mg protein) and CAT (169.04 nmol/min/ml) compared to other diets. There is no significant difference in 10g/kg diet compared to other diets. The finding on fatty acid profiling concludes that 10g/kg shows the best fatty acid profile for this study compared to control, 15g/kg and 5g/kg. Therefore, 10g/kg

enhanced the survival, growth performance as well as antioxidant value. Since there is no mortality recorded, the study concludes that MB is a potential natural dietary supplement in the fish feed which helps promote growth and boost the antioxidant activity in fish.

Keywords: *Oreochromis sp, Ganoderma lucidum*, growth performance, antioxidant activity, fatty acid profile

Fermentasi Miselium Biojisim daripada *Ganoderma Lucidum* sebagai Aditif untuk Juvana Tilapia Hibrid Merah

Abstrak

Kehadiran sebatian antibiotik dalam bidang akuakultur semakin mendapat perhatian di seluruh dunia kerana kemunculannya sebagai pencemar alam sekitar yang berbahaya dan kepada kesihatan manusia. Walau bagaimanapun, aditif makanan memainkan peranan penting dalam pertanian untuk mengurangkan penggunaan antibiotik dan prestasi produktif akuakultur. Oleh itu, polisakarida yang dihasilkan oleh *Ganoderma lucidum* diiktiraf kerana aktiviti biologi penting mereka. Biomas tumbuh dari miselium *Ganoderma lucidum* (MGL) melalui penapaian cecair terendam (SLF) telah menjadi alternatif yang mudah dan cepat berbanding dengan buah-buahan berbuah. Kajian ini bertujuan untuk melihat kemasukan biojisim dari MGL sebagai makanan tambahan kepada prestasi pertumbuhan, aktiviti antioksidan dan profil asid lemak tilapia hibrid merah (*Oreochromis sp*) (RHTO). Diet eksperimen adalah 5g /kg, 10g/kg dan 15g/kg MB dari MGL.Prestasi pertumbuhan diukur menggunakan parameter penunjuk pertumbuhan. Aktiviti antioksidan diukur untuk glutathione s-transferase (GST) dan catalase (CAT). Profil asid lemak ditentukan menggunakan kaedah kromatografi gas. Daripada penemuan ini, nilai tertinggi untuk berat akhir dan kadar pertumbuhan tertentu (SGR) masing-masing adalah 35.4g dan 2.14 dalam diet 15g/kg. Untuk nisbah penukaran makanan (FCR) dan pengambilan makanan (FI), hasil terbaik menunjukkan diet 15g/kg. Untuk antioxidant, 10g/kg menunjukkan hasil yang lebih baik seperti GST (protein 52.05nmol/mg) dan CAT (169.04 nmol/min/ml) berbanding diet lain. Tiada perbezaan yang signifikan dalam diet 10g/kg berbanding diet lain. Penemuan pada pengambilan asid lemak menyimpulkan bahawa 10g/kg menunjukkan profil asid paling lemak untuk kajian ini berbanding dengan kawalan, 15g/kg dan 5g/kg. Oleh itu, 10g/ kg meningkatkan

kelangsungan hidup, prestasi pertumbuhan serta nilai antioksidan. Oleh kerana tidak ada kematian yang direkodkan, kajian itu menyimpulkan bahawa MB adalah suplemen pemakanan semula jadi yang berpotensi dalam makanan ikan yang membantu mempromosikan pertumbuhan dan meningkatkan aktiviti antioksidan dalam ikan.

Kata kunci: *Oreochromis sp,* Ganoderma *lucidum,* miselium biojisim, Kadar pertumbuhan, antioksidan, Profil asid lemak

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LIST OF SYMBOLS AND ABBREVIATIONS

ºC	:	Degree Celcius
%	:	Percentage
ANOVA	:	Analysis of Variation
AOAC	:	Association of Official Analytical Chemical
BGW	:	Body Weight Gain
CAT	:	Catalase
DCP	:	Di-Calcium Phosphate
DHA	:	Docosahexaenoic Acid
EPA	:	Eicosapentaenoic Acid
FA	:	Fatty Acid
FAME	:	Fatty Acid Methyl Esters
FCR	:	Feed Conversion Ratio
FI	:	Feed Intake
FO	:	Fish Oil
g	:	Gram
G.lucidum	:	*Ganoderma lucidum*
GC	:	Gas Chromatography
GC-MS	:	Gas Chromatography Mass Spectrometry
GST	:	Glutathione S-Transferases
hrs	:	Hours
kg	:	Kilogram
MB	:	Mycelial Biomass
mg	:	Miligram
ml	:	Mililitre

SFA	:	Saturated Fatty Acid
SGR	:	Specific Growth Rate
SPSS	:	Statistical Package For The Social Sciences
SR	:	Survival Rate

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Fisheries and aquaculture remain relevant sources of food, nutrition, income, and livelihoods for hundreds of millions of people around the world. Tilapia is an important aquaculture species as a critical source of affordable animal protein, income to fish farmers and fishers, and domestic and export earnings. Tilapia has a high growth rate and suitable for the farm in a wide range of farming systems (Muin et al., 2016). Hence, according to the Fisheries Department of Malaysia (2016), tilapia has become the highest fish production in Malaysia next to catfish.

The antibiotic usage received much attention in media for the past few years due to the increasing number of diseases. The antibiotics used are often non-biodegradable and remain in the aquaculture environment for long periods. Antibiotics usage encourages the growth of bacteria, which can lead to an increase in infectious disease in fish, animals, and humans (Vignesh et al., 2011). The application of antibiotics and chemotherapeutics in aquaculture is restricted because of its negative impacts on the development of antibiotic-resistant bacteria, suppression of the host's immune system, destruction of the microbial population in the aquatic environment and bioaccumulation. Therefore, extra attention paid to the dietary supplementation of alternative friendly probiotics, prebiotics, and immunostimulants. The probiotics dietary supplementation improve the fish's immune response. Hence, reduce the susceptibility of fish to diseases (Gawad et al., 2016).

In aquaculture practice, fungal polysaccharides are the prebiotic substance that accepted as a nutritional component for regulating growth and health condition. Among these, prebiotics is non-digestible feed ingredients that benefit the host in several ways.

The benefits include stimulating the growth, abiotic stressors, and immune responses on defense mechanism against pathogenic microbes. Potential antioxidants such as carotenoids help to inactivate free radicals produced from regular cellular activity and biological and environmental stress (Kurutas, 2016). The need for antioxidants becomes even more critical as with the increased exposure to free radicals. Oxidative stress plays a significant role in the pathogenesis of several diseases in the past few decades (Carocho et al., 2013). It is the cellular toxicity caused by the overproduction of free radicals. Aquatic organisms have developed defenses to protect against reactive oxygen species (ROS) induced damage, including antioxidant enzymes such as Glutathione S-transferases (GST) (Snezhkina et al., 2019). Therefore, measurement of these antioxidant parameters may provide a hint of the antioxidant status in fish, and these parameters can serve as biomarkers for oxidative stress (Slaninová et al., 2009). The search for natural antioxidants as alternatives to synthetic products is of great interest (Snezhkina et al., 2019), particularly in the aquaculture industry.

The dynamic coupling and causal relationships have brought increasing attention to the health significance of fatty acids, especially polyunsaturated fatty acids (PUFAs), in human nutrition (Zhang et al., 2020). Fatty acids are essential for life due to their vital roles as a source of membrane constituents, energy, and metabolic and signaling mediators (Pereira et al., 2013). PUFAs are fatty acids that contain 18 or more carbon

atoms and more than one double bonds. Based on the position of the last double bond relative to the terminal methyl end of the molecule, the PUFAs are further divided into three classes: *n*-3 PUFAs, *n*-6 PUFAs, and *n*-9 PUFAs. Among them, *n*-3 PUFAs and *n*-6 PUFAs are essential fatty acids, which cannot be synthesized in mammals. The lipid pattern of their food strongly influences the fatty acid composition of fish. Feeding high-energy diets containing high amounts of fish oil in aquaculture results in marketable fish with substantial levels of n-3 polyunsaturated fatty acids. The marine fish species characterized by lipids containing low levels of linolenic acid and linoleic acid but having high levels of omega-3 such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) (Özogul et al., 2007; Ugoala et al., 2008). Further comparison of marine fish with freshwater fish species reveals the higher content of the omega-6 series fatty acids in freshwater fish and higher levels of omega-3 fatty acids in marine fish. The consumption of freshwater fish containing high levels of fatty acids is favourable for human health and beneficial effect in preventing cardiovascular diseases. (Jaya-Ram et al., 2018).

The previous study carried out by Chitra et al.(2016), shows that the *G.lucidum* can promote the feeding, followed by better survival and growth performance of fish. The tilapia fish industry is facing the issues within tilapia production due to several pathogenic diseases that could cause significant mortality in fish. Even though the vaccine and antibiotics introduced to overcome the diseases, but it turns up to overcome certain pathogenic diseases. Currently, there are no scientific studies conducted on mycelial biomass of *G.lucidum* as a dietary supplementation on antioxidant activity of red hybrid tilapia *(Oreochromis sp*.). Due to this reason, the *G.lucidum* is chosen as the species of interest throughout the project. The study aims to observe the inclusion of biomass from

the mycelium of *G.lucidum* on growth performance, antioxidative response, and fatty acid profile of red hybrid tilapia (*Oreochromis sp.).*

1.2 Objectives

1. To investigate the effect of fermented mycelial biomass from *Ganoderma lucidum* on the growth performance of red hybrid tilapia.
2. To determine the antioxidative response of red hybrid tilapia on feed with fermented mycelial biomass from *Ganoderma lucidum.*
3. To determine the fatty acid composition of red hybrid tilapia fed with fermented mycelial biomass from *Ganoderma lucidum.*

1.3 Hypothesis

The supplementation of fermented mycelium biomass of *G.lucidum* can affect the growth performance, fatty acid profile and antioxidant response of red hybrid tilapia (*Oreochromis sp.).*

CHAPTER 2: LITERATURE REVIEW

2.1 Tilapia Production

Tilapia is widely cultured in the tropical and subtropical regions and constitute the third largest group of farmed finfish. The first species of tilapia introduced to Asia was the Mozambique tilapia, *Oreochromis mossambicus*, in the 1940s. However, the production of *Oreochromis mossambicus* has replaced by Nile tilapia, *O. niloticus*. The production is due to their better growth performance in ponds. Furthermore, these fish sometimes referred to as 'aquatic chickens,' where they were cultured extensively within inland ponds. The production was rapidly increasing due to its beneficial characteristics. Tilapia is known for tolerance to crowding, high marketability, relative ease of captive spawning year-round, high disease resistance, success with polyculture, and the ability to accept lower-cost diets from terrestrial-based ingredients. (Ng & Ramono, 2013).

Figure 2.1: Red Tilapia

2.2 The usage of Antibiotics in Aquaculture

Aquaculture is one of the essential global production in the fight against malnutrition and poverty, particularly within developing countries. The aquaculture industry is an affordable source of high-quality animal protein, lipids, and other essential nutrients. (Tacon et al.,2010). Bacterial infections, which caused to raise the antibiotic resistance rate, especially in fishes and water environment of aquatic farms. Besides, the mode of "fish-to-fish" bacterial transmission in nursery ponds are usually occurring and spread in different environments of the fish breeding area from one area to another. Primary bacterial infections in tilapia fishes are *Aeromonas* species, *Streptococcus agalactiae, Streptococcus iniae*, *Flavobacterium columnare,* and *Francisella* species.

Hence, the farmers and hatchery operators have resorted to the use of various remedial measures, including the use of antimicrobials and drugs for controlling the disease. The frequency of utilizing these antibiotics and other chemicals is more in hatcheries and scientific farms than in the traditional farms. Research has proved that many chemicals manufactured and used today enter the environment, disperse, and persist in the environment for much longer originally expected (Kolpin, 2002). The usage of antibiotics in the fish and shrimp aquaculture has become a significant problem as it forms a sediment layer on the pond bottom. Moreover, it discharged as wastes and effluents mixing into runoff water and covering coastal mangroves destroying critical natural habitats for shrimps, fishes, and humans (Jusoff, 2013). The studies on fish farms have shown the majority of antibiotics added in feed. Hence, the residues may harm the ecosystems. (Le & Munekage, 2004).

2.3 Polysaccharide in fish feed

Higher fungi are excellent sources of a wide range of the most important natural products. Currently, considerable attention is generated in the polysaccharides produced by fungi. Polysaccharides are prebiotic substances, which could boost the immune responses, resistance to pathogens, and growth performance of fish and crustaceans (Mohan et al., 2016). Dietary polysaccharides are digested in the body and use as potential energy sources. The dietary polysaccharides were able to decrease the utilization of nutrients as an energy source and increase the storage of nutrients in organisms. **(**Chitra et al., 2016)

Table 2.1: Application of Mushrooms in different fish feeds and their effects

Mushroom and their compounds	Effects	Reference
Pleurotus florida	Improve growth rate of *(Oreochromis niloticus).*	Muin et al.(2015).
Pleurotus sajor-caju	Its stalk in the diet improved the specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and survival rates in red tilapia.	Van et al.(2019). Srichanun at al.(2017).
Pleurotus ostreatus	Different concentration of this mushroom extract shows increased growth rate, higher lysozyme activity and hematocrit contents in amour catfish. The polysaccharide extract increased in specific growth rate, weight gain, and hepatosomatic index (HSI) in Nile tilapia.	Ahmed et al.(2017). Katya et al.(2016).
Ganoderma lucidum	This mushroom ß glucan in the diet increases survival rate, weight gain, feed intake, specific growth rate in grass carp juveniles.	Chitra et al.(2016).
Innotus obliquus	It improved the growth performance of common carp.	Zou et al.(2016).

(Adapted from Srichanun et al., 2017)

2.4 *Ganoderma Lucidum (G.lucidum)*

The *G.lucidum* is a mushroom known as Lingzhi, which traditionally used in Chinese medicine for the prevention and treatment of human disease (Figure 2.2). Studies on *G.lucidum* and its products have reported containing beneficial biological, health-preserving, and therapeutic effects. Varieties of pharmacologically active chemicals have been extracted from *G.lucidum*. Similarly to *G. lucidum*, there are at least 140 different triterpenes that have been identified, which include ganoderic, lucidenic, ganodermic, ganoderenic, ganolucidic and applanoxidic acids, lucidones, ganoderals, and ganoderols (Phan et al., 2015). All these compounds have different medicinal values. Nowadays, they focused on polysaccharide produced by *G.lucidum* due to their important biological activities such as immunomodulatory, anti-oxidant, anti-cancer, and anti-inflammatory activities (Li et al., 2015).

Many medicinal and edible mushrooms are capable of being grown in the form of mycelial biomass in submerged cultures. Fungal fermentation in liquid medium ensures a high uniform quantitative biomass production as well as a high biological value, thus representing an alternative means to obtaining the various potential medicinal products. The fermentation technology has a role in maximizing the production of biomass and the level of biologically active components it contains (Vamanu, 2014). Growing mushroom mycelia in liquid culture on a defined nutrient medium has long been a simple and fast alternative method to produce fungal biomass (Zhong & Tang, 2004).

Some studies have already shown that the mycelial biomass of different medicinal mushrooms possesses pharmacologic properties comparable to those of mushroom

fruiting bodies (Vamanu, 2014). According to a study done by Vamanu (2014), the mycelia extracts of *G.lucidum* could prevent free radical attacks known to initiate membrane lipid peroxidation. It can defend against cellular malignization. Free radicals react with various molecules at the cellular level via oxidative stress and, as a result, bring about a perturbation of the normal cellular cycle. Hence, the mycelia extract is a natural defense mechanism against oxidative stress. The mycelia and their fermentation filtrate byproduct are alternative or substitute products of mature fruiting bodies because the production of fruiting bodies includes long cultivation. (Saltarelli et al., 2009).

Figure 2.2: The spoan culture of *G.lucidum*

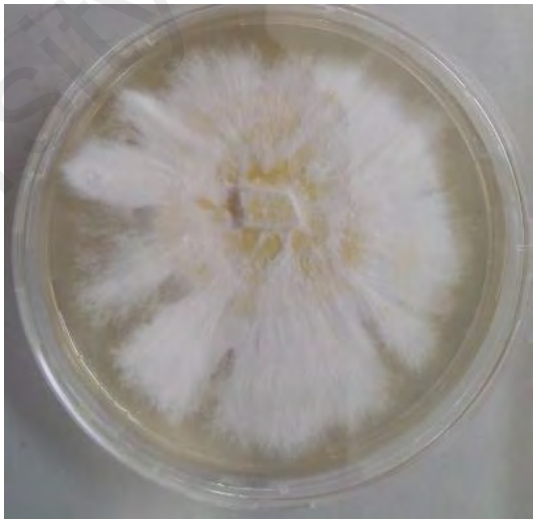


Figure 2.3: The mycelium of *G.lucidum*

Source: Adapted from (Supramani et al., 2018)

2.5 Antioxidant

Dietary supplements of prebiotics have shown great interest in aquaculture due to its growth performance enhancement and increasing resistance of fish to pathogens and environmental stressors through stimulating the host's immune response. According to Lobo et al.(2010), antioxidants are capable of stabilizing a free radical. When a free radical neutralized, it will prevent from damaging our cells. There are antioxidants that our body produces, including superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase, and glutathione S-transferases (GST).

However, the unrestricted production of oxygen-derived free radicals is hostile and damaging the cells. It can also cause a chain reaction resulting in the multiplication of new free radicals, as shown in a study by Mohan et al.(2015). According to Lundebye et.,2015), synthetic antioxidants are authorized for use in animal feed to protect against oxidation of unsaturated lipids. These include ethoxyquin (EQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), octylgallate and propylgallate. Thus, it is crucial to develop and utilize effective natural antioxidants. From the study conducted by Cellal (2019), the findings concluded that G.*lucidum* had high antioxidant potential and consumed as a natural antioxidant source. In the study done in mice fed Ganoderma, they were observed to improve anti-oxidation of plasma and increase the concentration of red blood cells. Therefore, when such blood circulates through the body, it facilitates the clearance of free radicals and reduces damage to tissues. As a result, organ survival longer as it was able to increase the lifespan of mice. The researcher also declares further experimental design to support the pathological organ evidence. (Chen, 2009)

2.5.1 Glutathione S-transferase (GST)

Glutathione S-transferases (EC 2.5.1.18) (GST), which catalyze the conjugation of glutathione with xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) play essential roles in both detoxification and bioactivation reactions (Dasari et al., 2018). According to Almeida et al.(2002), GST has detoxification properties. More studies conducted suggested that GST can detoxify endogenous toxic metabolites contamination and prevent peroxidative damage. Moreover, GST in some species of fish used as a biomarker for aquatic biomonitoring as well as defense against oxidative damage and peroxide products of DNA and lipids (Dasari et al., 2018) They play a significant role in protecting the cells from electrophilic compounds such as epoxides which are reactive intermediates produced during bioactivation of toxic organic contaminants.

2.5.2 Catalase

Catalase (EC 1.11.1.6) (CAT) is one of the dismutase enzymes. Catalase is a tetrameric heme-containing enzyme and is present in the cells of all aerobic organisms. It catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen (Menegazzo et al., 2019). The CAT is an important enzyme as it functions to protect and defend cells against oxidative damage (Taufek et al., 2016).

The *Oreochromis sp* is an omnivore that feeds on both plankton and aquatic plants. In a study conducted by Martínez-Álvarez et al.(2005), they compared the activity of antioxidant enzymes and the levels of lipid peroxidation in several tissues of omnivorous common carp, herbivorous silver carp, and carnivorous wels catfish (*Silurus glanis).* The outcome suggested that CAT activities in herbivorous fish were lower than

in omnivorous fish. Carnivorous species have the highest CAT activity in the liver and kidney. The antioxidant level in CAT activity was higher in livers of fish fed diets with a high lipid level and diets containing raw carbohydrates. (Rueda-Jasso et al., 2004).

2.6 Fatty Acid

Dietary lipids are vital nutrients affecting energy production in most fish and essential for growth and development. Most fish tissues contain a large portion of polyunsaturated fatty acids (PUFAs) essential for membrane function (Morales et al., 2005). However, fish are known to utilize protein preferentially to lipid or carbohydrate as an energy source. The diets containing low levels of lipid and digestible starch reduce the susceptibility of the fish towards oxidation and might enhance the growth rate (Rueda-Jasso et al., 2004). The fish oils have an essential benefit as a nutraceutical in our lives. Some fatty acids, especially omega-3 fatty acids, have known for their functions in the prevention of some artery diseases. The role of lipids in human nutrition is not only as of the source of energy, but lipids are also essential to maintain the human body.

Other vital functions of dietary lipids are as precursors of steroid hormones and prostaglandins, improving the flavor of feeds and affecting feed texture. (Global Aquaculture Advocate, November/ December 2009). According to a study done by Schulz et al. (2005), the fish are unable to synthesize long-chained polyunsaturated fatty acids (PUFA) as well as essential fatty acid (EFA). This EFA includes linoleic (18:2 n-6) and linolenic acid (18:3 n-3). Therefore, the addition of fish oil (FO) in feed enables the fish to obtain all the EFA.

The qualitative and quantitative EFA and FA requirements of fish vary among species. The fatty acids structure of fish tissue can be varied according to diet, size, age, reproductive cycle, salinity, temperature, season, and geographical location (FAO,2009). The FO contains about 25% saturated fatty acids and 75% unsaturated fatty acids. (Maruba et al., 2018). Next, a study from Ljubojevic et al.(2013) concludes that regardless of age and sex, the FA from RHTO's fillet may include Myristic, Palmitic, Stearic, Oleic, Linoleic, Linolenic, and Heneicosanoic. The freshwater fish like RHTO cannot biosynthesize C18 polyunsaturated fatty acids (PUFAs), linoleic acid (18:2 omega-6), or linolenic acid (18:3 omega-3) (Sokoła-Wysoczańska et al., 2018).

This statement is further supported by a study done by Pontoh & Tumiya (2018), the study showed that oleic acid is the highest fatty acids in tilapia in which oleic acid constitute approximately 39.7% and palmitic acid at 22.3% but slightly lower in EPA at only 3%. The polyunsaturated omega-3 fatty acids, such as EPA (Eicosapentaenoic acid) and DHA (Docosahexaenoic acid), have a widespread impact on preventing atherosclerosis diseases and can improve the human brain activity and eye visually (Kaur et al., 2015).

The fatty acid from various organism products is generally analyzed using the gas chromatography method. Gas chromatography-mass spectrometry (GC-MS) sample preparation is performed on smaller and more volatile samples as benzenes, alcohols, and aromatics, and simple molecules such as steroids, fatty acids, and hormones (Restek, 2019). The fatty acid methyl esters (FAME) analysis is a crucial tool both for identifying fats and oils as well as determining the total fat content in foods. Fats can be extracted

from a matrix using a nonpolar solvent and saponified to produce salts of the free fatty acids. It can also be applied to the study of liquid, gaseous, and solid samples. There are many advantages to using GC/MS for compound analysis, including its ability to separate complex mixtures, to quantify analytes, and to determine trace levels of organic contamination.(Restek, 2019).

CHAPTER 3: MATERIAL AND METHOD

3.1 *Ganoderma lucidum*

3.1.1 Preparation of biomass from the mycelium of *Ganoderma lucidum* (MGL)

The *G.lucidum* was obtained as fruiting bodies from Omics Lab in Universiti Malaya, and the strain used is QRS 5120 (Supramani et al., 2018). The fruiting bodies were sterile before proceeding to tissue culture to obtain the mycelium. The fruiting body was washed using 99.9% ethanol (Sigma-Aldrich, Dorset, UK) for 10 s and dried in a laminar flow. Then, cracked using a scalpel and the inner part of the fruiting body twisted and removed using forceps. Next, the tissue obtained placed on malt extract agar (MEA) (Sigma-Aldrich, Dorset, UK) and maintain at room temperature until signs of mycelium growth observed. The mycelium was sub-cultured onto fresh MEA to obtain pure mycelium (Supramani et al., 2018). Through submerged liquid fermentation (SLF), the suspended biomass grows as a cluster of mycelia that eventually stabilize to form pellets in the form of densely branched hyphae forming a compact ovoid shape. (Ortiz et al., 2016)

The harvesting process of mycelium biomass (MB) of MGL (Figure 3.1) took 20 days. This process includes two-cycle, which are called the first seed and second seed. Each cycle had 10 days each. On the first seed cycle, the media prepared in a 300ml for the 9 flask of 250mL Erlenmeyer flask. About 500ml media prepared according (Supramani et al., 2019) which consists of 1.0g of yeast extraction (Oxoid no. LP0021, Dardilly, France), 0.5g of magnesium sulphate ($MgSO_4$) (Bendosen Laboratory Chemicals no. C0481, Bendosen, Norway), 0.5g of mono- potassium phosphate (KH_2) (Bendosen Laboratory Chemicals no. C0637, Bendosen, Norway), 0.5g of dipotassium phosphate

(K_2H) (Bendosen Laboratory Chemicals no. C0680-2296192, Bendosen, Norway), and 4g of ammonia chloride (NH_4Cl_2) (Bendosen Laboratory Chemicals no.C0055, Bendosen, Norway). Then the glucose concentration was prepared for 400ml (400ml = 40g), and the pH for media and the glucose was adjusted to pH 4 by adding the hydrochloric acid (HCl). The mixture was then sterilized (121 °C, 30 min, 15 psi) in an autoclave. The mycelium in the agar was cut into cube size (5mm X 5mm) and place it into the media. The inoculum of were cultivated for 10-days in the following condition : Temperature :30 °C and agitation rate = 100 rpm (Wan et al., 2016) in a Rotary Shaker Incubator (New Brunswick, Edison, L.N., USA).

Next , the mycelium from the 1st seed culture was homogenised using a sterile Warring blender for 20s (Wan et al., 2016). This was used as the inoculum for the main culture in second cycle. For second seed cycle, 9 flask of 500mL Erlenmeyer flask were used. The inoculum was the measure for 20ml mixed with 100ml of media solution and 60ml of glucose in each flask. The inoculum of were cultivated for 10-days in the following condition : Temperature :30 °C and agitation rate = 100 rpm (Wan et al., 2016) in a Rotary Shaker Incubator (New Brunswick, Edison, L.N., USA). After 10 days, the culture proceeds for extraction of biomass.

3.1.2 Extraction of mycelial biomass (MB)

The method of extraction following Supramani et al.(2019) was used. The biomass extracted from the MB by using Bucher funnel filtrate to filtrate Ganoderma *lucidum* strains and fruiting bodies Ganoderma *lucidum*. The filtered mycelial biomass was mix with distilled water and washed three times to remove any impurities. The

supernatant was discarded, and the pellet placed in a food dehydrator at the lowest temperature (30ºC) until a constant weight achieve.

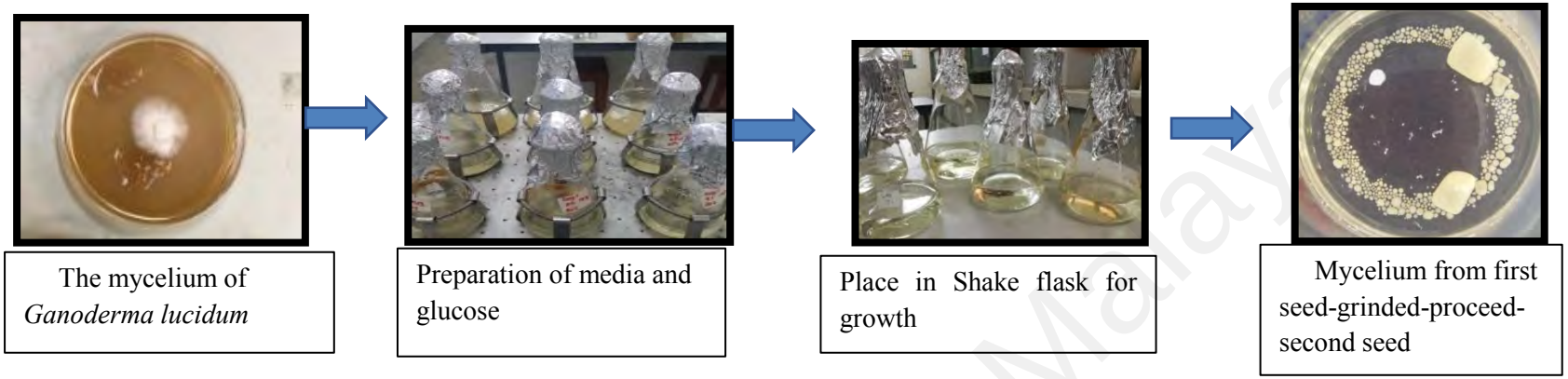
3.1.3 Preparation of biomass as the feed addictive

The dry mycelial biomass grounded to a fine powder. This fine ground powder was analysed for proximate composition to determine the protein, lipid, fibre and dry matter.

3.2 Preparation of experimental diets

The raw materials include fishmeal, corn meal, rice bran, soybean meal, vitamins, mineral and Di-calcium Phosphate (DCP) were purchased from a local supplier. All the raw ingredients were finely grounded samples and analysed in duplicate using the standard method for dry matter, moisture, crude protein, crude lipid and ash content. (AOAC, 2012) before being used formulating the experimental diet. All the proximate analysis data are shown in Table 3.1. The proximate results were used to formulate the experimental fish feed using WinFeed version 2.8 (Table 3.2). All the experimental diets were prepared using, a mini pelleting machine to produce standard-sized pellets of 0.3cm in diameter. Next, the feed were dried in the oven at 60ºC for 24 hrs. The dried feed kept inside plastic ziplock and stored in a cold room at 4ºC to prevent fungus infestation (Figure 3.2). Moreover, the diets were proximate for crude protein, crude lipid, crude fibre, ash and moist. Table 3.3 shows proximate compositions of experimental diet and control feed which been used throughout the experimental week. The experimental diets were 5g/kg, 10g/kg and 15g/kg of MB from MGL.

Preparation of first Seed



Preparation of Second Seed

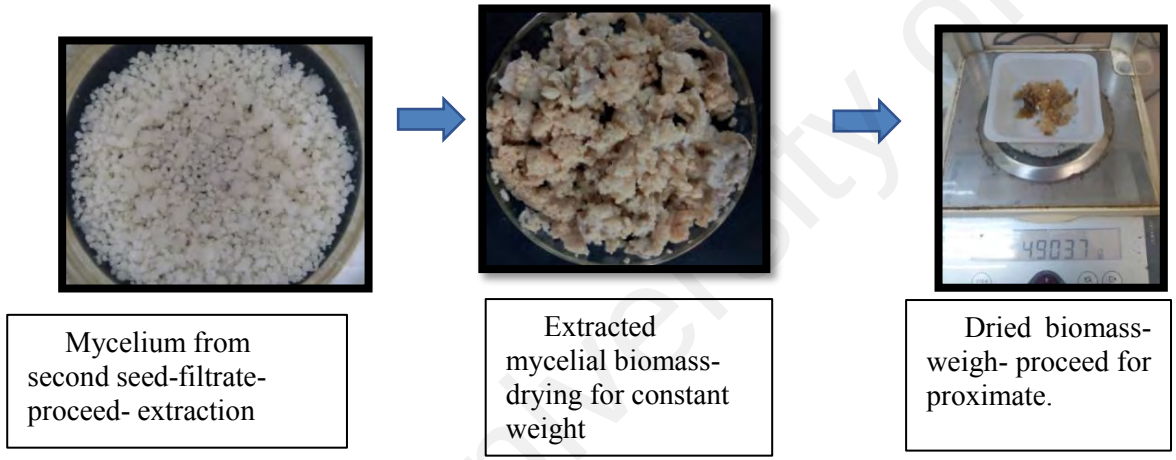
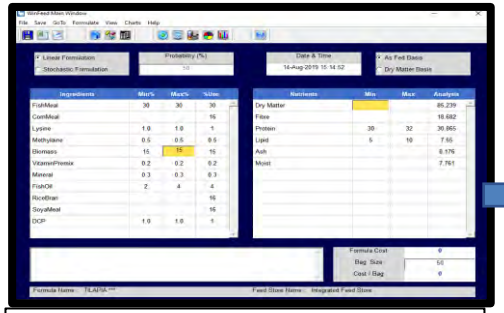
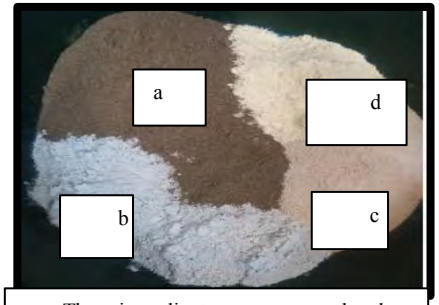


Figure 3.1: Production of MB from *Ganoderma lucidum*

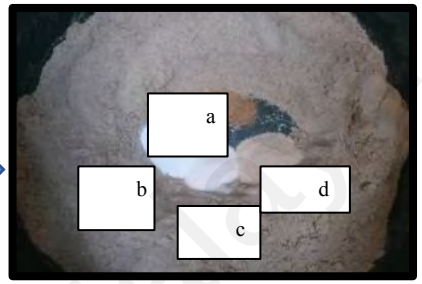
Preparation of Diet



The feed is formulated using WinFeed version 2.8(Least Feed Cost Formulation computer program



These ingredients were measured and mixed well. The alphabets indicate as below:a: fishmeal b: rice bran c: Soya meal



These ingredients were measured and mix well. The alphabets indicate as below: a: VitaminPremix b: Lysine/Methionine c: DCP d: Mineral

The required mycelial biomass was added to the mixture. An arrow indicates the biomass.

40ml of fish oil and 350ml of distilled water added. The mixture was mixed thoroughly until the semi-moist dough formed.

The feed was prepared using mini pelleting machine to produce standard sized pallet of 0.3cm in diameter

The feed was sieve before dried in the oven to remove the excess of feed powder form. The feed was dry in the oven for 24 hours at 60ºC

The feed was measure. It is packed in zip lock for each experiment diet throughout the week.

Figure 3.2: Preparation of diet

Table 3.1: Proximate analysis of raw materials

Components	Protein	Fibre	Carbohydrate	Ash	Lipid
MB	32.23±0.3^{e}	13.8± 7.1^{a}	48.38 ± 8.1^{b}	1.14 ± 1.1 ^{a}	4.45 ± 0.2^{c}
FM	54.27± 1.1^{c}	14.54 ± 11.3^{a}	5.6 ± 9.9^{a}	23.16 ± 0.5^{b}	2.41 ± 0.1^{ab}
CM	6.64± 0.3^{a}	9.81± 0.9^{a}	79.23 ± 1.6^{c}	1.73 ± 1.1^{a}	2.6 ± 0.01^{b}
RBM	11.23±0.3^{b}	19.4 ± 8.2^{a}	55.30 ± 9.2^{bc}	5.3 ± 1.4^{a}	8.76 ± 0.1^{d}
SBM	43.01 ±0.2^{d}	9.64± 3.3^{a}	40.04± 1.4^{b}	5.16 ± 1.6^{a}	2.14 ± 0.1^{a}

[1]Analysed in duplicate using the standard method for dry matter, moisture, crude protein, lipid and ash content. (AOAC, 2012).

[2]The results represent mean ± SEM, all percentage reports on a dry matter basis.

[3]Means in the same row with different letters are significantly different ($p< 0.05$)

[4] MB: Mycelia Biomass, FM: Fishmeal,CM :Corn meal ,RBM : Rice Bran Meal,SBM: Soy Bean meal.

Table 3.2 : Formulation of the experimental diets

Ingredients	Control	5g/kg	10g/kg	15g/kg
FM(g)	300.0	300.0	300.0	300.0
CM(g)	193.9	193.3	192.6	192
RBM(g)	199.3	198.3	197.4	196.4
SBM (g)	236.8	233.4	230	226.6
Biomass	0.0	5.0	10.0	15.0
Lysine (g)	10.0	10.0	10.0	10.0
Methionine(g)	5.0	5.0	5.0	5.0
Vitamin	2.0	2.0	2.0	2.0
Mineral(g)	3.0	3.0	3.0	3.0
DCP(g)	10.0	10.0	10.0	10.0
Fish Oil (ml)	40.0	40.0	40.0	40.0
Total	1000	1000	1000	1000

[1] Preparation of feed for 1000g per diet. FM: Fishmeal;CM :Corn meal;RBM : Rice Bran Meal;SBM: Soy Bean meal; DCP: Di-calcium phosphate

[2]The vitamin premix supplied the following per 100g diet: Vitamin A,500IU;Vitamin D3,100IU;Vitamin E,0.75mg;Vitamin K, 0.02mg;Vitamin B1,1.0mg;Vitamin B2,0.5mg;Vitamin B3,0.3mg;Vitamin B6, 0.2mg;Vitamin B12,0.001mg;Vitamin C, 0.1mg; Niacin,0.2mg;Folic Acid,0.1mg;Biotin,0.235mg;Pantothenic acid,1.0mg; Inositol,2.5mg

[3]The mineral premix supplied the following per kg diet:
Selenium,0.2.mg;Iron,8mg;Manganese,1.0mg;Zinc,8.0mg;Copper,0.15mg;Potassium Chloride, 0.4mg; Magnesium Oxide,0.6mg;Sodium Bicarbonate,1.55mg;Iodine, 1.0mg; Cobalt,0.25mg;

Table 3.3: Proximate composition of experimental diet and control feed

Diets	Control	5g/kg	10g/kg	15g/kg
Protein(%)	37.86	33.51	35.36	31.78
Lipid (%)	6.29	5.25	5.26	5.82
Fibre (%)	1.63	1.22	1.28	1.65
Ash (%)	12.00	12.20	12.61	12.59
DM (%)[3]	87.25	69.36	73.75	72.76
NFE (%)[4]	42.22	47.82	45.49	48.16
GE (kJ/g)[5]	18.98	18.51	18.55	18.38

[1]Analysed in duplicate using the standard method for dry matter, moisture, crude protein, lipid and ash content. (AOAC, 2012).

[2]The results represent mean.

[3]DM: Dry matter

[4]NFE: Nitrogen Free Extract, NFE= % Dry matter - (% crude protein + % crude fat + % crude ash+ % crude fiber)

[5]Gross energy for every diets was calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g (Schulz et al., 2005).

3.3 Experimental Fish Setup

A total of 120 RHTO fingerlings were purchased from the local farm located at Sg. Buloh, Selangor. The fishes were transported to the Freshwater Aquarium Laboratory located in the Institute of Biological Sciences. Next, eight tanks were used and filled with ¾ of water, added few drops of anti-chlorine to dechlorinate the water. The tanks were equipped with filtration and pump systems. The tanks were aerated for two days before the arrival of the tilapia fingerlings.

All the RHTO fingerlings were acclimatized to environmental conditions for two weeks before the feeding trials and fed with a commercial diet (Dindings) twice per day. All the uneaten food were collected and dried after feeding to determine the feed intake. The eight plastic tanks (32cm x 62cm x 34 cm) were filled with ¾ of water from an 80L capacity tank with the closed re-circulation system used in these feeding activities. About 20-30% of dechlorinated water was used to replace the water every once in two days to maintain the water quality. The RHTO fingerlings randomly divided into four groups, each in duplicates cultures as justified by Wan Mohtar et al.(2020), about 15 fishes per tank with an weight average of 15.4g ± 1.4.The RHTO were in a range weight of 10 - 20g. Three groups of RHTO fingerlings were fed with diets of either 5g/kg, 10 g/kg, and 15 g/kg biomass of MGL. The remaining group was served as a control with basal feed without any supplementation of MGL biomass. The feeding trial was conducted for 42 days. (Caldini et al., 2011).

Next, the experimental RHTO fingerlings were fed twice daily at 0900h and 1500h. The feed was given at a rate of 3% of their Body Weight (BW) ratio at the beginning of

the feeding trial at week 1 and week 2. The feed further adjusted to 2.8% for weeks 3 and 4 and 2.6% for week 5 and week 6 due to insufficient production of MB. Any mortality was observed and recorded every day throughout the feeding trial. Next, the experimental RHTO measured as the individual weight record (per fish). The level adjusted according to the BW after weighing them once in two weeks. At the end of the feeding trial, all ten fishes from the eight tanks were selected randomly for further analysis.

The water quality was maintained and measured according to the American Public Health Association (1992). The water temperature maintained around 26ºC - 29°C, followed by pH at 6.5-7.5 and dissolve oxygen 6.5-8.0mg/L. Next, the ammonia and nitrate were measured using the HI96786 nitrate portable photometer and HI96715 ammonia portable photometer. The values were recorded and maintain around 0.5-0.8mg/ml for ammonia and 0.5-1.9mg/ml for nitrate.

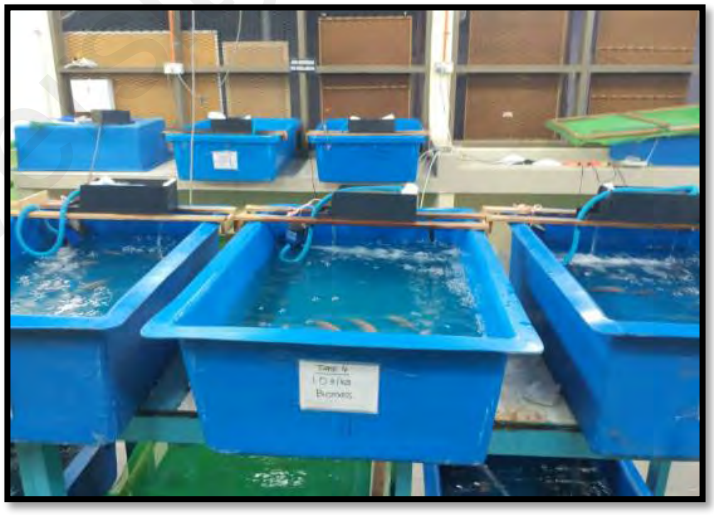


Figure 3.3: Tank setup

3.4 Proximate and chemical analysis of diets

The experimental diets and ingredients were analysed for the proximate composition, according to the Association of Official Analytical Chemist method (AOAC, 2012).

3.4.1 Crude Protein

The crude protein content was determined by the Kjeldahl method (Method 981.10, adapted from AOAC, 2012). Approximately, 0.15g of the sample was weighed and added into the Kjeldahl digestion tube. One tablet of 100mg Selenium Kjeltabs Catalyst followed with 6ml concentrated sulphuric acid was added into the tube before digested in FOSS Tecator Digestor Auto for 1 hour at 420ºC. After one hour of digestion, the tube was left to cool down for 15 minutes before the distillation process. The digested sample was hydrolyzed with 80ml of deionized water and 50ml of sodium hydroxide (NaOH), distilled with 25ml of 4% boric acid and 7-8 drops of titration indicator. The titration indicator was prepared by dissolving 100mg of bromocresol green in 100ml of methanol and 70mg of methyl red in 100ml of methanol. The distillation process was done in a programmable unit of the Vapodest50 equipped with a titrator with an automatic addition of water and NaOH. All samples and blanks analyzed in duplicates. Next, 0.01 M of hydrochloric acid (HCl) was used to titrate the distillation product in the conical flask, and the values were recorded. All samples and blanks were analysed in duplicates.

The protein content of the sample and blanks were calculated as :

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

Where, S = titrate of HCL

B = Titrate HCL for blank

N= Normality of HCL => 0.0996

Percentage of Protein (%) = Percentage of Nitrogen (%) x 6.25

3.4.2 Crude lipid

The total amount of crude lipid in the diet was obtained by using petroleum ether extraction as principle of Soxhlet method (method 945.18, adapted from AOAC, 2012). A sample weighed approximately to 4g was added into the timber. About 80 ml of petroleum ether added into the beaker-containing sample. The sample was immersed in petroleum ether as well as cover with aluminium foil and left it overnight. The solution (petroleum ether + lipid) were poured into a round bottom flask (diameter: 29/32). The solution was further rotavap using a Buchi Rotavapor R-200 with Buchi heating Bath B-490 (Temperature: 45°C) to vapourised out petroleum ether and left with lipid. The sample in timble was extracted for lipid continuously until a clear petroleum ether obtained. This clear petroleum ether indicates as the entire lipid extracted completely from the sample. An empty beaker was measured and the extracted lipid was poured into the beaker as the beaker placed in an oven at 80°C until a constant weight obtained. The dried sample (de-fatted sample) was kept in a dry environment and used for crude fiber analysis. All the samples analyzed in duplicate.

$$\text{Percentage of Lipid (\%)} = \frac{(W3-W2)}{(W1)} \; X \; 100$$

Where, W1 = Sample weight (g)

W2 = Initial weight of extraction cup (g)

W3= Final weight of extraction cup (g)

3.4.3 Crude Fiber

Crude fibre analysis was carried out by using a fibre cap analysis with alkali and acid digestion (Method 962.09, adapted from AOAC, 2012). The fibre capsules with lids were weighed individually. Approximately, 0.5g de-fatted sample obtained from defatted lipid sample was added in the capsule. The capsule + sample was placed in the carousel with a stopper to lock the capsule in place. For the first cycle, the sample was washed with 350ml of 1.25% of sulphuric acid for 30 minutes followed with second cycle with 350ml of 1.25% NaOH for another 30 minutes, which boiled on a hot plate. Next, the sample washed three times in boiling water in between each cycle. On the last 30 minutes cycle, the sample was washed in 1% of hydrochloride acid (HCL) and followed with water. The sample was dried at 130°C for 2 hours. The weighed sample was placed in a pre-weighed crucible and was placed in muffle furnace at 600ºC for 4 hours. All the samples were analyzed in duplicate.

$$\text{Percentage of Fibre (\%)} = \frac{W3-(W1\ X\ C)-(W5-W4-D)}{(W2)}\ X\ 100$$

Where, W1 = Weight of empty capsule and its cap(g)

W2 = Weight of sample (g)

W3= Weight of capsule (g) after 130°C

W4 = Weight of empty crucible (g)

W5 = Weight of crucible after 600ºC

C = Black correction for capsule solubility, given 0.9995

D = ash of empty capsule (g) given, 0.007

3.4.4 Dry matter, Moisture and Ash

The pre-dried crucible was weighed and 2g of the sample in the crucible. The weight of the crucible + sample was recorded before drying. The sample was dried in the oven at 105°C for 24 hours (Method 934.01, adapted from AOAC, 2012). The crucible was left cooled for 30 minutes before being weighed. Next, the crucible was placed in a muffle furnace at 600ºC for 4 hours. Once cooled, the crucible was weighed to determine the ash content (Method 942.05, adapted from AOAC, 2012). All the samples were analyzed in duplicate reading

Percentage of Dry matter (%) $=\frac{(W3-W1)}{(W2-W1)} X\ 100$

Percentage of Moist (%) =100- % dry matter

Percentage of Ash (%) $=\frac{(W4-W1)}{(W3-W1)} X\ 100$

Where, W1 = Weight of empty crucible (g)

W2 = Weight of crucible + sample (g)

W3= Weight of crucible + sample (g) after 105°C

W4 = Weight of crucible + sample (g) after 600ºC

3.4.5 Nitrogen free extract (NFE) or carbohydrates

The NFE values were calculated as following : (Taufek et al.,2016)

NFE : 100 – (% Protein + % Lipid + % Fiber + %Ash)

3.4.6 Gross energy

Gross energy for every diets was calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g (Schulz et al., 2005).

3.5 Growth Performance analysis

Survival and growth parameters such as body weight gain (BWG), feed intake (FI), and specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR) were calculated according to the following formulas: (Muin et al., 2016)

1. BGW (%) = (final weight – initial weight) /final weight) x 100
2. FI (g) = Total feed for the feeding period / Number of alive fish
3. SGR = (In final weight of fish – In initial weight of fish) / time of the experiment
4. FCR = FI/ body mass gain
5. SR (%) = (final number of alive fish / initial number of alive fish) x 100

3.6 Antioxidant Analysis

After 6 weeks of feeding trials, five RHTO were randomly collected from each tank after anesthetizing with clove oil (40 mg/L of water) (Raji et al., 2018). The sacrificed RHTO was marked individually for body weights, and liver weights were recorded accordingly.

3.6.1 Sample preparation

A total of 1.0 g of each liver was homogenized in 10ml buffer containing 25 mM sodium phosphate buffer (pH 7.4), 0.1 mM protease inhibitor, 1.0 mM EDTA, 0.1 mM dithiothreitol (DTT) and 0.1 mM phenylthiourea (PTU). The homogenates were centrifuged at 100,000 ×g (Beckman 80Ti) for 30 minutes at 4°C. The supernatants were then transferred to a tube and stored at -70°C for further analysis.

3.6.2 Liver Protein Concentration

The liver protein concentration were determined using the Bradford assay. This assay contains against their average absorbance. The protein content of the samples was estimated from Coomassie Brilliant Blue G reagent, and bovine serum albumin (BSA) as the standard (Bradford, 1976). A 100mg of Coomassie Brilliant Blue (CCB) G-250 was dissolved in 50 ml of 95% ethanol. Next, 100 ml of 85% (v/v) phosphoric acid was added to the mixture, distilled water was added until 1L. The Bradford reagent solution was filtered with Whatman No.1 filter paper and left overnight before use.

For the preparation of standard, Bovine serum albumin (BSA) stock solution with a concentration of 2.0 mg/ml was prepared in distilled water. From the stock solution, six standards solutions were prepared from the range of 50 to 300 µg/µL. In a 96 well plate, 5µl of diluted protein sample was mixed well with 150µl Bradford reagent. The Bradford reagent was immediately changed to bright blue colour due to the presence of protein. The absorbance was read at 595 nm. The amounts of BSA in the standards were plotted the standard curve.

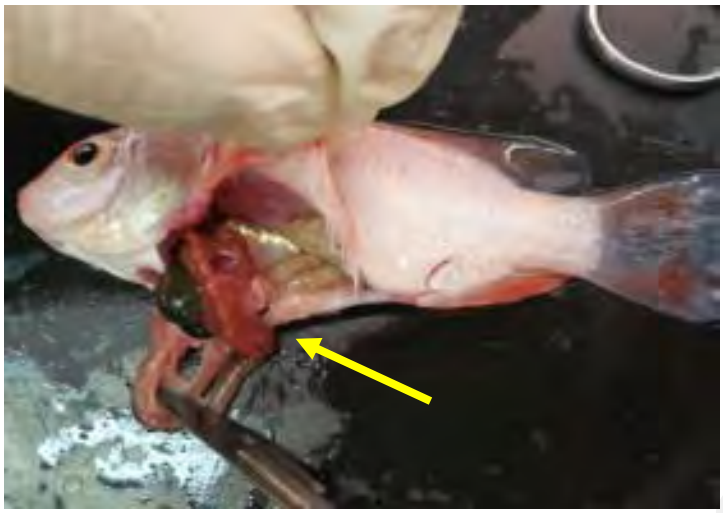


Figure 3.4 Indication of RHTO's Liver (An Arrow indicates the liver)

3.7 Oxidative Stress assay

3.7.1 Glutathione S-transferases (GST)

Glutathione S-transferases (GST) was evaluated by measuring the activity towards 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, as described by Habig et al. (1974) and Taufek et al.(2016). In a 96-well plate, the assay contains 94µL of 100 mM sodium phosphate buffer with pH 7.6, followed by 2µl of 60 mM glutathione (GSH). An 18.7mg of GSH were dissolve in 1mL of sodium phosphate buffer. Next, 2µl of diluted samples and 2µl of 60 mM of 1-di-2,4-dinitrobenzene (CDNB), were dissolved in ethanol, and later added in the well accordingly. Each sample was monitored in triplicate. One unit of GST activity was calculated as the amount of enzyme catalyzing the conjugation of 1µ mol of CDNB with GSH per minute at 25°C (ε_{340}nm = 9.6 mM^{-1} cm, with pathlength = 0.552cm), the result were expressed as a nmol/min/mg/protein. The calculation for the specific activity is adapted from Glutathione S-Transferase (GST) Assay Kit Technical bulletin (2007) as follow:

Enzyme activity: $\frac{(A340nm/\min)\ x\ (\ sample-\ blank)\ x\ 3\ x\ df}{9.6\ (0.552) x\ sample\ (ml)}$

Specific activity (nmol): Enzyme activity/protein concentration of the sample

3.7.2 Catalase (CAT)

Catalase (CAT) (EC 1.11.1.6) activity was assayed, according to Claiborne.(1985). The 50 mM sodium phosphate buffer was prepared with pH 7.0. Next, 19mM hydrogen peroxide (H_2O_2) was prepared by using cold sodium phosphate buffer. In a 1.25ml cuvette, 240µL of sodium phosphate buffer, 100µl of diluted samples, and 100µl of H_2O_2 were mixed together. The reaction was quantified at 25°C by measuring the disappearance of H_2O_2 at 240 nm within 5 minutes (Taufek et al., 2016) CAT activity was reported in terms of nmol H_2O_2 nmol/min/ml (ε 240 nm = 0.0436 mM/cm). The CAT activity was calculated using the following formula.

Enzyme activity: $\frac{(A340nm/\min)\ x\ (\ sample-blank)\ x\ 3\ x\ df}{0.0436\ x\ sample\ (ml)}$

3.8 Fatty Acid Profiling

3.8.1 Sample preparation

Seven RHTO were selected randomly for this study from each tank (10 for each diet). The RHTO were cleaned and all the organs were removed. Next, they were dried in oven at 60ºC for 48 hours. The dried RHTO were blend and weighed at 10g for lipid extraction. The oil from the fillet were extracted using a Soxhlet method with petroleum ether as the solvent. (AOAC, 2012). (Brief discussion on paragraph 3.4.2). The preparation of fatty acid methyl esters (FAMES) for GC analysis were done using sodium methoxide method. The determination of fatty acid was done by comparing the relative retention times of fatty acid metyl ester (FAME) peaks of samples with standard from Nanocat, University Malaya.

3.8.2 Gas Chromatography- Mass Spectrum(GC-MS)

The fatty acid methyl esters were analyzed using a Agilent Techonologies 6890N gas chromatograph powered with a HP Innowax 30m x 0.025mm x 0.25micron (Agilents Technologies), in Nanocat, Universiti Malaya. Next, the helium gas was used as the carrier gas. About 0.05g of sample were diluted into the test tube with 10ml of hexane. The test tube were secured with (screw cap) or reaction vial. The tube or the vial was closed and vortex for 30s, and followed by centrifugation of the sample. Later, the clear supernatant were transferred into a 2-mL autosampler vial. Lastly, the automated split injection was performed using an Agilent 7683 autosampler.

3.9 Statistical analysis

The data of study were analysed using a one-way analysis of variation (ANOVA) (Ahmed et al., 2020) using the statistical package for the social sciences (SPSS) computer software (version 25.0). Duncan's multiple range tests at a significant level of 0.05 % used to determine the significant difference between treatments. Data are presented as mean ± standard error expression (SE).

Production of Biomass from mycelium Ganoderma *lucidum* (App.35g- 45g in 6 Months)
(Refer to Figure 3.1)

↓

Preparation of feed using Winfeed Software 2.8
(Refer to Figure 3.2)

↓

Control Diet – 0% MB	Diet 5g/kg of MB	Diet 10g/kg of MB	Diet 15g/kg of MB

↓

Feeding Period – 42 days Each diet- 15 fishes per tank (duplicate)

↓

Growth Analysis -Calculating the growth parameter

1. BGW (%) = (final weight – initial weight) /final weight) x 100
2. FI (g) = Total feed for the feeding period / Number of alive fish
3. SGR = (In final weight of fish – In initial weight of fish) / time of the experiment
4. FCR = FI/ body mass gain
5. SR (%) = (final number of alive fish / initial number of alive fish) x 100

Number of sample n =15 (duplicate)

Antioxidant Analysis

- <u>GST analysis</u>

Enzyme activity:

$$\frac{(A340nm/\text{min})\ x\ (sample - blank)\ x\ 3\ x\ df}{9.6\ (0.552) x\ sample\ (ml)}$$

Specific activity (nmol):
Enzyme activity/protein concentration of the sample

-<u>CAT analysis</u>

Enzyme activity:

$$\frac{(A340nm/\text{min})\ x\ (sample - blank)\ x\ 3\ x\ df}{0.0436\ x\ sample\ (ml)}$$

Number of sample n =5 (duplicate)

Fatty Acid Profiling

- The oil from fillet (10g) were extracted using Soxhlet Method principle

- Gas Chromatography- Mass Spectrum method

- The determination of fatty acid was done by comparing the relative retention times of fatty acid methyl ester (FAME) peaks of samples with standard from Nanocat, University of Malaya.

Number of sample n =7 (duplicate)

↓

Statistical analysis
One-way analysis of variation (ANOVA) (version 25.0). Data are presented as mean ± standard error (SEM)

Figure 3.5: Experimental design

CHAPTER 4: RESULTS

4.1 The effect of fermented mycelial biomass from *Ganoderma lucidum* on the growth performance of red hybrid tilapia (*Oreochromis* sp.)

The growth performance of the RHTO is shown in Table 4.2. The experiment trial started with 15 fishes per tank for each diet, respectively. At the end of the experiment, the survival rate for each diet was 100%. Therefore, no mortality was recorded during the feeding trial period.

During the six weeks of the feeding trial, the RHTO utilized the feed by eating them all as feed intake reported in Table 4.2. According to Caldini et al. (2011), the growth of fish were able to access after maintained in the experimental system for six weeks. The weekly weight gain of RHTO was made every two weeks once on Friday. The initial mean value for the fish for control (17.45g ± 1.05), 5g/kg (14.40g ± 0.5), 10g/kg (16.2 ± 0.9), and 15g/kg (16.2 ± 0.9), as shown in table 4.1. At the end of the feeding trial, the highest body weight gain performance was recorded in the group fed with 15g/kg (35.4g±2.9), next 10g/kg (34.4g±1.1), followed by control (29.8g±0.2) and 5g/kg (28.60g±1.10), as shown in Figure 4.1.

From the finding, the data obtained for the biometrics indices have no significant difference in the experiment diet (5g/kg, 10g/kg, 15g/kg) compared to the control diet. By referring to the BGW and SGR values, the experiment diets (5g/kg, 10g/kg, 15g/kg) has no significance different compared to the control diet. Moreover, 15g/kg gave the

highest value for BGW and SGR compared to other diets where the values were 59.19% ± 3.59 and 2.14±0.21, respectively. The value for FCR indicates the lowest value in the experimental fish compared to the control fishes. The lowest value is 15g/kg (1.13±0.19), 10g/kg (1.4±0.06) and 5g/kg (1.61±0.04). Furthermore, the FI values indicate the highest in diet 10g/kg (25.5g± 1.42), which is not signed with the control diet as well as the 5g/kg and 15g/kg diets. The lowest FI values observed in diet 5g/kg (22.88g ± 1.52), followed by 15g/kg (23.72g ± 4.38) and control diet (24.05g±1.32). The finding can be concluded as a positive growth rate and a 100% survival rate for RHTO throughout the feeding week.

Figure 4.1 and Figure 4.2 shows the weekly weight gain of red hybrid tilapia with a different diet. The weight of each diet is increasing every week. The weight of each tilapia has no significant differences between each treatment in Week 0, Week 2, Week 4 and Week 6. The highest weight gain was observed in week 6 is 15g/kg (35.5g± 3.0), and the lowest is 5g/kg (28.6g± 1.1). Figure 4.3 shows the total MB production from MGL throughout the experimental period.

Table 4.1: Growth performance and feed efficiency of RHTO fed with different diets

	Control	5g/kg	10g/kg	15g/kg
Initial Weight (g/fish)	17.45 ± 1.05^{a}	14.40 ± 0.5^{a}	16.2 ± 0.9^{a}	14.55 ± 2.45^{a}
Final weight (g/fish)	29.8 ± 0.2^{ab}	28.60 ± 1.10^{a}	34.4 ± 1.1^{ab}	35.4 ± 2.9^{b}
BWG (g/fish)	41.42 ± 3.92^{a}	49.64 ± 0.19^{ab}	52.94 ± 1.11^{b}	59.191 ± 3.58^{b}
FI (g/fish)	24.05 ± 1.32^{a}	22.88 ± 1.52^{a}	25.5 ± 1.42^{a}	23.72 ± 4.37^{a}
SR (%)	100	100	100	100
SGR	1.28 ± 0.16^{a}	1.63 ± 0.01^{ab}	1.8 ± 0.06^{ab}	2.14 ± 0.21^{b}
FCR	1.98 ± 0.31^{b}	1.61 ± 0.04^{ab}	1.4 ± 0.06^{ab}	1.13 ± 0.19^{a}

[1]The results represent mean± SEM of 15 fishes per tank (duplicate)

[2]Means in the same row with different letters are significantly different ($p< 0.05$)

[3]BWG :Body Weight Gain, FI:Feed Intake, SR:Survival Rate, SGR: Specific Growth Rate, and FCR:Feed Conversion Ratio

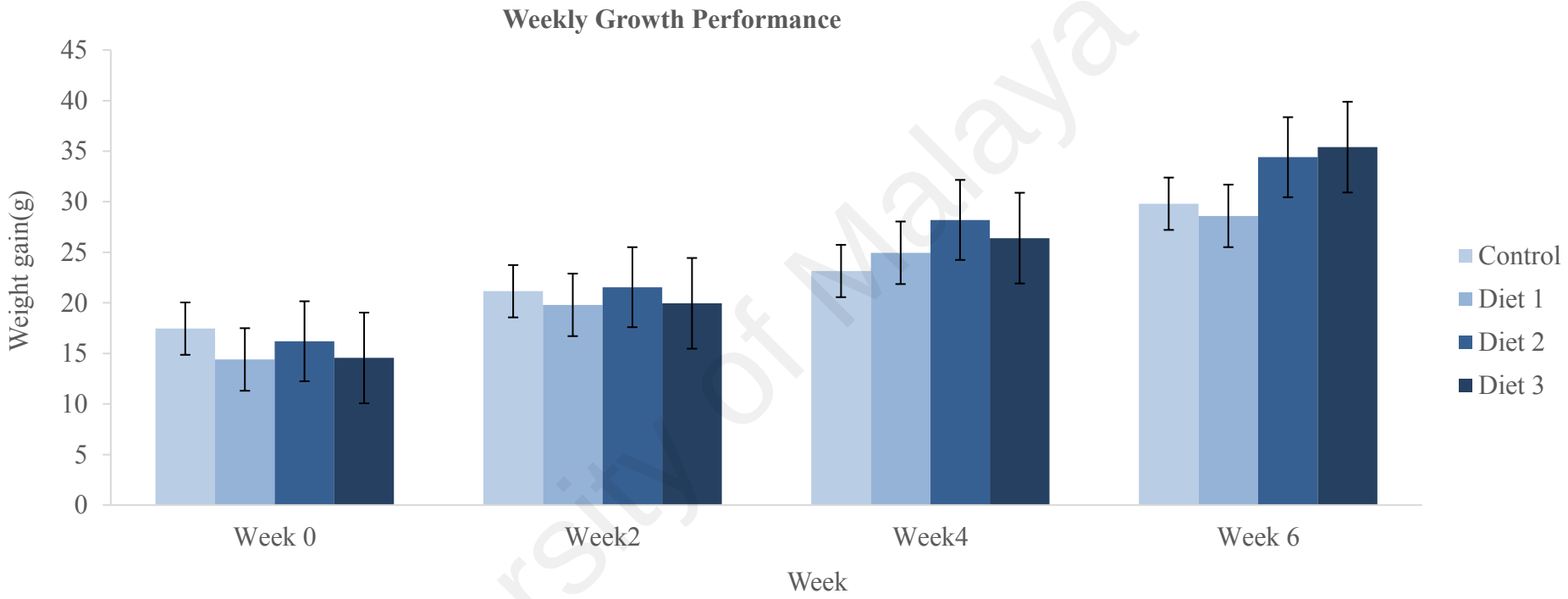


Figure 4.1 The weekly growth performance of RHTO

[1]The results represent mean of 15 fishes per tank (duplicate)

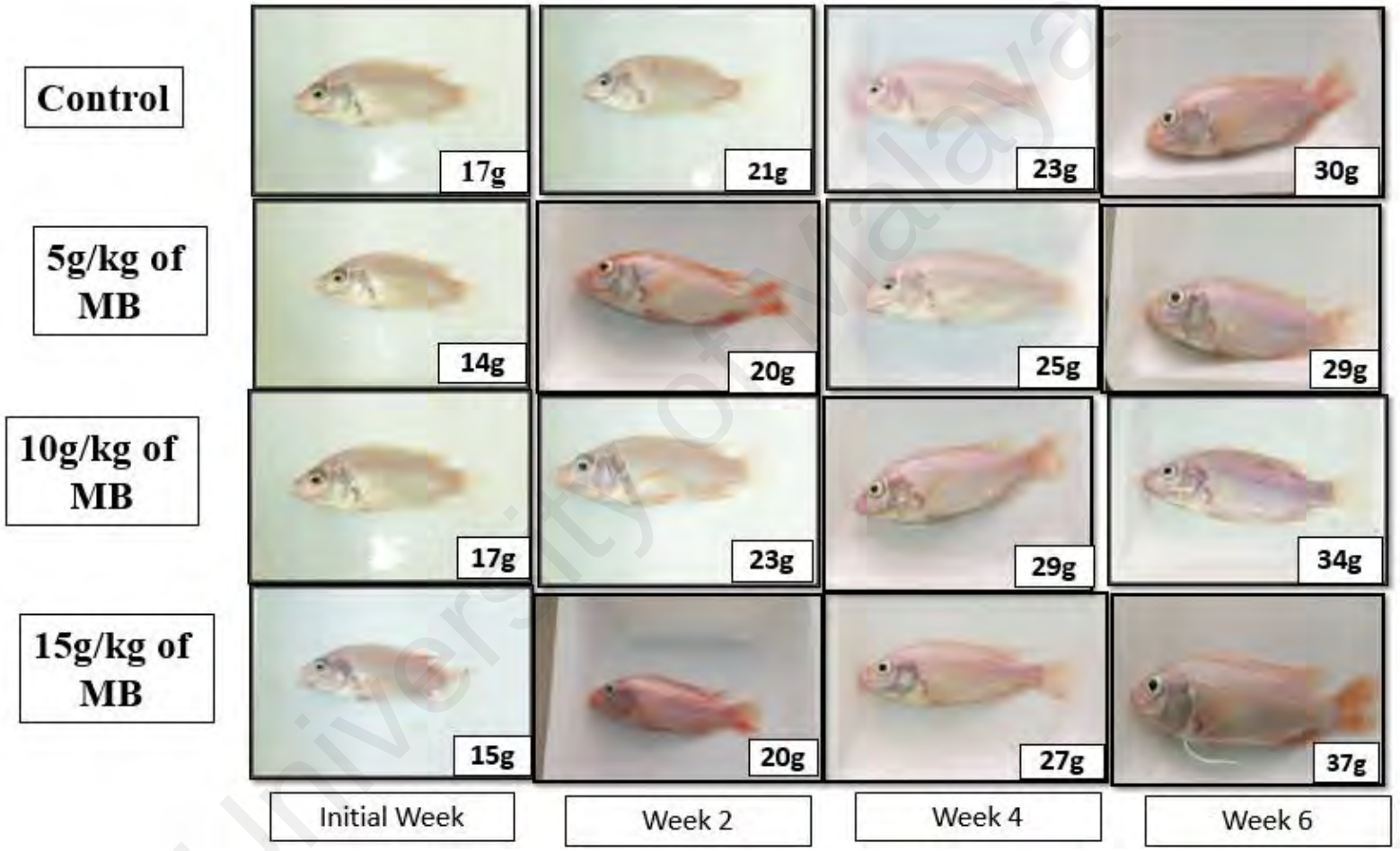


Figure 4.2 Weekly growth performance by RHTO during the feeding week.

[1]The results represent mean of 15 fishes per tank in duplicate culture

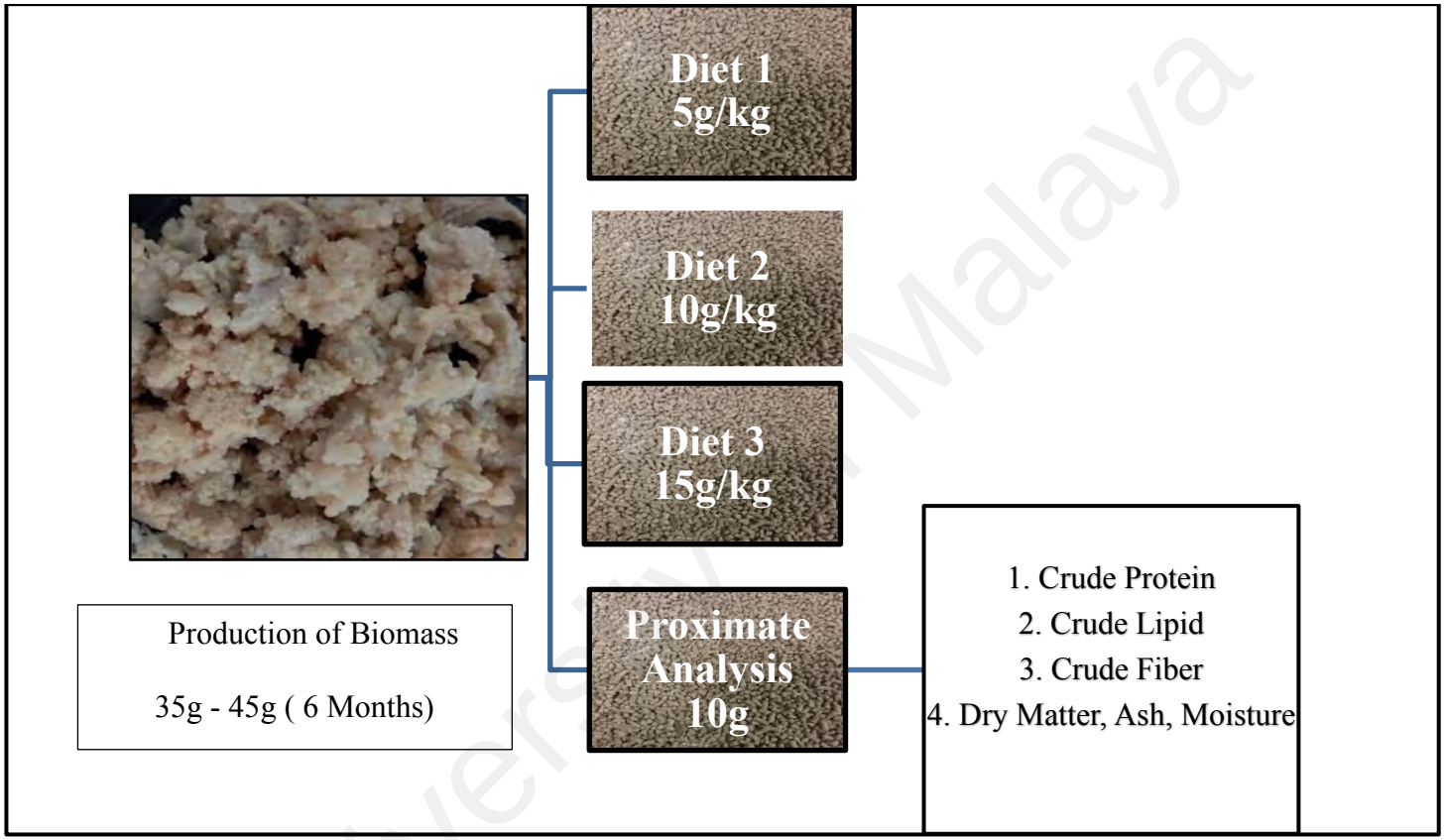


Figure 4.3 The total biomass production for the experimental diet and proximate.

4.2 The antioxidative response of red hybrid tilapia (*Oreochromis* sp.) on feed with fermented mycelial biomass from *Ganoderma lucidum*

The enzyme activities were assessed from the liver of RHTO fed with the experimental diets and the results is tabulated in Table 4.1. The liver protein in the control diet (0.273± 0.1 mg/ml) shows the highest value among the experimental diet. There are no significant differences among all the experimental diets when compared with control. On the other hand, the CAT activity shows significantly higher value in 15g/kg diet (233.58 ± 2.9 nmol/min/ml), followed by 10g/kg (169.04 ± 7.2 nmol/min/ml) and 5g/kg (138.3± 7.2nmol/min/ml). Moreover, the GST activity shows the highest value in 15g/kg (113.83 ± 5.0 nmol/mg protein) while the lowest value in 5g/kg (7.39 ± 0.97 nmol/mg protein).

Table 4.2 shows Liver protein, Catalase (CAT), and Glutathione S-transferase (GST) activity of RHTO experimental diets.

Enzyme and liver protein	Experiment diets			
	Control	5g/kg	10g/kg	15g/kg
Liver protein (mg/ml)	0.273± 0.1[a]	0.269 ± 0.04[a]	0.18 ± 0.004 [a]	0.17± 0.3[a]
CAT (nmol/min/ml)	117.814± 7.2[a]	138.3± 7.2[b]	169.04 ± 7.2[c]	233.58 ± 2.9[d]
GST (nmol/mg protein)	20.773± 0.99[a]	7.39± 0.97[b]	52.05± 1.5[c]	113.83 ± 5.0[d]

[1]The results represent mean± SEM of 5 fishes per tank (duplicate)

[2]Means in the same row with different letters are significantly different ($p < 0.05$)

[3]GST means Glutathione S-transferase, and CAT means Catalase

4.3 The fatty acid composition of red hybrid tilapia (*Oreochromis* sp.) feed with fermented mycelial biomass from *Ganoderma lucidum*

The results obtained show the fatty acid profiling of RHTO fed with MB as a feed additive. Table 4.3 presents the results obtained from GC-MS. From the finding, 10 peaks of fatty acid (FA) were identified from the analysis. The data comprise of 6 saturated fatty acids (SFA), and 3 unsaturated fatty acids were present, which includes 3 monounsaturated fatty acid (MUFA) and 1 polyunsaturated fatty acid (PUFA). The FA include palmitic acid, oleic acid, myristic acid, palmitoleic acid, linoleic acid, stearic acid, pentadecanoic acid, nonadecanoic acid, and tridecanoic acid. The polyunsaturated, linolenic, and linoleic fatty acids in lipids are dietary essentials for tilapia because the fish cannot biosynthesize them. Lipid levels of 5-12% are optimum in tilapia diets (Lim et al., 2009). By interpreting the results, the typical FA present in all diet (Control and experimental diet) is palmitic acid, oleic acid, myristic acid, and linoleic acid. Besides, nonadecanoic acid only present in the control diet, while tridecanoic acid only present in 10g/kg diet. For pentadecanoic acid, this FA present in both control and experimental diet (5g/kg,10g/kg). Meanwhile, the stearic acid and elaidic acid only found in the experimental diet (5g/kg, 10g/kg, and 15g/kg). Moreover, palmitoleic acid only found in the diet of 5g/kg and 10g/kg.

Table 4.3 shows the fatty acid profiling for RHTO using GS-MS

Fatty acid	Control	5g/kg	10g/kg	15g/kg	Level of saturation	Omega	CD
Linoleic acid	*	*	*	*	PUFA	6	18:2
Elaidic acid	-	*	*	*	MUFA	-	18:1
Myristic acid	*	*	*	*	SFA	-	14:0
Nonadecanoic acid	*	-	-	-	SFA	-	1
Oleic acid	*	*	*	*	MUFA	9	18:1
Palmitic acid	*	*	*	*	SFA	-	16:0
Palmitoleic acid	-	*	*	-	MUFA	7	16:1
Pentadecanoic acid	*	*	*	-	SFA	-	15:0
Stearic acid	-	*	*	*	SFA	-	18:0
Tridecanoic acid	-	-	*	-	SFA	-	13:0

[1]SFA : Saturated fatty acid, MUFA-Monounsaturated fatty acid, PUFA- Polyunsaturated fatty acid

[2] CD indicates carbon atoms of the fatty acid, and the number of double bonds in it.

[3] (*) indicates the present of Fatty acid, (-) indicates the absent of the fatty acid

[4]The data shows results on seven fishes.

CHAPTER 5: DISCUSSION

5.1 The effect of fermented mycelial biomass from *Ganoderma lucidum* on the growth performance of red hybrid tilapia (*Oreochromis* sp.)

Feed additives play an essential role in agriculture to enhance the efficiency of feed utilization and aquaculture productive performance. The feed additives are supplemented in small amounts to RHTO to improve their immune systems and induce physiological benefits beyond traditional feeds. (Bharathi et al., 2019). From a study done by Barrows et al., (2000), the study suggested the feed additives can be categorized into (1) additives that affect fish performance and health (functional feed additives) and (2) additives that affect feed quality and feed uptake. The usage of medicinal mushrooms such as *G.lucidum* in aquaculture is due to their properties, which enhance the health of RHTO. The various range of mushrooms which include fruiting bodies and mycelia, are rich in food nutritional value. Based on the study done by Ulziijargal & Mau, (2011), the proximate composition and energy conclude the fruiting bodies and mycelia are low in fat and calories but rich in protein and dietary fibre.

In the study, significant improvements in survival, weight gain, length gain, feed intake, and specific growth rate were reported in the experimental diet compared to control. Hence, the study on MB as a feed additive can promote the feeding, followed by better survival and growth performance of fish. The usage of MB as feed additive further supported by studies on G.*lucidum,* which have shown that an improvement in survival, increase performance, feed intake, and specific growth rate of prawn and fish (Chitra et al., 2016). Besides, all the fishes with the experimental diets survived well. Hence, no

mortality recorded throughout the experiment. Several factors influence the high survival rate, such as proper handling practices and water quality management during the experimental period may affect the survival rate of fish. (Muin et al., 2017),

The study was conducted to determined biometric indices, which include body weight gain (BWG), specific growth rate (SGR), feed conversion ratio (FCR), feed intake (FI), and survival rate (SR). On the other hand, FCR indicates the amount of feed consumed per unit body of body weight gain. The FCR value plays an essential role as an indicator of feed utilization efficiency as a low value of FCR is a good indicator of the high quality of feed. Hence, in this study, the 15g/kg shows a low FCR value (1.13 ± 0.19) compared with the typical FCR values (1.4 to 2.5) for RHTO on the previous studies (Beveridge, 2004; Ofori et al., 2010). The obtained FCR suggests more efficient food utilization through the extraction of nutrients from the feed and converting it into flesh that reflected on the growth of the fish. (Alhassan et al., 2018).

From the study by Muin et al.(2017), the specific growth rate (SGR), which indicates the optimum growth achieved as the fish consumed dietary feed nutrient. In this study, the higher SGR value was observed in 15g/kg (2.14% ± 0.21), which indicates the feed utilizes fully by the fish. In contrast, the lowest SGR in the control diet (1.28% ± 0.16) may be due to the effect of different environmental conditions and as increasing age of RHTO (Alhassan et al., 2018).

Whereas for the feed intake (FI), 10g/kg shows, the highest significant value with 25.5g±1.42 and the lowest FI in 5g/kg diet (22.88g ± 1.52). The FI in experiment diet (5g/kg,10g/kg,15g/kg) maybe related to some bioactive compounds embedded in *G.lucidum* can potentially affect the digestibility of nutrients. The high fibre content of mushroom may explain the result of the lower nutrient intake in the present study.(Vetter, 2007). In general, the 15g/kg showed improved results in terms of BWG, FCR, SGR, and FI with no significant difference compared to 10g/kg.

5.2 The antioxidative response of red hybrid tilapia (*Oreochromis* sp.) on feed with fermented mycelial biomass from *Ganoderma lucidum*

The antioxidant enzyme is biomarkers of reactive oxygen species (ROS) that mediate exposure to contaminants and prospective environmental risk evaluation instruments (Kohen & Nyska, 2002). These antioxidant enzymes are necessary to protect against oxidative stress and tissue-specific damage (El Gazzar et al., 2014). The ROS detoxified by a set of antioxidant enzymes that protect macromolecules such as proteins, lipids, and nucleic acids against damage (Lushchak et al., 2001). The antioxidant enzymes may reflect the level of fish metabolism depending on the behavior, particularly natural mobility and ecological peculiarities (Gatlin & D.M, 2002). According to Atli et al., (2016) studies, fish with various ecological needs may have different antioxidant capacities. The antioxidant enzyme often related to reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical, hydroxyl radical, which could lead to oxidative stress in fish (Atli et al., 2016). In this study, the GST and CAT activity been observed

on the liver of RHTO, as the liver is the site to eliminate xenobiotic as a site for multiple oxidative reactions and maximal free radical generation.

The GST activity shows the highest value in 15g/kg (113.829 ± 4.951 nmol/mg protein) with no significant effect compared to 10g/kg diet. Contrastingly, elevated GST activities was correlated with diets of higher MB content. This result may imply that MB contains compounds that could be stimulating the biotransformation pathway in RHTO liver. Since MB is rich with antioxidant properties (Chen, 2009), the diet 5g/kg shows the lowest GST activity (7.386 nmol/mg protein ± 0.971) compared to control and experimental diet. The dietary supplement might protect the cell from injury by scavenging ROS and toxic metabolite. The GST activities contribute to the detoxification of oxidative stress products. The detoxification happens by catalysing the conjugation of a variety of metabolites, including xenobiotic metabolites and lipoperoxidation products, with GSH and by transforming the toxic compounds into more easily dischargeable ones (Taufek et al.,2016). According to Sinha et al.(2015), antioxidant defence's in fish are also dependent on feeding behavior and nutritional factors. Furthermore, dietary levels of lipids and some vitamins have been reported to influence antioxidant defence's and oxidative status of fish. Diets containing low levels of lipid and digestible starch reduce the susceptibility of the fish to oxidation and may enhance the growth rate (Rueda et a1.,2004)

On the other hand, CAT activity shows significantly higher values in experimental diets compared to control diets. The obtained results shows 15g/kg diet (233.578 ± 2.898

nmol/min/ml) have significant effect compared to 10g/kg (169.037 ± 7.244 nmol/min/ml) and 5g/kg (138.303± 7.244 nmol/min/ml).

According to Ahmed et al.(2014), the increasing CAT activity was found in 0.5% of hot water extract (HWE) mushroom stalk supplementation due to the high supplementation of beta-glucan, which may be toxic for fish (Qing et al.,2007). Similarly, work was done by Lakshmi et al., 2006 with groups of mice fed Ganoderma showed a significant increase in the glutathione (GSH) and antioxidant enzyme activities (SOD, GST, CAT). The higher CAT activity is supported by Wdzieczak et al. (1982), where the peroxidation in erythrocytes and liver of different younger fish species showed high antioxidant activity.

In this study, the increased level in both GST and CAT activities in the experimental diet shows no effect on the growth of the RHTO. The GST and CAT activity is further support by the intake of mycelia biomass from MGL which enhances antioxidation capability. The antioxidant activity function as the body defense system within the cells is strengthened and causes the free radical to decompose. The cells decompose free radical into safe substances utilizing complicated internal biochemical reactions within the cell. (Chen, 2009).From the obtained results, there is significant differences between all the experimental diet (5g/kg,10g/kg, and 15g/kg) compared to the control diet for GST and CAT value.

5.3 The fatty acid composition of red hybrid tilapia (*Oreochromis* sp.) feed with fermented mycelial biomass from *Ganoderma lucidum*

The RHTO, which is a freshwater fish, are more abundant in n-6 FA compared to n-3 FA for the maximal growth (Alam et al., 2014). The food scientists and nutritionists need the data on fatty acid composition to help them formulate process and develop products to ensure the health of consumers (Suloma et al., 2008). Further study was done by Ng et al.(2004), showed that fillet lipid level in Mozambique tilapia diet containing 3% - 8% of dietary lipid. Lipids are fundamental to the growth, physiological functions, and body maintenance of the RHTO (Ng et al., 2004).

The most prominent FA are the polyunsaturated fatty acids (PUFA), which aid in reducing the risk of cardiovascular diseases (Suloma et al., 2008) To date, research on the fatty acid requirements of tilapia has produced contradictory results. The variation in the FA profile could be due to various factors such as length of the experiment, nutritional history of the experimental fish, size of fish, source of dietary lipids, and water temperature.

Linoleic acid is an essential FA in the human body and can promote growth and development. A previous study has also shown that linoleic acid may have a role in the development of vision and intelligence in infants (Stoneham et al., 2018). The research on fatty acid requirements has shown that linoleic series fatty acids are dietary essentials for tilapia (Chen et al., 2013; Al Souti et al., 2012). The optimum dietary levels of the n-6 acids have been estimated at 1% for redbelly tilapia, Tilapia *zillii*, and 0.5% for Nile tilapia, *Oreochromis niloticus*. (Lim et al., 2009)

The most common FA found in RHTO in this current study is palmitic acid (16:0). This palmitic acid is considered the most abundant saturated and predominant (Suloma et al..2008) fatty acid in nature in the lipid amount of animals, plants, and lower organisms. From research was done by Sci et al.(2017), the finding suggests that palmitic acid is a crucial metabolite not influenced by diet in fish.

Next, myristic acid (14:0) is a long-chain saturated fatty acid and the omnipresent component of lipids in most living organisms. Myristic acid consumption has a positive impact on cardiovascular health. This statement is agreeable with the study reported that moderate myristic acid consumption improves long-chain omega-3 fatty acids levels in plasma phospholipids, which could exert improvement of cardiovascular health parameters in humans (Azab et al., 2020). The behavior largely influenced by the balance between saturated fatty acid and simple dietary carbohydrates in the diet (Ruiz-Núñez, Dijck-Brouwer & Muskiet, 2016).

Next, the stearic acid (18:0) is the second most abundant saturated fatty acid in nature (Sci et al., 2017), and found in the lipid of most living organisms (Azab et al., 2020). This stearic acid only found in the experimental diet (5g/kg, 10g/kg,15g/kg), which similar to research done by Lv et al. (2012). The researcher concludes that palmitic acid, linoleic acid, oleic acid, stearic acid are primary fatty acids in *G. lucidum*. Fatty acids are essential constituents of fungal cells with recognized roles as storage material and as components of the plasma and cell organelle membranes. In fungi, the primary fatty acids that typically occur in membrane phospholipids and storage triacylglycerols are palmitic

and stearic, and their unsaturated derivatives palmitoleic, oleic, linoleic and linolenic acids (Hasan et al., 2013).

Furthermore, oleic acid is the primary monounsaturated fatty acids (MUFA) in RHTO. The presence of oleic acid further supported by the research done by Agbo et al.(2014), where the study concludes that the oleic acid (C18:1) had the highest concentration (31.77 – 32.37%) among the unsaturated fatty acids present in the RHTO. Furthermore, oleic acid is a MUFA that functions in the regulation of blood sugar, blood fat, and cholesterol. (Neuringer, 2000).

The current study did not detect any DHA in RHTO oil. The finding was supported by a research done by Navarro et al. (2012), whereby DHA is derived from EPA, which, in turn, derives from linolenic acid (18:3, x-3), which was detected at low levels in the carcasses. Therefore, the activity of desaturase and elongase enzymes involved in the synthesis of the omega-3 PUFA series might be low.

In conclusion, by comparing the experimental and control diets, 10g/kg shows the most abundant fatty acid profiling for this study. The FA include palmitic acid, oleic acid, elaidic acid, myristic acid, palmitoleic acid, linoleic acid, stearic acid, pentadecanoic acid, and tridecanoic acid.

CHAPTER 6: CONCLUSION

6.1 Conclusions

Based on the results of the study, the findings reported here shed new light on:

1. The findings of this research provide insights for growth study, as there is no significant in all the experiment diet compared to control. The experimental diet, which consists of 5g/kg, 10g/kg and 15g/kg of MB, improved the growth and biometric indices compared to control diets. Since there is no significant difference in 10g/kg diet compared to control. Therefore, 10g/kg enhanced the survival, growth performance and biometric indices throughout the feeding week. Moreover, there is no mortality recorded in both the control and treated groups. Throughout six weeks of the feeding trial, the fish ate all the feed without rejection. From this observation and finding, it can conclude that MB is a potential natural and non-toxic feed addictive in the fish feed (Taufek et al., 2020), which helps to promote growth.

2. This project is the first comprehensive investigation on antioxidant results. The CAT and GST activity showed that all the experiment diets (5g/kg, 10g/kg and 15g/kg) are significantly higher than control. Since there is no significance in 10g/kg compared to control and 5g/kg,15g/kg Hence the finding can be concluded as 10g/kg of MB diet able to enhance the antioxidant activity as it protects the cell from damage. A further should be done by concerning the impact of MB of MGL on other antioxidative enzymes.

3. Generally, these results suggest that 10g/kg shows the best fatty acid profile compared to control, 15g/kg and 5g/kg. This includes palmitic acid, oleic acid, myristic acid,

palmitoleic acid, linoleic *acid, s*tearic acid, pentadecanoic acid and tridecanoic acid. Fatty acids contents of lipids in fish were reported to vary considerably, both within and between species. The differences observed in the contents of fatty acids can be related to the percentage of MB in each diet, which acts as a saturation point for the fatty acid production in RHTO.

6.2 Recommendations and future works

Overall, this study strengthens the idea that MB could be a potential feed additive for enhancing growth, antioxidant and improved fatty acid profiling. Concerning the research methods, some limitations need to be acknowledged:

The period for the experiment- About six weeks of feeding will provide with minimum period to observe the effect and growth, as this is a preliminary study. By prolonging the experiment, the observation might be more detailed.

The fatty acid profiling –The current study only provides with qualitative data of FA. Further work needs to be done on quantitative data of FA to determine the percentage of FA obtained in the sample. It is suggested that the association of these factors is investigated in future studies to investigate the effect of MB as a dietary supplement. Further studies need to be carried out in order to validate on immunological parameters such as phagocytic activity, respiratory burst, and bactericidal activity to gain more clarification on non-specific defense mechanisms. Moreover, challenging the tilapia on their resistance to bacterial, viruses, and parasites will be able to gain more knowledge of MB as an immune system booster. Additionally, the feeding trial could be carried out

batch by batch. This data could distinguish the relationship between weight gain and antioxidant activity.

Moreover, the present study only observes CAT and GST to determine oxidative stress when fish supplied with MB meal. As we know, MB is rich with antioxidant properties, further investigation in lipid peroxidation and other antioxidative enzymes will be an advantage for further research. Next, further researches are recommended to study the antioxidant level on a different part of a fish organ such as kidney, gills, gonad, and fillet on MB as a dietary supplement.

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