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Evaluation of underutilised plants and agro-industrial by-products as potential substrates for rearing black soldier fly larvae, Hermetia illucens (Diptera: Stratiomyidae) for inclusion in Asian seabass (*Lates calcarifer*) diets

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Thesis Submitted to the University of Nottingham for the Degree of Doctor of Philosophy

December 2022

ABSTRACT

The current model, which uses fish meal (FM) as the primary source of protein for fish, cannot be maintained indefinitely. As a result, there is a need for further research into alternative sources that are both high in protein and fat. The larvae of the black soldier fly (BSFL, *Hermetia illucens*) have attracted research attention due to their nutritional values and ability to be reared on a wide variety of organic waste. This study aims to assess the potential of BSFL meal (BSFLM) as a substitute for FM in the diet of Asian seabass (*Lates calcarifer*). Two underutilised plant species, *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG), and four agro-industrial by-products (AIBPs), including soybean waste (SBW), wheat pollard (WP), rice bran (RB), and milk-extracted coconut meat (MECM), either single or in combination (MECM:SG), were evaluated as feed substrates for rearing BSFL. Different substrates significantly affected ($p < 0.05$) the final weight, size, larval development time, and nutrient composition of BSFL. Larvae fed with RB had the highest weight gain, with high concentrations of protein and fat (50.8 and 31.8 g/100g DM, respectively). The highest wet weights of larval biomass (659 – 682 g) were achieved on high protein substrates (MO, SG, and SBW). Larvae reared on AIBP had higher levels of essential amino acids (around 27 g/100g DM) than those reared on leaf-meals (around 23.5 g/100g DM), particularly WP- and RB-fed larvae. Meanwhile, larvae had a high content of saturated fatty acids (SFA), ranging from 54 to 93% of total FA. Larvae fed WP and RB had the highest levels of polyunsaturated fatty acids of about 26% of total FA. In the present study, based on the optimal nutrient composition and growth performance of the larvae, day 17 was selected as the best time for harvesting BSFL for all single feed substrates. On the mixed feed substrates (MECM:SG), the crude protein (CP) and fat of larvae increased with the increase of SG and MECM, respectively, ranging from 33.5 – 54.3 g/100g DM and 12.9 – 33.8 g/100g DM. The optimum day to harvest the mixed substrate-fed larvae was on day 19, due to the larval wet weight, higher larval CP content, and the best conversion of feed substrate into larval biomass (23%), particularly for the BSFL fed on the 1:1 ratio of MECM:SG substrate. The BSFL on

this mixed substrate was further evaluated for its potential to replace FM in the diet of Asian seabass.

The Asian seabass was fed diets containing 70% FM in the reference diet (RD) and 30% BSFLM and 50.4% FM in the test diet (TD). Each diet contained 0.5% chromium oxide as a digestible marker to determine the apparent digestibility coefficient (ADC) of BSFL. The ADC of protein and fat of the BSFL in juvenile Asian seabass were 71% and 88.7%, respectively. Three test diets with varying levels of BSFL inclusion (20, 40, and 60% of the FM replacement) were prepared to evaluate the growth performance of the Asian seabass, which equated to 74, 148, and 222 g BSFL per kg of diet, respectively. A total of 280 juvenile (46.77 ± 0.08 g/fish) Asian seabass were randomly assigned to 20 tanks (300-L), with five replicated groups for each treatment. Fish were fed for 90 days, and the bulk weight of the fish was recorded every fifteen days. There was no significant difference ($p > 0.0839$) in weight gain of fish across treatments, but a decreasing trend of weight gain was observed as BSFL levels increased in the diets. Fish fed with BSFL20 showed the best weight of 113.53 ± 5.68 g, followed by those fed on a control diet (111.22 ± 2.89 g), and the lowest was in fish fed BSFL60 (98.77 ± 4.52 g). Likewise, there were no significant effects of BSFLM inclusion on the survival rate, final weight (FW), average daily gain (ADG), serum biochemical parameters, nutrient composition, or sensory test of the flesh. The only exception was the feed conversion ratio, which increased with the increase of BSFLM in diets ($p = 0.0257$). The study revealed that the optimal FM replacement in the diet of Asian seabass was greater than 20% but less than 30% without affecting fish performance. The findings of this study show the production of BSFL with high nutritional value using underutilised local plants and agro-industrial by-products has the potential to be used in the diet of Asian seabass. Similar research on other cultured species could be conducted to develop a novel and sustainable source of nutrients for future aquafeed formulations.

ACKNOWLEGEMENTS

The accomplishment of this study is due to the efforts of many people, and I am grateful to each of them. This has been in the works for five years. Without the support of my supervisors, coworkers, friends, and family, I would not have been able to come this far. Special thanks go to the MARDI that offered me the fellowship and study leave for four years to develop my career and improve my competence as a researcher.

To Assistant Professor Dr Susan Azam-Ali, who serves as my major supervisor, I would like to extend a heartfelt thank you for taking me on as a student, believing in my capacity, and supporting me in all aspects throughout the entirety of this course of study. Your ideas and the countless discussions we have are extremely valuable, and I thank you for them. I am grateful to my co-supervisor, Dr. Noraini Samat, who also serves as my boss at work, for all her help, understanding, and ideas. Thank you for giving me the opportunity and time to finish the writing. To the second co-supervisor, Mr Giva Kuppusamy, the first person I got in touch with when I decided to further my education in the field of entomology. I am grateful to you for providing me with many pleasant things during the beginning stages of my studies. Also, I would like to express my gratitude to Professor Dr Syed Azam Ali, the former CEO of Crops For the Future (CFF), for allowing me to carry out my research at CFF and use the facilities and resources that are located there. To the former farm manager of CFF, Mr Oii Gin Teng, who always pleases me when I ask for assistance, considers all my requests to obtain leaves to rear larvae and provides workers for the harvesting larvae. Thanks also to Khirubalini for always being with me while doing work at CFF. Without you all, I would never have been able to complete this arduous task. Thank you so much. You have all been so helpful; may everyone who has contributed receive an indescribable reward for your kindness.

I owe special gratitude to my friends Farahiyah Ilyana Jamaludin, Yong Su Ting, and Dr. Saadiah Jamli for the purchase of laboratory goods and chemical analysis and understanding my situation. This work would not have been possible without the advice, ideas and statistical support from my former Director, Dr Shanmugavelu and

proofreading the thesis by Dr Musaddin Kamaruddin and Dr Ariff Omar. To Dr Hazila, Norman and Elyana from FT Centre, MARDI, who treated me well with my rather hasty plan to hold a sensory test in their laboratory. Not forgetting the technician under the School of Bioscience, where protein analysis is carried out. Thanks to Ms Sharon Aziz, the PGR Operations Coordinator, for your assistance and consideration that you gave me.

My family deserves special appreciation. To my husband, Muhamed Shamsul Anuar, there are no words to describe what you've given me through this journey. The one that couldn't be forgotten is my mother for her constant *doa* and affection. Always be a good listener, even though occasionally she doesn't understand what I'm doing, and mother-in-law, siblings, and in-laws for their prayers and moral encouragement. Finally, my boys, "MDH" who made me happy and at the same time worried, particularly during the Covid-19 Pandemic are gems that could not be quantified.

And last, to all my friends who helped me directly and indirectly to complete my studies, especially to Norazila Kassim Shaari, Zainal Abidin, Zainan Abidin, Aina Kahar, Rosniza Kassim, Zulfar, Zahrul, Fadhil, Hafizzudin Ayob, Nik Siti Mariani, Maisarah, Azlina Daniel, Wan Abd Ghani, Jameah, Siti Hajar, Siti Masidayu, Nurulhayati, Dr Thayalini, Mardhati and Shariza. I appreciate all of you.

Thank you

Dedication

Above all, I am grateful to God, the creator of all beings, for bestowing upon me the strength, health, and grace necessary to see this success.

This thesis is dedicated to my children, Danish, Daris, and Daiyan, you are aware of the amount of effort I invested in this 'present'.
I really hope that it can inspire you in the future.

To my dear husband, M. Shamsul Anuar, whose faith in me provided me with the impetus to continue forward and keep my gaze fixed on God. There are no words to describe what you've given me through this journey.

To my devoted mother, who has always been by my side with her 'doas' for my success and performance. And to my family's unwavering love and support throughout my journey.

Finally,

A little knowledge in this book is expected to serve as a reference for researchers, entrepreneurs, and farmers.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µL	Microlitre
%	Percentage
AA	Amino acid
ADG	Average daily gain
ADF	Acid detergent fibre
AIBP	Agro-industrial by-products
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BSF	Black soldier fly
BSFL	Black soldier fly larvae
BSFLM	Black soldier fly larvae meal
CF	Crude fibre
Cfat	Crude fat
CP	Crude protein
DM	Dry matter
FAO	Food and Agricultural Organization
FA	Fatty acid
FAME	Fatty methyl esters
FCR	Feed conversion ratio
FM	Fishmeal
FO	Fish oil
g	Gram
GC	Gas chromatography
GE	Gross energy
GLM	General linear models
h	hour
HCL	Hydrochloric acid
H_2SO_4	Sulphuric acid
kg	Kilogram
L	Litre
M	Molarity
MECM	Milk-extracted coconut meat
mg	Milligram
MJ	Megajoule
min	minutes
mL	Millilitre
mm	Millimetre

MO	*Moringa oleifera*
N	Normality
NaOH	Sodium hydroxide
NDF	Neutral detergent fibre
NPN	Non-protein nitrogen
PER	Protein efficiency ratio
PUFA	Polyunsaturated fatty acid
RB	Rice bran
RD	Reference diet
SBM	Soybean meal
SE	Standard error
SG	*Sesbania grandiflora*
SBW	Soybean waste
TD	Test diet
WP	Wheat pollard

CHAPTER 1

BACKGROUND, OBJECTIVES AND LITERATURE REVIEW

1.1 Background of Study

The world population is predicted to reach 9 billion in 2050, which requires a tremendous increase in food production from the various agro-ecosystems, thus imposing enormous pressure on the environment (FAO, 2011). With a rising world population and progressively demanding consumers, the production of sufficient protein from livestock, poultry and fish represents a severe challenge in the future. At the same time, food security and sanitation are expected to decline globally (FAO, 2013). Apart from focusing on agriculture for food production, aquaculture, and the animal food-producing sector, are prioritised to increase production to accommodate the world population's demand for animal protein resources. Global fish production from the fisheries and aquaculture sectors reached approximately 179 million tonnes (MT) in 2018 (FAO, 2020), decreased slightly in 2019, and is anticipated to reach 200 MT by 2029, an increase of 25 MT (or 14%) over ten years (OECD/FAO, 2020). In comparison, the total capture was 91.7 MT (1999) and 93.3 MT (2019) per decade, which does not differ much despite the ups and downs between times (Figure 1). However, the aquaculture sector is growing rapidly to meet the increasing demand.

The aquaculture sector has developed to a remarkable level where the total fish supply grew at an average annual rate of 3.2% from 1961 to 2013, which was double that of the human population growth, 1.6% and surpassed meat from all terrestrial animals, which was 2.8% (FAO, 2016; FAO, 2018). Fish production from this sector is forecast to drive the most increases in output, reaching 105 MT in 2029, which is 10 MT more than the marine capture sector or a 20% increase over 2019 (Figure 1). Aquaculture will

continue to meet the growing supply-demand gap for protein sources due to increasing population growth and income levels. Out of the total fish captured, around 18% is currently converted into fishmeal (FM) and fish oil (FO) to produce fish feed for aquaculture. It is predicted that this proportion is expected to increase by 10 and 17% during the next decade, making the production increase from 24 to 28% and from 41 to 45%, respectively, owing mostly to the increased use of fish waste in their manufacture (OECD/FAO, 2020).

Demand for fish for human consumption has grown dramatically over the years. Approximately 90% of fish produced from capture fisheries and aquaculture are projected for human consumption, growing from 155 MT in 2017 to 2019 and increasing to 180 MT in 2029. Another 10% of captured fish is for non-food uses such as FM and fish oil FO productions. Aquacultural activities produced more fish for human consumption than wild capture operations in 2017-2019 and the projected year, 2029 (Figure 2). Currently, aquaculture provides around 53% of seafood production for human consumption and is expected to increase to 58% in 2029, compared to 41.6% from capture fisheries (OECD/FAO, 2020). Meanwhile, world fish consumption per capita is expected to reach 21.5 kg in 2030, up from 20.3 kg in 2016 (FAO, 2018). With the rapid development of the aquaculture sector, millions of tonnes of feed are needed. Therefore, using stable, abundant, and sustainable ingredients that do not compete with human food and do not cause environmental damage is a goal for the aquaculture industry to achieve. Many factors must be considered in mitigating environmental impacts on sustainable seafood production both from marine and land sources, without reducing productive natural resources and damaging the valuable aquatic environment. Overfishing of fish stocks, such as capture fisheries for FM production, must be discouraged to ensure a continuous supply of fish for human consumption (FAO, 2017; 2018).

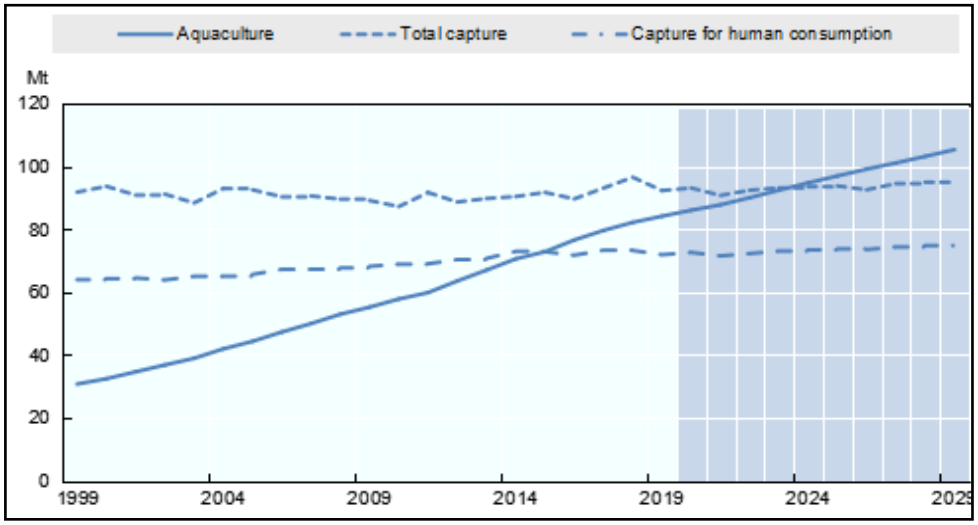


Figure 1: World aquaculture and capture fisheries.

Source: OECD/FAO (2020)

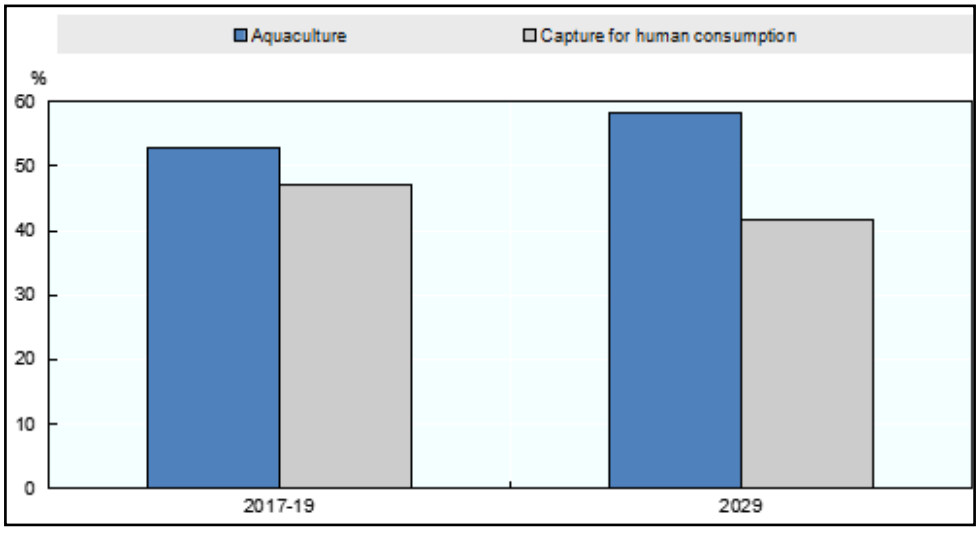


Figure 2: Share of aquaculture and capture fish in total fish source for human consumption.

Source: OECD/FAO (2020)

Feed is the single most crucial input in aquaculture and livestock production. Both content and quality are crucial factors in feed formulations. Protein is the most expensive component of animal feed, owing to its high demand and, to some extent, scarcity. The rising cost of essential feed ingredients such as wheat, maize, soybeans, and FM is one of the most critical constraints on the animal feed industry's future development. It is worth emphasising that aquaculture's sustainability relies on many aspects, including cost-effective feed. Feed costs were estimated to account for around 40 – 70% of total production costs in aquaculture and livestock production (FAO, 2009; van Huis *et al*., 2013; Ssepuuya *et al*., 2017), while variable expenses ranged from 33 – 75% (Ashley *et al*., 2018). Concerns about food security have necessitated the search for more sustainable protein sources for human consumption and animal feeding. FM is a significant conventional protein source in formulating a diet for fish due to its superior nutritional quality and palatability properties (Sánchez-Muros *et al*., 2016). The crude protein (CP) content of FM can reach as high as 70%, and its high digestibility and amino acid profile make it an ideal protein source in aquafeed, which is difficult to replace (Xu *et al*., 2020). Simultaneously, a plant-based protein, soybean, which has between 40% and 49% CP content (Banaszkiewicz, 2011), is widely used in terrestrial animal feed with some limitations. The plant proteins are of a lower overall nutritional quality. They have an imbalance between essential and non-essential amino acids (Refstie *et al*., 2000). The proteins sourced from plant-based foods may also contain anti-nutritional factors (ANFs) (Bondari and Sheppard, 1981; van Huis *et al*., 2013; Nguyen *et al*., 2015; Daniel, 2018) that can affect the nutrient utilisation or food uptake by organisms and could have an impact on the taste, smell and aroma of meat products (Arshad *et al*., 2018). The deficiencies in plant protein sources have caused an increase in the demand for animal-based protein in animal feed, particularly aquafeed. However, animal-based protein meals are anticipated to decline in production in future years in farmed seafood. Its usage is unsustainable as

the aquaculture sector expands and pressure on wild fisheries grows in tandem with the human population.

Being a monogastric species, fish require a high quantity and quality of protein in their diets. FM and FO, typical components of aquaculture feeds, are produced from captured whole wild fish, fish trimmings, trash fish and by-products of the fish processing industry (FAO, 2016). There are several fish species used for the production of FM and FO, which include pelagic fish such as Peruvian anchoveta (*Engraulis ringens*), menhaden (*Brevoortia patronus*), sardine (*Clupea sp.*), mackerel (*Scomber sp.*), and Alaska pollack (*Gadus chalcogrammus*) (Miles and Chapman, 2006). It is currently estimated that about 25 – 35% of the FM and FO produced globally is derived from fish processing waste, which has lower protein levels but is richer in minerals and amino acids (AAs) than FM processed from whole fish (FAO, 2019). FM is frequently manufactured utilising fish processing by-products rather than entire fish due to competition from human consumption. Many aquaculture feed formulations still use FM as a significant protein ingredient where the inclusion level in feed is normally between 5% and can exceed 50% (Table 1) (Miles and Chapman, 2006; Glencross *et al*., 2007; Tacon *et al*., 2011). Given the predicted large and continued expansion of aquaculture, the overall quantity of FM devoted to aquaculture is expected to increase. However, the inclusion rate of FM in feed has declined based on the fish species (FAO, 2020). Diseases such as white syndrome and myonecrotic virus in shrimps in Indonesia and Brazil and infectious salmon anaemia virus in Chile have affected fish and seafood production such as shrimp, squid, and crabs (Walker and Winton, 2010). Besides, the early death syndrome has caused significant losses in farmed shrimps in Asian countries, including China, Vietnam, Malaysia, and Thailand (Leaño and Mohan, 2012; de Schryver *et al*., 2014). These diseases have affected the worldwide demand for FM and FO but have been under control since 2014. The El Niño phenomenon elevated fish prices to significant highs through late 2014 but slightly declined in mid-2015 while

decreasing the FM production. Similar circumstances affected the price of FO, which increased due to lower production in Chile and Peru (FAO, 2016). These conditions threaten the sustainability and growth of the aquaculture industry. However, the supply of FM and FO recovered from El Niño in late 2016 and increased global production has been recorded since 2017. Also, the price of FM has stabilised due to an improvement in supply (OECD/FAO, 2016). However, in 2019, the demand for FM declined due to the outbreak of African Swine Fever (ASF), which caused the collapse of the piglet industry in China (Figure 3). Although the inclusion level of FM in pork feed is only 5% or less, the large-scale pig business has influenced FM prices globally. Also, China is the largest importer of FM in the world for aquafeed and livestock (OECD/FAO, 2021). After the outbreak of the ASF disease, FM prices returned to stability, varying between USD1,400 MT in May 2020 and gradually increasing to USD1,500 MT by May 2021 (World Bank, 2021). However, fluctuations and instability in FM prices because of the above problems or supply from the world's major FM producers, namely Peru and Chile (Tacon *et al*., 2011), have affected the animal feed industry worldwide and ultimately directly affected the development of the aquaculture and livestock industries.

Table 1: Percentage of fishmeal inclusion in compound aquafeed in fish and crustacean species.

Species Group	Fishmeal (%)	Reference
Marine finfishes	20 - 65	Tacon *et al*., 2011
Salmons	25 - 40	
Trouts	18 - 40	
Marine shrimps	5 - 40	
Freshwater prawns	20 - 65	
Tilapia	5 - 7	Miles and Chapman, 2006
Asian Seabass	61	Siddik *et al*., 2019
	62.7	Suhaimee *et al*., 2005

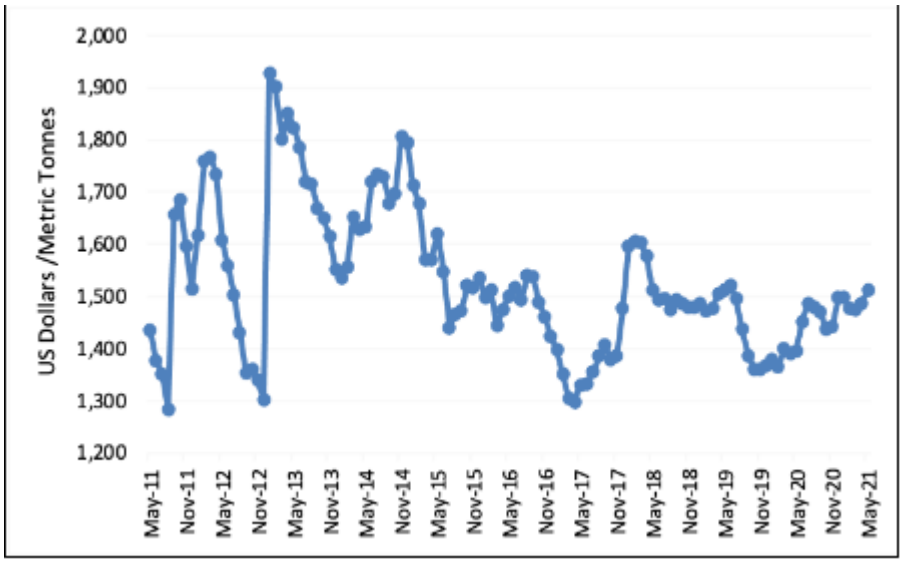


Figure 3: Peruvian fishmeal/pellets pricing at 65% protein in 10 years.
(Source: World Bank, 2021)

The utilisation of insects as an ingredient in animal feed has gained considerable interest in recent years. It is often described as a solution to unsustainable overfishing for FM production. Insects can convert various organic materials, such as animal and plant wastes, into valuable proteins and lipids for animal feed (Barragan-Fonseca *et al*., 2017). Furthermore, they can generate high value-added secondary products such as biodiesel (Surendra *et al*., 2016; Ishak and Kamari, 2018), chitin (Finke, 2007), and organic fertilisers (Nguyen *et al*., 2015). Insects have an advantage over other animal-derived ingredients due to their high growth and feed conversion rates, and rapid reproduction while at the same time not affecting the quality of the environment along their life cycle due to lower production of greenhouse gases and ammonia emissions (Oonincx *et al*., 2010; van Huis, 2013). Insects contain a high amount of energy, quality proteins, vitamins, fatty acids (FAs), amino acids (AAs) and micronutrients for human and animal growth (Verkerk *et al*., 2007; Rumpold and Schlüter, 2013). It has better amino acid profiles than plant protein meals and could be a better candidate to replace

FM (Tran *et al*., 2015). In many countries, insects are already being farmed for commercial use in aquafeed to substitute for FM and soybean meal (SBM). In addition, the European Commission (No. 2017/893) has approved the use of insect protein for aquafeed, and the types of feed allowed for seven species of insects in July 2017. This regulation prohibits using organic by-products such as manure and food waste as feed substrates for these insects to minimise feed and food safety issues since the insects are akin to 'farmed animals'. These insect species, such as the black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*) are regarded as non-pathogenic. They do not adversely affect animals, plants, or human health. Of the seven species allowed by European Union (EU) legislation, the black soldier fly larvae (BSFL) are considered a potential candidate for animal feed due to its ability to convert low-value organic substances into high-quality protein biomass (Danieli *et al*., 2019). In Malaysia, Entofood, Unique Biotech, Protenga, and Nutrition Technologies have been involved in insect-based animal feed production. While overseas, some of the renowned insect-based animal feed companies include Protix (The Netherlands), Entomo Farm (France), Bioflytech (Spain), Hermetia Baruth GmbH (Germany), Agriprotein (South Africa), Enviroflight (US), Hexafly (Ireland), Enterra (Canada) and Entobel (Vietnam). The market value of BSFL has had rapid growth from US$108.7 million in 2019 and is expected to reach US$539.4 million by 2025 (Meticulous Research, 2020). In the current market, the price of insect meal remains high compared to FM. With the increasing interest in and availability of insect-derived protein, the Brabant Development Company forecasts the price of insect protein to converge with FM by 2023 (Arru *et al*., 2019).

A variety of organic materials, such as animal manure, kitchen scraps, and agricultural waste byproducts, can be consumed by BSFL (*et al.*, 2015). Different substrates

generated larvae that varied in performance, nutritional makeup, and time is taken to reach the prepupae stage (Hopkins *et al*., 2021; Fitriana *et al*., 2022; Siddiqui *et al*., 2022). Current research has focused on increasing knowledge of the ideal feed substrates by studying the nutrient composition and growth performance of BSFL in composting organic waste into sustainable products through bioconversion (Bosch *et al*., 2019; Jucker *et al*., 2020; Galassi *et al*., 2021). To date, there is no detailed research into the exact macronutrient and micronutrient requirements of BSFL. Available information suggests that the rearing substrates influence the larvae's life cycle, development time and nutrient composition (Tschirner and Simon, 2015; Bava *et al*., 2019; Scala *et al*., 2020).

In the present study, a range of substrates from locally available underutilised plant species and agro-industrial by-products (AIBPs) were used to rear black soldier fly larvae. The suitability of the substrates as feed was evaluated through their impacts on larval development time, growth rate and nutrient composition. Neglected or underutilised plant species (NUPS) usually refer to plants that researchers or consumers have not fully exploited. Little is known about their potential use, functionality, or commercial values (Azam-Ali, 2010; Mabhaudhi *et al*., 2017; Baa-Poku, 2018), although they may have better potential as food sources (Mayes *et al*., 2012). Some have little economic value and may be used as animal feed (Stefoska-Needhan *et al*., 2015). Utilising underutilised plant species through the BSFL bioconversion cycle offers the potential to produce feed for fish production. Using plant meals for BSFL production reduces the reliance on other plants that can be eaten directly by humans and other animals. Underutilised plant species such as *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG), which are not widely used in Malaysia, were selected as feed substrates for feeding BSFL because they contain high levels of nutrients such as proteins, amino acids and minerals (Hasan *et al*., 2012; Odetola *et al*., 2012). Several substrates, alone and in combination, were evaluated for their potential use as feed

substrates for BSFL. The Asian seabass (*Lates calcarifer*) was selected for the fish growth and feeding trials using BSFL because it is a commercially valuable and important fish species used in aquaculture in Southeast Asia, including Malaysia (Ganzon-Naret, 2013). It is a popular fish with superb meat texture and taste. With the widespread use of black soldier fly larval meal (BSFLM) as an alternative source of protein in animal feeds, mainly in aquafeed, the rate of over-exploitation of small fish for FM production can be controlled and reduced substantially. Additionally, the usage of FM can be minimised by substituting BSFLM for FM in the aquafeed, which reduces the total reliance on conventional protein sources.

The present study hypothesises that partial substitution of FM with BSFLM in the diet of Asian seabass has no adverse effect on feed acceptance and performance. The research sought to answer the following fundamental questions:

1. What is the impact of different feed substrates on the performance and nutrient composition of black soldier fly larvae (BSFL)? Is there an impact of feed substrate on the ability of BSFL to convert substrate meal into larval biomass, considering factors such as waste reduction, bioconversion, and protein conversion ratio?
2. Which feed substrate is optimum for larval production? When is the best time to harvest larvae, considering the development and nutrient composition factors, such as protein, fat and total larval weight?
3. Is using a combination of feed substrates superior to using a single substrate for larval development and nutrient composition? What is the impact on the larval ability to convert feed substrate into biomass?
4. Following on from question 3, what is the optimum mix of different substrates on larval development and nutritional value?
5. Can black soldier fly larval meal (BSFLM) replace fish meal in the Asian seabass diet? What is the ideal percentage of BSFLM replacement in the diet that does not affect the growth and development of Asian seabass?

1.2 Justification of Study

Malaysia ranked 15th in the world as a fish producer in 2014, with an estimated production of 521,000 tonnes (FAO, 2016). In 2015, fisheries sector in Malaysia contributed to the national GDP at 1.1% (Mohamad Omar, 2017). In addition, Malaysia is one of the top fish-consuming countries in Asia, with more than 40 kg/person/year, nearly double the averages in Thailand and China, but still falls short of Japan and South Korea. The average consumption of fish from year to year was increased and was expected to increase to 64 kg/person/year in 2020 (Teh, 2012, Figure 4). However, the contribution of capture fisheries to the country's fish food is decreasing from 2016 to 2019 by 7.6%, which is from 1.57 to 1.46 million metric tons, respectively) (DOF, 2016-2019). In addition, the aquaculture sector, with contributions from three sources, freshwater fish, brackish water, and seaweed, has also shown a reduction of 21%, from 520,215 to 391,465 tonnes in the last six years (2014 to 2019). The huge drop in total fish production in Malaysia was due to pollution, climate change, invasion of foreign vessels, and local fishing vessels entering the restricted zone (DOF, 2015). In addition, depletion of fish stocks has increased food prices; national environmental issues such as floods and the *El Nino* phenomenon) and diseases have hampered the growth of aquaculture (Yue and Wang, 2017). Malaysia imports 600,000 tonnes of aquaculture feed annually which is expected to rise to 1.5 million tonnes by 2025. Commercial production of fish and crustaceans relies heavily on imported feedstuffs such as FM, wheat, and soybean meal (SBM) (Suhaimee, 2017). These feeds are expensive and unsustainable, which causes fish feed prices to soar (Tacon and Metian, 2015), posing further vulnerability to the sustainability of the aquaculture industry. As a result, it is very important to develop alternative materials to replace major protein sources from local resources. In recent years there has been a decrease in the rate of incorporation of fishmeal (FM), traditionally a major ingredient of aquaculture feed, into compound feeds of herbivorous and omnivorous fish (Hardy, 2010). This trend is projected to

continue, with a reduction from 50 to 10%, including in the diets of marine fish which require high protein in the diet (Tacon and Metian, 2008; Figure 5). This situation arose due to a major decrease in availability of FM, a high demand for the product, and increased market pricing (De-Silva and Hasan, 2007). It is conceivable that a diet free of FM will become popular soon (Daniel, 2018), which is expected to come true when the use of FM in compound feed shows a declining trend (FAO, 2020).

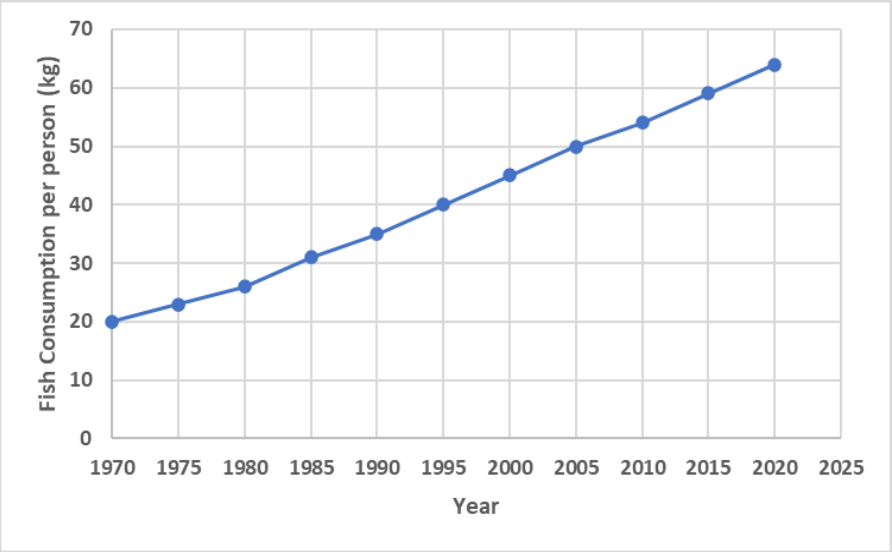


Figure 4: Fish consumption of an average Malaysian from 1970 to 2010 with projections for 2015 and 2020.

(Source: Teh, 2012)

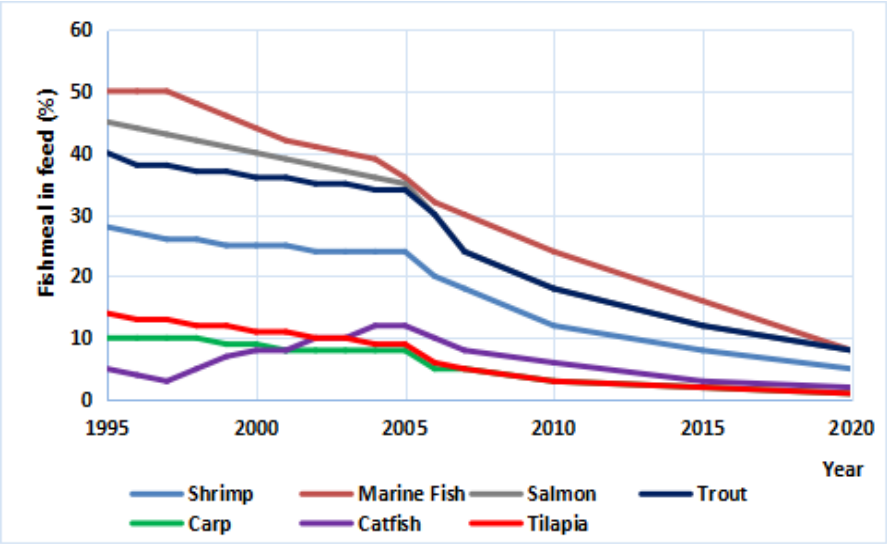


Figure 5: Percentage of fishmeal used in aquafeed on different species.
(Source: Tacon and Metian, 2008)

The search for an alternative protein source for aquafeed is important to reduce the growing demands and dependence on conventional protein sources. An insect-based protein source is a possible alternative to replacing FM and SBM in animal feed (Wang and Shelomi, 2017) due to their nutritional profiles and low environmental footprint (Bessa *et al*., 2020). Compared to traditional livestock and some crops, edible insects have higher feed conversion efficiencies and reproductive rates, a short lifespan and smaller space requirements (Kumar *et al*., 2017). As a natural resource, insects are the focal point of one of the growing research areas in animal feeding. Studies have shown that insects have been used in animal feeding to produce more food for human consumption (Loponte *et al*., 2017; Magalhães *et al*., 2017; Khan, 2018).

Unlike other insects such as house flies, the black soldier fly (BSF, *Hermetia illucens*) is not a pest and a vector of diseases for humans and animals (Erickson *et al*., 2004). Besides, the larvae of this insect can consume a wide range of organic materials due to their robust digestive system and can recapture nutrients from feed and convert them into body growth (Barragan-Fonseca *et al*., 2017; Wang *et al*., 2017). Furthermore, the

larvae can convert biowaste efficiently into BSF larval biomass with high protein and lipid content ranging from 32 – 58% and 13 – 39%, respectively, on a dry weight basis (Gold *et al.*, 2018). Besides the highly nutritious biomass, the remaining substrate, called residue or frass, is value-added through the bioconversion activity of larvae and can be used as fertiliser which is safe for crop farming (Banks *et al*., 2014; Nguyen *et al*., 2015; Beesigamukama *et al*., 2020). Furthermore, the rearing of black soldier fly larvae (BSFL) is not complicated because it can be grown without dedicated facilities, allowing small farmers to carry out BSFL farming on a small-scale basis without incurring significant capital expenditure (Diener *et al*., 2009).

The increasing demand for meat and seafood has raised awareness of BSF as an alternative protein source in the animal feed industry. The global BSF market is forecasted to reach $2.57 billion by 2030, with a compound annual growth rate (CAGR) of 33.3% from 2019 to 2030 (Mediculous Research, 2020). Legal permission authorising the use of insect meals by the Food and Drug Administration (FDA), the Association of American Feed Control Officials (AAFCO) and the Canadian Food Inspection Agency (CFIA) in animal feed (Globe Newswire, 2018) further boosts the demand for the BSF products (Globe Newswire, 2018). In 2019, countries in the Asia-Pacific region, such as China, South Korea, Japan, Vietnam, Malaysia, and Thailand, dominated the BSFL market. The demand for BSF products is projected to grow tremendously in Europe after the EU government approved the use of BSFL meal as aquafeed ingredients in 2017. Additional contributing factors to the rapid growth of BSF farming include the growing market for protein-rich animal feed, increased investment, and financing in the BSF industry, and a massive supply of organic waste from agriculture and the food and beverage industries.

Farming BSFL on organic waste provides solutions for waste management and global shortage of protein supply. Numerous studies have reported the production of BSFL as

an alternative protein source for animal feed using the abundant daily supply of food and kitchen waste (Nguyen *et al*., 2015; Cammack and Tomberlin, 2017; Salomone *et al*., 2017; Lalander *et al*., 2019). The use of organic waste for BSFL feeding is value-added since it involves recycling materials that are usually discarded and have no further benefit to humankind. Furthermore, for some entrepreneurs, the effort of rearing BSF has been a source of revenue (Mutafela, 2015; da Silva and Hesselberg, 2019). BSFL can be reared using a variety of organic materials, therefore, a need to understand the effect of different substrates on the performance and nutritional composition of BSFL, and how these parameters can be manipulated by changing the substrate and rearing conditions. This is very important to produce the BSFL with high quality content in order to replace the major ingredient in the animal feed. The use of BSFL in fish feed has drawn the interest of many researchers. BSFL meal has successfully been used to substitute FM in the diets of many fish species, either partially (Bondari and Sheppard, 1981; St-Hilaire *et al*., 2007b; Kroeckel *et al*., 2012; Fawole *et al*., 2020) or up to 100% in a salmon diet, without causing detrimental effects on the fish performance (Belghit *et al*., 2019). Thus, the purpose of this study is to use selected feed substrates to produce alternative insect-derived proteins which is BSFL that can be used to replace FM in the diets of Asian seabass.

1.3 Objectives of Study

Therefore, the specific objectives of this study were:

1) to assess the performance and nutrient composition of BSFL reared on the six different feed substrates.

2) to determine the best substrate for rearing BSFL with optimum harvesting time.

3) to assess the potential of BSFL meal as a substitute or replacement for FM in Asian seabass (*Lates calcarifer*) diets.

The research was organised into four sequential phases, each with specific objectives.

Phase 1. Screening the nutrient composition of potential feedstuffs for feeding black soldier fly larvae.

(a) to determine the nutrient composition of six feedstuffs, including two underutilised plants, *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG), and four agro-industrial by-products soybean waste (SBW), wheat pollard (WP), rice bran (RB) and milk-extracted coconut meat (MECM) as feed substrates for rearing BSFL.

Phase 2. The growth performance and nutrient composition of black soldier fly larvae reared on underutilised plants and agro-industrial by-products.

(a) to evaluate the effects of selected feed substrates on the growth performance and nutrient composition of BSFL.

(b) to determine the optimum time for harvesting the BSFL (between days 7 and 21) based on their growth performance and nutrient composition.

(c) to assess the efficiency of BSFL in consuming feed substrates for larval biomass production by determining feed reduction, feed-to-biomass conversion efficiency (BCE) and protein conversion ratio (PrCE).

Phase 3. Production of black soldier fly larvae on standardised feed substrates.

(a) to evaluate the effects of mixed substrate on the performance and nutrient composition of BSFL.

(b) to determine the best-mixed feed substrates for BSFL rearing based on their capacity to convert feed substrates into larvae biomass.

(c) to identify the optimal time to harvest larvae based on larval performance and nutrient composition.

Phase 4. Feeding trials of black soldier fly larvae meal in Asian seabass (*Lates calcarifer*) diet.

(a) to determine the apparent digestibility coefficient (ADC) of nutrients in the reference and test diets containing BSFL.

(b) to assess the effects of partial replacement of dietary FM with BSFLM on seabass growth performance and nutrient utilisation.

(c) to examine the serum biochemistry and sensory attributes of Asian seabass fed with diets containing BSFLM.

(d) to determine the optimum percentage of BSFLM replacement in the diet without affecting the growth and performance of Asian seabass.

1.4 Significance of Study

The exploitation of natural resources has resulted in significant negative impacts on the environment, increased food supply competition, and higher costs for livestock farming. According to Rumpold and Schlüter (2013) and Pinotti *et al*. (2019), insects are an emerging source of high-quality protein that can contribute to the safety of the world's food supply. The introduction of BSFL biomass rather than farmed crops, such as soybeans, would also mean less ground and water use (van Huis *et al*., 2017) and reduced cost of fish production (Rana *et al*., 2015). Besides, reducing FM utilisation in animal and aquaculture feed contributes to protecting wild fish from being over-exploited (Tacon and Metian, 2008). Mass-produced BSFL made from underutilised side stream or organic waste, such as agro-industrial by-products, reduce the potential for pollution caused by these materials (Li *et al*., 2011; Gold *et al*., 2018; Lalander *et al*., 2019; Kim *et al*., 2021).

Exploring neglected or underutilised crops or plant species opens up new branches for food security. The underutilised plant species are an essential source of vitamins, minerals, and nutrients (Okigbo and Anyaegbu, 2021). The crops are not widely used for human food and can be grown on marginal land close to aquaculture production areas in tropical climates, increasing the sustainability of the process. Misuse of these plants for commercial, cultural, genetic, and agricultural purposes has been linked to their extinction. Therefore, besides being able to play a role as a future crop, the exploitation of these plants for the production of animal food provides opportunities for diversification of food system. The use of underutilised plants can reduce the risk of over-reliance on limited sources of high nutrients of organic waste for rearing larvae. In addition, the use of agro-industrial by-products for animal husbandry has been widely used because BSFL is recognised to be able to convert low-value organic substrates into highly nutritious macromolecules, making it a good alternative for waste management and strengthening in a circular economy (Cappellozza *et al*., 2019; Jucker *et al*., 2020). Through bioconversion technology, BSFL reared on meals derived from underutilised plants, and AIBPs provide indirect benefits related to their contribution to food production, filling the gap in increased food and feed security.

Furthermore, BSFL is more straightforward to produce than other animal food sources, that require comprehensive care and processing. Adopting BSF rearing technology from low-cost materials for biomass production can increase the income and living standards of smallholders and entrepreneurs, as BSFL can be produced for their use or commercial-scale production, respectively. In addition to producing larval biomass, frass fertiliser or residue from bioconversion as a secondary value-added product would increase insect farmers' profit margins (FAO, 2011). Thus, this investigation will evaluate the potential of including underutilised plant species and organic waste as feed substrates in the rearing of BSFL. In the future, these crops may provide a cheap source of substrate for rearing BSFL and could be more cost-effective than substrates

with other applications and uses. In addition, using organic waste that may otherwise pollute the environment if disposed of in landfills, for rearing BSFL will significantly reduce waste by producing high-grade protein-containing insect biomass and other nutritional values.

1.5 Review of Literature

1.5.1 Neglected or Underutilised Plant Species

About 30,000 edible plant species have been identified worldwide, of which over 7,000 are grown for food (FAO, 1998). One hundred and fifty crop species are grown commercially today, while approximately 30 species are cultivated as food to feed about 90% of the world's population. Four main crops, namely rice, wheat, maize, and soybeans, provide about 60% of human food requirements, and the remaining plant species are underutilised and not commonly planted widely (Li *et al*., 2020). Nevertheless, the number of NUPS remains unknown, as there is no scientific consensus on the overall number of edible plant species (Hunter *et al*., 2019). Underutilised crops or plant species refer to understudied, neglected, orphaned, lost, or disadvantaged crops/plant species. They may have the potential to significantly impact food and nutritional security as many of them are high in nutrients and can grow in resource-poor soils. Typically, they are produced by small-holder farmers and the rural poor in developing countries (Massawe *et al*., 2015). The neglected or underutilised crops or plant species are distinguished by their rarity, the scarcity of scientific information and knowledge in terms of geographical, property, social and economic importance, and their actual sporadic usage is insufficient compared to their economic potential (Gruère *et al*., 2006). NUPS face investment challenges as government, private and international agencies focus on the major crops (Shiferaw *et al*., 2013). Preparing a comprehensive list of underutilised plants is difficult because plant species' descriptions vary from location to location. Regarding geographical distribution, plant species that are not utilised in some regions may be used extensively elsewhere (Padulosi *et al*., 2002; Tontisirin, 2014). Sorghum (*Sorghum bicolour*), for instance, is referred to as an underutilised crop in Australia and the United States but is a staple food in some regions of Africa, Asia, and Central America (Taylor *et al*., 2006). For

example, the chickpea (*Cicer arietinum*) is a major pulse crop in Syria and other Western Asian countries; however, many Italian experts believe it is an underutilised plant in their region (Tontisirin, 2014).

1.5.2 Roles of Neglected and Underutilised Plant Species in Food Security

In the current situation, the production rates of four major food crops, such as maize, rice, wheat, and soybeans, increased by 1.6%, 1.0%, 0.9%, and 1.3% per annum, respectively, which are less than the 2.4% per annum rate required to double global production by 2050. These results are very worrying, given that the yields achieved are well below the estimated level required to meet the predicted global demands for food in 2050 (Ray *et al*. 2013). Several studies have indicated that maize and wheat yields might no longer increase in some parts of the world due to climate change and variability (Brisson *et al*., 2010; Ray *et al*., 2012). Unpredictable environmental conditions and variability render these major crops unable to survive, thus causing changes in agricultural production areas and the yield of the crops to be compromised and destroyed over a while.

NUPS contribute significantly to essential nutrient components such as proteins and minerals often deficient in staple food crops in certain populations (Ebert, 2014). For decades, underutilised plant species have been used as traditional foods. They have sadly been forgotten as more productive crops are available in the agricultural systems, although their nutritional values are equivalent to or higher than conventional food crops. Currently, underutilised food sources such as minor grains, pulses, root and tuber crops, fruits and vegetables, and non-timber forest products have the potential to substantially increase food and nutrition security, improving sustainability (Keatinge *et al*., 2010). Growing NUPS increases genetic diversity and potentially

improves food security (Chivenge *et al*., 2015) and alleviates poverty (Ravi *et al*., 2010), which can be accomplished by promoting these plants through global and domestic research institutions. Many underutilised food crops can be used to supplement young children's nutrient deficiencies, which can be promoted by developing healthy recipes that add these nutrient-dense crops to supplemental food (Andrias *et al*., 2019). Six species of small millets, including finger millet (*Eleusine coracana*), small millet (*Panicum sumatrense*), Italian or foxtail millet (*Setaria italic*), barnyard millet (*Echinochloa crusgalli*), proso millet (*Panicum miliaceum*), and kodo millet (*Paspalum scrobiculatum*), have been phenotyped, sequenced, and genotyped for crop improvement while developing high yield varieties with blast disease, fodder quality, maturation, head smut, and shoot fly resistance (Vetriventhan *et al*., 2020).

However, important genetic and genomic resources are still lacking in NUPS, which might be exploited and conserved to accelerate their development and make their cultivation viable (Kamenya *et al*., 2021). The Crops For the Future Research Centre (CFFRC), which was established in Malaysia in 2011, was the world's first and only research centre dedicated to research solely on underutilised crops. Prior to its closure in 2020, CFFRC carried out a wide variety of research activities on the genetic characterisation and nutritional properties of several underutilised crops (Gregory *et al*., 2019) and developed new food products utilising the crops (Mayes *et al*., 2019). Crops such as bambara groundnut (*Vigna subterranea* L.), winged beans (*Psophocarpus tetragonolobus*), amaranth (*Amaranthus spp.*), moringa (*Moringa oleifera*), proso millet (*Panicum miliaceum*), and sesbania (*Sesbania grandiflora*) were evaluated for their potential to contribute to food and nutritional security under future climates (Gregory *et al*., 2019). Two underutilised plants that grow in abundance in Malaysia, *Moringa oleifera* and *Sesbania grandiflora*, were selected for this study to determine their nutrient composition and potential for use as substrates for rearing BSFL.

1.5.2.1 *Moringa oleifera*

Moringa oleifera (MO) is an unexploited and underutilised tropical plant (Bashir *et al*., 2016) and belongs to the *Moringaceae* family (Figure 6). All components of MO, including the leaves (Figure 7), flowers, and seeds, are edible (Makkar and Becker, 1997) and can be consumed by humans. The National Institute of Health (USA) has named it the "Botanical of the Year 2007" (Gupta *et al*., 2017). MO leaves have a protein content of between 20 and 29% on a dry matter basis (Makkar and Becker, 1996) and are rich in minerals (Abbas *et al*., 2018). Moringa has been touted as a potentially beneficial animal feed due to its high nutritional content (Nouman *et al*., 2014) for livestock (Mendieta-Araica *et al*., 2011), poultry (Mahfuz and Piao, 2019), and fish (Billah *et al*., 2020). MO was used to substitute for fishmeal in the Bocourti's catfish (*Pangasius bocourti*) diet (Puycha *et al*., 2018), and soybean meal in the fancy carp (*Cyprinus carpio*) diet (Yuangsoi and Masumoto, 2012). Due to its many useful properties, MO was used as a supplement in diets and as an antimicrobial to increase fish health (Brilhante *et al*., 2017). In a study by Chen *et al*. (2021), the Nile tilapia (*Oreochromis niloticus*) diet) containing 8 – 10% moringa leaves may be beneficial for enhancing resistance against *Streptococcus agalactiae* infection in addition to the low mortality rate recorded with 10% of moringa leaves.

Kingdom Plantae
Division Magnoliophyta (Flowering plant)
Class Magnoliopsida (Dicotyledons)
Order Capparales
Family Moringaceae
Genus *Moringa*
Species *oleifera*

Figure 6: The taxonomic classification of *Moringa oleifera* (Kumar *et al*., 2021)

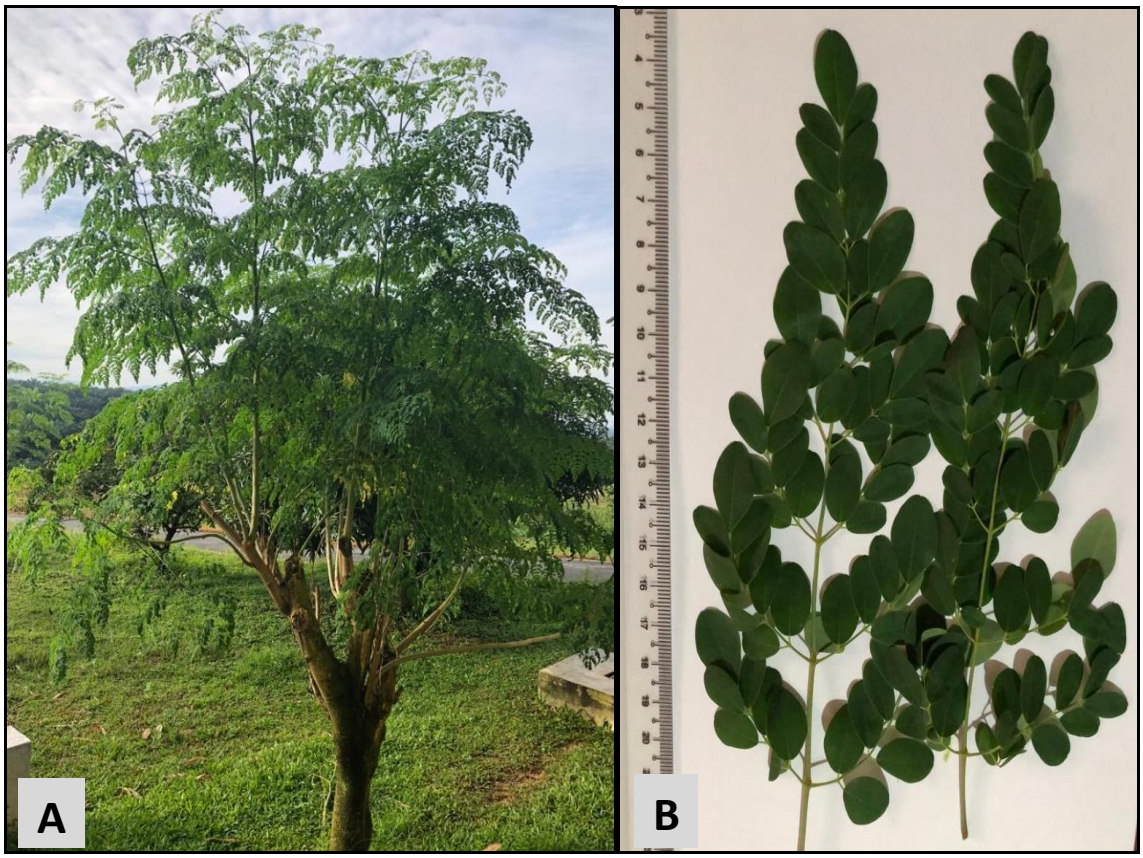


Figure 7: *Moringa oleifera* tree (A) and leaves (B).

1.5.2.1 *Sesbania grandiflora*

Sesbania grandiflora (SG), commonly known as a vegetable hummingbird or turi, is a leguminous fodder plant in the *Fabaceae* family (Figure 8) native to tropical Asia, including Malaysia (Hasan *et al*., 2012). The flowers and leaflets (Figure 9) are rich in essential amino acids (EAAs), vitamins, minerals, and other bioactive compounds (Thamboli, 1996). Sesbania leaves were found to have a high crude protein (CP) content, around 22 – 30% (Kaitho *et al*., 1998). Apart from its nutritional value, only a few studies have been reported on the use of this plant's leaves in fish feed. A study by

Firmani *et al*. (2015) reported that adding turi leaf flour to black tilapia (*Orechromis niloticus*) increased growth. Feed added with 0 – 9% turi leaf flour produced the most excellent results, with daily specific growth rates ranging from 1.57 to 1.66% and feed efficiency ranging from 39.82 to 45.57%.

Kingdom Plantae
Division Magnoliophyta
Class Magnoliopsida
Order Fabales
Family Fabaceae
Genus *Sesbania*
Species *grandiflora*

Figure 8: The taxonomic classification of *Sesbania grandiflora* (Hai, 2015).

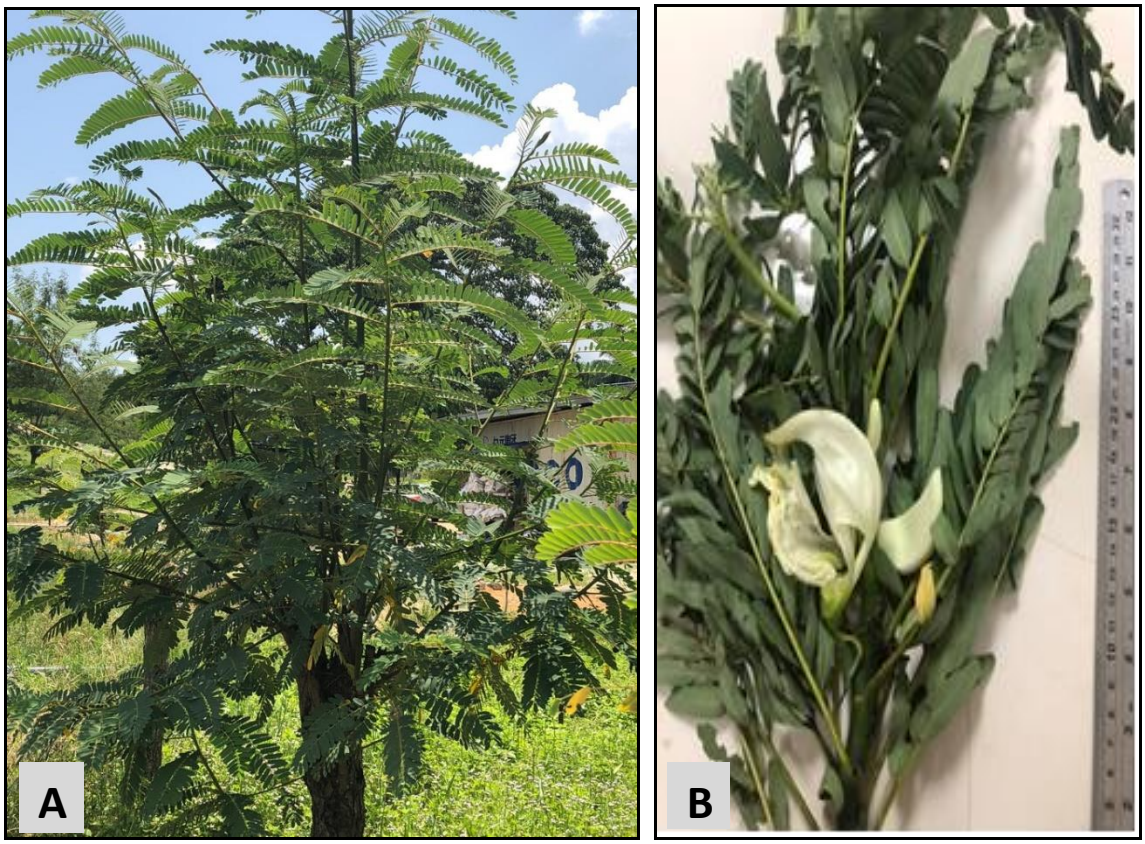


Figure 9: *Sesbania grandiflora* tree (A) with leaves and white flower (B).

1.5.3 Asian Seabass

The Asian seabass or barramundi (*Lates calcarifer*) was first described by Bloch (1790). It is a carnivorous fish of the Perciformes order (Arockiaraj and Appelbaum, 2010) and belongs to the *Centropomidae* family with seven other species, all of which are of African descent (Greenwood, 1976). *Lates calcarifer* dominates a diverse geographical range and is broadly dispersed in the coastal and fresh waters throughout the Indo-West Pacific region, including India, Burma, Sri Lanka, Bangladesh, the Malay Peninsula, Java, Borneo, Celebes, the Philippines, Papua New Guinea, northern Australia, southern China, and Taiwan (Greenwood, 1976). The Asian seabass is also known as 'Barramundi', giant perch, palmer, and cock-u in Australia, sea bass and 'bhekti' (India), 'bulgan' (The Philippines), 'kakap' (Indonesia and Malaysia), 'akame' (Japan), and 'anama' (Papua New Guinea) (Rabanal and Soesanto, 1982). However, it is more commonly referred to as the Asian seabass. Schipp *et al*. (2007) suggested that it is an economically important aquaculture species. It is widely reared in extensive or intensive fresh, brackish, and marine water bodies in South-East Asia, such as Malaysia, Indonesia, Thailand, Taiwan, and Australia (Harpaz *et al*., 2005; Glencross, 2006). Most production of seabass are from the Asian countries such as China, India, Thailand, Malaysia, Vietnam and Philippines (FAO, 2006). The Asian seabass is a diadromous species in the wild and travels to estuarine or coastal water for spawning (Greenwood, 1976). This species has a complex life history that reflects the nature of its hermaphroditic sequence. This fish develops first as females and then changes to males at 3-4 years of age. Most mature individuals are males, but almost all large specimens are females (Ganzon-Naret, 2013). The demand for seabass increased worldwide due to its excellent quality and delicately flavoured white flesh (Anil *et al*., 2010). Among the advantages of seabass is its voracious feeding habit, which can be fed with artificial food or fish waste. Consequently, it has a rapid growth rate and can be bred in captivity, making it an ideal candidate as an aquaculture species

(Boonyaratpalin *et al*., 1998; Singh, 2000). Furthermore, seabass is regarded as an ideal aquaculture species due to its ability to thrive in a wide range of water salinities, including seawater, brackish water and freshwater (Harpaz *et al*., 2005). The harvest size of seabass is between 0.4 and 4.0 kg, depending on the production system and market demand (Glencros, 2006). In terms of price, it sells for about USD3.80/kg, which is still affordable compared to groupers priced at USD5.95/kg (Fisheries Development Authority of Malaysia, Weekly Price Report 2021, LKIM, 2021).

1.5.3.1 The Nutrient Requirements of Asian Seabass

The nutrient requirements of fish differ according to their growth stages: larvae, juvenile, and adult. Larvae have higher food needs than juveniles, thus, their requirements are not comparable to other stages of growth. A dietary regime that maintains healthy youth growth may inversely contribute to poor larval growth and survival performance. Proteins and amino acids (AAs) are the essential nutrients that all animals need for protein synthesis and subsequent development (Wu *et al*., 2014). The density of dietary energy provided, and the size of fish reared appear to be the primary factors influencing the levels of protein required for seabass. Crude fat or lipid is also an important dietary macronutrient in seabass, providing energy and essential fatty acids (Glencross, 2006).

The nutrient requirements of seabass are well understood. Asian seabass requires high protein and fat because it is a carnivorous feeder (Table 2). Over the last three decades, researchers have made significant efforts to develop nutritionally balanced and cost-effective feeds for rearing Asian seabass. The study on the nutrient requirements of the Asian seabass was initiated by Cuzon and Fuchs (1988). This effort was followed by Sakaras *et al*. (1988) and Sakaras *et al*. (1989) through factorial studies

that showed protein levels in seabass diets of 45 and 50%, and lipid levels of 18 and 15% produced the best growth performance. The protein requirements in Asian seabass diets are relatively high because of the carnivorous/piscivorous nature of the fish (Ambasankar *et al*., 2009). Catacutan and Coloso (1995) also undertook a factorial study investigating the inclusion levels of 5%, 10% and 15% of lipids in three protein levels at 35%, 42.5% and 50%. Their study indicated that the highest growth rate was observed in the fish group with 15% lipid and the highest protein level of 50%. However, fish development was not substantially higher than those fed 10% lipids and 42.5% protein. Glencross *et al*. (2003) showed that feeding Asian seabass with extruded commercial diets with a similar protein level but at different lipid levels of 16% and 22% resulted in comparable fish growth. In contrast, higher lipid levels result in a lower feed conversion ratio. Williams *et al*. (2003) examined the protein and energy requirements using two different fish weights. Four protein levels, which ranged from 38 to 52%, performed at three different energy levels on 230 g fish indicated that the best growth was observed amongst fish fed with the highest protein and energy levels (52% and 21 MJ/kg). In contrast, diets containing higher protein levels ranging from 44 to 65% and energy of 20.9 – 22.8 MJ/kg using 80 g fish indicated that the optimal protein and energy levels were 60% and 22.8 MJ/kg, respectively (Williams *et al*., 2003). The efficiency of protein utilisation of Asian seabass was approximately 46% (Glencross, 2006) in a 470 g/kg protein diet fed to 222 g fish. However, the efficiency was reduced to 34% when supplied with a lower protein diet of 370 g/kg of similar fish (William and Barlow, 1999). Tucker *et al*. (1988) found that fish fed with either 90 or 130 g/kg of lipid showed similar growth, but the feed conversion ratio (FCR) was significantly lower at the higher lipid levels. Also, somatic fat deposition has been shown to increase in direct proportion to dietary fat levels. Later, Glencross *et al*. (2003) indicated that two diets that contained similar protein levels but different lipid levels of 160 and 220 g/kg showed the same growth performance of 555 g fish. However, the FCR of fish fed a higher lipid diet was significantly lower than those fed a lower lipid level.

Coloso *et al*. (1999; 2004) worked out the essential amino acids (EAAs) required by Asian seabass. Four out of ten fish EAAs have been determined, including methionine/total sulphur AA (Coloso *et al*., 1999), lysine and arginine (Murillo Gurrea *et al*., 2001), and tryptophan (Coloso *et al*., 2004). Glencross (2006) described that the estimation of EAA required by Asian seabass was based on the composition of the relative ratio of AA to lysine in the body tissues, which is usually considered the first limiting AA in formulated diets. Methionine is essential and must be consumed in the diet as it is not endogenously synthesised. Cysteine, on the other hand, is unnecessary because fish can synthesise it. However, cysteine can constitute up to at least 40% of methionine without significant effects on seabass growth, whereby the amount of methionine required was between 10.5 and 13.6 g/kg in a diet (Poppi *et al*., 2017). As for tryptophan, Coloso *et al*. (2004) suggested that Asian seabass required only about 4.1 g/kg of dietary protein. No studies have reported the efficacy of AA use in Asian seabass, but it is believed to be close to that of protein (Glencross, 2006). It is also noted that there are no studies conducted to determine the fatty acid requirements for the Asian seabass.

Table 2: Summary of nutrient requirements of Asian seabass (*Lates calcarifer*).

Size of fish used (g)	Optimal Crude Potein (%)	Optimal Crude Fat (%)	Optimal Gross Energy (MJ/Kg)	Temperature (°C)	References
n/d	55	n/d	13.4 - 16.4	n/d	Cuzon and Fuchs, 1988
7.5	50	15	n/d	n/d	Sakaras, *et al*., 1988
n/d	45	18	n/d	n/d	Sakaras, *et al*., 1989
n/d	40 - 45	n/d	n/d	n/d	Wong and Chou, 1989
1.3	50	15	50	29	Catacutans and Coloso, 1995
76	46 - 55	-	18.4 - 18.7	28	Williams and Barlow, 1999
230	52	7, 13, 19	17.8 - 21.0	28	Williams, *et al*., 2003
80	60	18	20.9 - 22.8	28	Williams, *et al*., 2003
<200	50	14	15	n/d	Glencross, 2006
>200	46	20	17	n/d	Glencross, 2006

1.5.3.2 Feed Sources for Asian Seabass

Diet supply is critical in the culture of Asian seabass because it requires high protein levels. It is estimated that more than 50% of the variable costs for Asian seabass are feed costs (Yong *et al*., 2017). The total expense of growing out an Asian seabass in 210 days at an average body weight of 1.1 kg was USD3.61/tail (Mohd Nor *et al*., 2019). To supply juvenile fish with the nutrients they need, a feed formulation combines multiple feed components in the quantities required to give the nutrients needed by the fish at a specific growth stage at an affordable price. Besides that, the nutrient requirements, palatability, acceptability, digestibility, and toxicity of the feed components also need to be determined (Coloso, 2014). Generally, all culture systems for Asian seabass employ trash fish as feed due to low cost and availability (Sros *et al*., 2020). However,

the supply of trash fish has become more scarce and costly, besides causing pollution to the water (Boonyaratpalin *et al*., 1998; Scros *et al*., 2020). Boonyaratpalin *et al*. (1998) discovered that feeding Asian seabass only on trash fish decreased feeding efficiency and resulted in a slower growth rate. Studies are conducted to replace the use of trash fish meals and FM in the seabass diet. Among those used are using insect meals such as cricket and BSFL. Sorphea (2019) reported that these insects could be FM and trash fish replacers in the seabass diet. The use of green pea *Pisum sativum* as a substitute protein source for FM in *Lates calcarifer* demonstrates that up to 30% substitution results in fish with the lowest weight increase values, ascribed to the poor palatability of material (Ganzon-Naret, 2013). Feeding *Spirulina platensis* to juvenile Asian seabass in a freshwater culture system found that it could replace up to 10% of FM protein in diets without affecting growth. In the feed trial, a higher inclusion rate of spirulina in the diet impaired fish growth, feed efficiency, and health. The result was possibly due to a reduction in the digestibility of spirulina (Yong *et al*., 2017). Asian seabass has also been fed animal by-products, such as chicken waste, to replace FM. The results show that chicken waste could be supplied only up to 10% (Nandakumar *et al*., 2013). Various feed ingredients have been tested for multiple purposes in the seabass diet, such as measuring the digestibility of protein and energy. The results indicate that soybean, lupin kernel meal, wheat gluten, barley, sorghum, and fishmeal are highly digestible by Asian seabass (Glencross *et al*., 2012), whereas poultry offal meal is the least digestible (Glencross *et al*., 2014).

On the other hand, palm kernel meal (PKM) was used to replace soybean meal and wheat meal mixtures in juvenile seabass. Replacement with 15% PKM gave higher growth than 30% PKM (Suhaimee *et al*., 2005). The use of various types of feedstuffs in the seabass diet showed different results in the acceptance of such materials due to palatability factor, nutrients and digestibility of the material. The ingredients chosen for Asian seabass diets may be insufficient to satisfy and maintain seabass growth due to

the carnivorous species' requirements for high protein and specific EAA. Therefore, using other sources, such as insects in the feed ingredient of seabass, may reduce the constraints in feedstuffs used mainly in replacing the primary protein source in fish feed.

1.5.4 Protein Sources in Aquafeed

Protein is the most important essential nutrient required for fish growth and development, but it is expensive, and therefore increases the production costs. As a result, it is critical to precisely establish the protein requirements for each fish species and the growth stage. A complete diet formulation should include proteins with known amino acid content and a digestibility value reflecting nutrient quality. Aquaculture, where animals are fed high-protein aquafeeds, accounts for over 70% of all aquatic-based animal production. Aquafeeds rely on fishmeal (FM) and fish oil (FO) derived from wild-caught small pelagic fish as protein, amino acids (AA), and fatty acids (FA), respectively (Hua *et al*., 2019). Aquafeed typically has crude protein levels ranging from 20 to 55% (Ayadi *et al*., 2012). Different types of fish require different amounts of protein, herbivorous (plant-eating) and omnivorous (plant and meat-eating) fish have lower protein requirements compared to carnivorous (flesh-eating) fish (Craig, 2017, Table 3). The dietary inclusion level, quality of protein, and protein ratio to other nutrients (fat and carbohydrate) significantly impact the growth performance of fish (Shukla *et al*., 2019). The source of ingredients and nutritional composition ratios typically dictates the price of aquafeed. A diet high in protein will be costly compared to those with low dietary protein levels.

Various additional resources have been used as protein sources in aquafeed, either from animal-based or plant-based sources, to replace FM and FO in diets; however,

these substances fall short of the benefits provided by the primary protein source, FM. FM alternative components, in any case, cannot match FM in terms of nutritional value, such as polyunsaturated fatty acid; nevertheless, using high-quality locally available feed ingredients as a replacement that less expensive but compatible alternative protein content can reduce the costs of production, therefore cost of fish can be reduced and increase profits to fish farmers. These ingredients are still under study to overcome some constraints, including the presence of anti-nutritional factors (ANFs) and unbalanced nutrient profiles such as AA and FA, as well as product variations, especially the quality and quantity of materials.

Table 3: Protein requirement of fish species based on diet type.

Seafood	Protein Requirement	Type of diet	Reference
Catfish	28 – 32%	omnivorous	Craig, 2017
Shrimp	30 – 35%	omnivorous	
Nile tilapia	35 – 40%	herbivorous	
Hybrid striped bass	38 – 42%	carnivorous	
Trout & other marine finfish	40 – 45%	carnivorous	
Asian Seabass	45 – 50%	carnivorous	Glencross, 2006
Salmonids	40 – 50%	carnivorous	Hardy, 1996

1.5.4.1 Fishmeal

Fishmeal (FM) is a nutrient-dense feed ingredient mainly used in domestic animal diets, particularly aquafeed. FM can be made from almost any form of seafood but is traditionally made from wild-caught, small marine fish, fish trimmings and by-products of the fish processing industry that contain a high proportion of bones and oil, often considered unfit for direct human consumption (Miles and Chapman, 2006).

Approximately 20% of FM is produced from fishery by-products (FAO, 2018). The FM industry uses approximately 20 million tonnes (MT) of raw materials per year to produce 15 million tonnes of FM and FO, whereas 5 MT is for direct feeding in aquaculture and livestock, pharmaceutical activities, and ornamental purposes (FAO, 2018). FM has several characteristics that distinguish it as an excellent source of protein in aquaculture organisms and farm animals (Sánchez-Muros *et al*., 2014). Its high quality, the abundant amount of essential nutrients and energy, vitamins, well-balanced AAs and essential fatty acid content, lack of ANFs, and ease of availability makes it an indispensable component of animal and aquaculture diets for most fish species (Tacon and Metian 2008; Daniel, 2018; Shukla *et al*., 2019).

For the above reasons, FM is the standard ingredient in aquaculture diets, besides being highly digestible and palatable (Miles and Chapman, 2006). Generally, the high-quality FM uses whole fish, which contains crude protein (CP) ranging from 66 –74%, 8 – 11% lipid, and <12% ash (Heuzé *et al*., 2015). In contrast, FM produced from by-products contains 52 – 67% CP, 7 –14% lipid, and 12 – 23% ash. Since most of the oil is usually removed during the processing of the FM, the residual lipid content is typically between 6 and 10%. FM provides an excellent supply of essential polyunsaturated fatty acids (PUFAs), namely, the omega-3 and omega-6 fatty acid families (Turchini *et al*., 2019). The amino acid content in FM is well-balanced, which meets the nutrient requirements, especially for carnivorous fish species. FM enhances animal feed efficiency and growth by increasing food palatability and nutrient intake, digestion, and absorption. Combining balanced AA, notably essential AAs, FAs, and phospholipids of the FM, with other elements derived from animal and plant proteins, promotes rapid growth for optimal larval and broodstock development, growth, and reproduction (Miles and Chapman, 2006).

FM has traditionally been the preferred dietary protein source for many farmed fish species. Continuous growth in aquaculture production and expansion of other feed businesses generate significant demand for FM and FO in commercial feed, resulting in price increases (Tacon and Metian 2008). As a result, FM is no longer an attractive ingredient because of its soaring prices due to its high demand and diminishing supply. Thus, identifying inexpensive, non-conventional feed protein sources would increase the long-term sustainability of the fish industry, both in terms of resource utilisation and cost savings associated with feeding (Mmanda *et al*., 2020). The alternative feed sources are vital to fulfilling the protein and nutrient requirements without significantly and adversely affecting fish growth (Hua *et al*., 2019). Modern processing techniques today allow the use of numerous new resources. Plant proteins are the most often used substitutes for FM in aquafeeds. They are cost-effective and often preferred due to consumer opposition to land animal by-products for fish feeding (Collins *et al*., 2013). Some of the animal-based protein sources that have been evaluated as potential candidates to replace FM include beef and bone meal (Moutinho *et al*., 2017), poultry by-products (Siddik *et al*., 2019), and feather meal (Yong *et al*., 2018). The alternative feed ingredient for replacing FM should be high in protein and highly digestible to assist feed producers in achieving their amino acid requirements, particularly for fish with exceptionally high protein and amino acid requirements. Among other significant criteria for alternative protein sources is the source's long-term viability in production.

1.5.4.2 Soybean Meal

Soybean meal (SBM) is the most common plant protein source in aquafeed. SBM is commonly considered the most nutritious plant-protein feedstuff for aquatic animals and has been used for many years in the diet formulations of numerous fish species (Tantikitti *et al*., 2005; Rubbani *et al*., 2011). It is considered a cost-effective alternative

to the high-quality FM in diets for various aquaculture species because of its protein content, well-balanced AA profiles, reasonable price, consistent quality, and availability (Daniel, 2018). SBM, which contains between 44 and 49% protein, is commonly used in aquaculture feed (Banaszkiewicz, 2011). It is a low-cost feedstuff used up to 50% replacement in the freshwater fish diet (Yue and Zhou, 2008). Besides, it has one of the best amino acids (AA) profile of any plant protein feedstuff, containing large quantities of EAA (approximately 45% of total AAs). SBM contains an adequate amount of lysine and tryptophan but lacks sulfur-containing AA such as methionine and cysteine (Arru *et al*., 2019; Castro *et al*., 2019). However, a diet high in SBM will be nutritionally deficient in methionine, lysine and tryptophan (Yue *et al*., 2012; Craig, 2017).

Numerous authors have stated that plant ingredients can be used to substitute for FM in animal diets without impairing animal performance (Kpundeh *et al*., 2015; Dorothy *et al*., 2018). Silva-Carrillo *et al*. (2012) used SBM to replace FM in rose snapper (*Lutjanus guttatus*) diets. Their study suggested SBM can be used only up to 20% of the diet to replace FM because the SBM will affect fish performance at higher substitution levels. Abdel-Warith *et al*. (2020) reported that the growth of *Clarius gariepinus* was significantly reduced when the replacement of FM by the full-fat SBM (FFSBM) level in the fish diet was increased. The fish could tolerate the control diet, but the palatability of the test diets declined with the increasing *substitution* of FFSBM. A 12-week feeding experiment on *Oreochromis niloticus* was found that SBM can partially or fully replace FM with methionine supplementation without affecting the fish's development, nutrient consumption, or health status (Ajani *et al*., 2016). In addition to the problem of palatability (Refstie *et al*., 2000; Watanabe, 2002), soybeans contain ANFs such as trypsin inhibitors (decreases protein digestibility) and lectins, but the levels will be greatly reduced during the processing of soybeans into SBM (Gu *et al*., 2010). ANFs impair fish digestion (Iwashita *et al*., 2008) and cause gastrointestinal problems such as

enteritis (Refstie *et al*., 2000). Ancillary processing, such as fermentation, is carried out to increase the nutritional value of soybeans in fish (Voorhees *et al*., 2019).

Meanwhile, livestock and aquaculture industries compete for soybean with humans because it is a staple diet in some regions. This problem is worsened by soybean growers worldwide experiencing yield reductions due to climate change and variation (Sharma *et al*., 2022). Additionally, crop cultivation for animal feeding necessitates deforestation, which likely destroys forest biodiversity and necessitates primary ecosystem management services (Foley *et al*., 2011). As a result, the food sector is predicted to be threatened by rising prices of soybean and possibly supply disruptions (Khusro *et al*., 2012). Therefore, apart from SBM, some alternative plant-based protein sources have been evaluated as potential protein replacements in animal feed, including groundnut oil cake, canola meal, sunflower meal, cottonseed meal, distillery by-products, brewery by-products, corn gluten, and sugar industry by-products/wastes (Shukla *et al*., 2019).

1.5.4.3 Insect Meals

With the limitations on plant meal sources, it is vital to look for alternatives. In this sense, the use of insects in the diet of aquatic animals could be an alternative. Insect-derived animal proteins are the most likely candidates to meet the search for alternative protein sources in animal production. Insects have become an important food source for many types of animals whereby feeding insects, especially fish and birds, occurs more quickly, owing to their habit of eating insects. Many nutritious insects are available worldwide, and their production has less impact on the environment than other conventional animal protein sources. Besides, they have high feed conversion efficiency, a short life cycle period and superior protein and fat

contents (Oonincx *et al*., 2010; van Huis *et al*., 2013). The chemical makeup of edible insects reveals a wide variety of available nutrients depending on species, lifestyles, types, diets, and developmental stages (Ramos-Elorduy *et al*., 2002). Table 4 shows the nutrient composition, such as protein and fat in several insects compared to FM and SBM. Insects have been used to substitute partially for conventional protein sources such as FM and SBM in the poultry and aquaculture feed industries (Makkar *et al*., 2014; Stamer, 2015). Several studies have indicated that insect-derived feed sources can replace the conventional protein and fat ingredients used in feed formulations without adverse effects on animal performance and product quality (Anand *et al*., 2008; Sealey *et al*., 2011). Available evidence suggests that insect-based feeds result in fish performance comparable to fish fed with FM, FO, or soybean-based diets (Dabbou *et al*., 2018; Belghit *et al*., 2019; Rawski *et al*., 2020).

Housefly maggots have been used as a supplement in the diets of tilapia (*Oreochromis niloticus*) (Ezewudo *et al*., 2015) and African catfish (*Clarias gariepinus*, *Heterobranchus longifilis*) and its hybrid (Adewolu *et al.*, 2010; Ossey *et al*., 2012). The results showed that housefly maggots could substitute up to 30% of FM without affecting the fish performance (Adewolu *et al*., 2010; Ossey *et al*., 2012). Another study by Wang *et al*. (2017) reported that the housefly maggot meal could replace FM by up to 75% in Nile tilapia (*Oreochromis niloticus*) feed without any adverse effect on fish performance. Ogunji *et al*. (2021) determined the growth and production of male and female gonads in a study on *Clarias gariepinus* fingerlings by partly replacing FM with housefly maggot meal. Their study found that feeding fish with maggot-meal enhanced the diets, facilitating growth and accelerated gonadal development. According to Aniebo *et al*. (2009), fish fed with maggot meal-based diets containing 0%, 50%, and 100% FM displayed no substantial variations in African catfish (*Clarias gariepinus*) growth and nutrient consumption indices. The performance of catfish fingerlings fed with earthworm (*Eisenia fetida*) and maggot-based (*Musca domestica)* diets showed better

growth than the control diet (FM). Monebi and Ugwumba (2013) suggested that replacing FM with earthworm meal at a 50 to 75% inclusion level was adequate for optimal growth performance and nutrient utilisation of *Heteroclarias* fingerlings. Substituting FM with cricket meal demonstrates that the latter can be used as an additional protein source in the diet of farmed African catfish without affecting their body structures or feed consumption. However, fish fed with 100% cricket meal as a protein source showed a slightly lower feed conversion ratio than fish fed with lower inclusion levels of the cricket meal (Taufek *et al*., 2018).

The larvae and pupae of the mealworm beetle (*Tenebrio molitor*) have been studied extensively, primarily for feeding fish and birds. This insect has been selected due to its high-quality protein, short-lived, easy-to-breed and highly palatable (Ramous-Elorduy et al., 2002; FAO, 2013). Mealworm (MW) has been used to replace FM in a variety of fish diets, including African catfish (*Clarias gariepinus*) (Dedeke *et al*., 2013), tilapia (*Oreochromis niloticus*) (Sánchez-Muros *et al*., 2016), gilthead seabream (*Sparus aurata*) (Piccolo *et al*. 2017), and European seabass (*Dicentrarchus labrax L.*) (Gasco *et al*. 2016). The weight gain of rainbow trout (*Oncorhynchus mykiss*) was not compromised when fed with FM-substituted diets containing mealworm meal at 50% (Belforti *et al*., 2015) and 14% (Jeong *et al*., 2020). Overall, the actual volume of MW meal that can be incorporated into the feed without impairing fish output depends on the quality of the MW meal, which varies according to the diet composition and rearing conditions, as well as the specific nutritional requirements of a particular fish species (Jeong *et al*., 2020).

Table 4: The protein and fat content of selected insect species compared to fishmeal and soybean meal.

Species	Crude Protein (%)	Crude Fat (%)	Reference
Metamasius spinolae (cactus weevil)	69.1	17.4	Sánchez-Muros *et al*., 2014
Rhantus atricolor (cactus weevil)	71.1	6.4	
Drosophila melanogaster (fruit fly)	70.1	12.6	
Sphenarium histrio (grasshopper)	74.8	8.6	
Boopedon flaviventris (grasshopper)	76.0	8.4	
Melanoplus mexicanus (grasshopper)	77.1	4.2	
Musca domestica (House fly, larva)	60.0	20.0	Hussein *et al*., 2017
Gonimbrasia belina (caterpillar larvae)	57.0	10.0	Siulapwa *et al*., 2012
Ruspolia differens (grasshopper)	44.6	49.0	
Hermetia illucens (black soldier fly larvae)	50.5	36.4	Liu *et al*., 2018
Allomyrina dichotoma (beetle larvae)	54.1	44.2	Ghosh *et al*., 2017
Teleogryllus emma (cricket larvae)	55.7	25.1	
Tenebrio molitor (mealworm larvae)	53.2	34.5	
Rhynchophorus phoenicis (palm weevil)	50.1	21.1	Jonathan, 2012
Fishmeal	61.9	8.9	Ween *et al*., 2017
Soybean meal	45.10	7.9	Karr-Lillenthal *et al*., 2006.

1.5.5 The Black Soldier Fly

Hermetia illucens (Linnaeus, 1758), the black soldier fly, is a fly (Diptera) belonging to the *Stratiomyidae* family (Figure 10). This insect originates from America but is found worldwide in tropical and temperate regions, including Asia, Europe, and the South-East of the United States (Cicková *et al*., 2015; Sideris and Tsagkarakis, 2017). The fly is a non-pest insect or vector for transmitting diseases to humans and animals but rather

is known as an organic waste converter (Diener *et al*., 2009; Lalander *et al*., 2015; Nguyen *et al*., 2015) and as a source of animal food supplements for fish (Bondari and Sheppard, 1981), swine (Ipema *et al*., 2021), poultry (de Souza *et al*., 2021) and pet food (Bosch and Swanson, 2020). The black soldier fly larvae (BSFL) are sometimes referred to as latrine larvae, which naturally occur around poultry, pigs, and cattle manure piles. Black soldier fly (BSF) can solve environmental problems regarding handling manure and other organic wastes by reducing manure mass, moisture content and belligerent odours (van Huis *et al*., 2013). BSF can be used as a biological control for houseflies (*Musca domestica*) since BSF can make manure more liquid and inhibit housefly oviposition (Sheppard *et al*., 1994). BSFL, on the other hand, can modify manure microflora by reducing harmful bacteria such as *Escherichia coli* 0157: H7 and *Salmonella enterica* (Erickson *et al*., 2004) due to containing an antibacterial peptide such as defensin-like peptide 4 secreted by fungi (Li *et al*., 2017), which makes organic farming safer (Banks *et al*., 2014). BSF technology also plays a role in biodiesel production, chitin utilisation and its derivatives in medical, pharmaceutical, food processing, and packaging cosmetics, textiles, and agricultural products (Bondari and Sheppard, 1987; Wang and Shelomi, 2017).

Kingdom Animalia (Animals)
Phylum Arthropoda (Arthropods)
Class Insecta (Insects)
Order Diptera (Flies)
Family Stratiomyidae (Soldier flies)
Genus *Hermetia*
Species *illucens* (Black Soldier Fly)

Figure 10: The taxonomic classification of the black soldier fly, *Hermetia illucens,* as reported by Linnaeus (1871).

Hermetia illucens can convert a wide variety of organic materials derived from plants (Manurung *et al*., 2016), animals (Wang and Selomi, 2017), humans (Banks *et al*., 2014) and food waste (Nyakeri *et al*., 2017; Wang and Shelomi, 2017) into edible biomass (Wang and Selomi, 2017). Larvae are considered high-value feedstuffs because of their superior protein content and well-balanced critical amino acid profiles comparable to FM (Barroso *et al*., 2014; Makkar *et al*., 2014; Henry *et al*., 2015; Liland *et al*., 2017). Due to its superior nutritional qualities, BSFL has been suggested as a viable candidate to substitute costly conventional protein and fat sources such as FM and SBM in poultry, aquaculture, and livestock feeds (Makkar *et al*., 2014; Sánchez-Muros *et al*., 2014; Liu *et al*., 2017). According to Nyakeri *et al*. (2017), BSFL was an alternative source of low-cost protein in poultry and aquafeeds that may be invaluable and significant to meet the rising global meat demand, which was projected to double (58%) by 2050 relative to 2005 (Tilman *et al*., 2011).

1.5.5.1 The Life Cycle of Black Soldier Fly

A growing BSF experiences successive mounting over five instar stages, namely egg, larva, prepupa, pupa and adult stage (Song *et al*., 2021), as shown in Figure 11. The five instar stages can be between two weeks to six months, depending on the availability of food supply and environmental conditions, before transforming into pupae (Tomberlin *et al*., 2009). However, Ferrarezi *et al*. (2016) reported that the whole life cycle of a BSF is about 45 days under perfect conditions. The prepupa stage lasts two to three weeks before the prepupae moult into pupae (Ferrarezi *et al*., 2016). The colour change from creamy to dark grey indicates the transformation process of prepupae to pupae respectively. The adult BSF is black and wasp-like, measuring 15 – 20 mm long (Sheppard *et al*., 2002).

The optimal temperature for rearing black soldier flies is between 26 – 27°C, and relative humidity is 60 – 70% (Barragan-Fonseca *et al*., 2017). While mating and oviposition can occur at temperatures ranging from 24 to 40°C (Sheppard *et al*., 2002), the ideal temperature for mating is around 27.7°C (Zhang *et al*., 2010). The larvae become inactive when the temperature is less than 10^{o}C. Mating occurs two days after emergence, and oviposition occurs four days after fertilisation (Tomberlin and Sheppard, 2002). Besides environmental temperature, relative humidity plays a vital role in rearing black soldier flies, with the best moisture conditions between 30 and 90% (Sheppard *et al*., 2002). Higher relative humidity in the environment makes the feed too wet, an unconducive condition for larvae to have adequate oxygen, consume the feed substrate and even crawl on the feed substrate.

A BSF produces about 500 to 1200 creamy white-colour eggs of approximately 1 mm in length. The hatching process is relatively slow and may take about four days (102 to 105 hours) to complete at 24°C (Diclaro II and Kaufman, 2009). Under laboratory temperature conditions of about 27°C, egg development to the prepupae stage takes about 22 – 24 days or approximately 40 - 43 days from eggs to adults (Tomberlin *et al*., 2002). Newly hatched larvae are light cream; at this stage, they consume feed at a great quantity and speed. Larvae can reach up to 27 mm in length and 6 mm in width and weigh up to 220 mg in the last larval stage, depending on the diet given (Salomone *et al*., 2017). The larval stage has been demonstrated as the most successful phase in turning feed substrates into biomass (Nguyen *et al*., 2015; Manurung *et al*., 2016; Dortmans *et al*., 2017). The high digestibility and superior protein content, coupled with low chitin level make larvae a potential alternative to replace FM as a protein source in animal feed (Nguyen *et al*., 2015; Park, 2015; Spranghers *et al*., 2017). Fully grown larvae produce biomass that contains 40 – 45% protein and up to 35% fat on a dry matter basis (Sheppard *et al*., 1994; Stamer, 2015).

The larvae are referred to as the last cycle of BSF before metamorphosis or the transformation into an adult fly (Dortmans *et al*., 2017). At this stage, the larvae have not yet hardened like the pupae or prepupae stage, which contains high chitin levels, and is often considered an anti-nutritional factor in animal feeding (Diener *et al*., 2009). The creamy colour becomes darker before transforming into prepupae (Diener *et al*., 2009; Dortmans *et al*., 2017). On the other hand, prepupae is the final larval stage that emerges from the feed substrate (usually a wet substrate) to search for a dark and dry place to pupate (Tomberlin *et al*., 2009; Diener *et al*., 2011). Compared to larvae, prepupae have a higher chitin concentration which is less digestible by fish and chicken (Dortmans *et al*., 2017). Adults (flies) do not feed on substrates except water because they do not have functional mouthparts, which have evolved into hook-like organs used for moving. Their energy needs are met solely by the fats stored during the larval stage (Newton *et al*. 2005; Diener *et al*., 2011; Li *et al*., 2011).

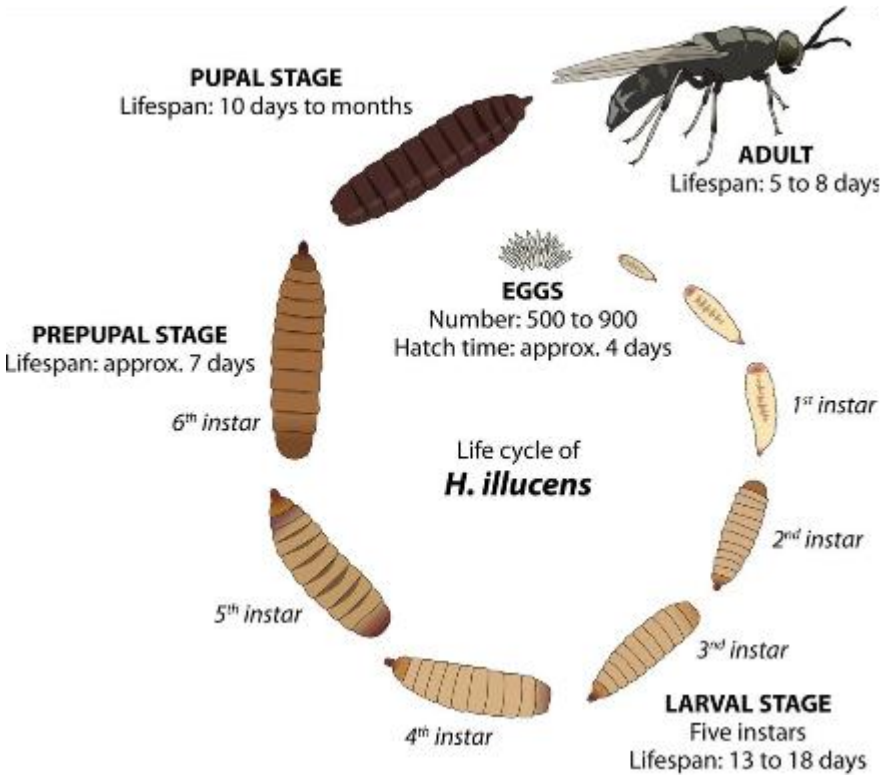


Figure 11: The lifecycle of a black soldier fly.

1.5.6 Black Soldier Fly Larvae Feed Substrates

BSFL is known to feed on various biological wastes due to its strong digestive tract and voracious appetite, and thus has the potential to transform large volumes of organic waste into highly valuable larvae biomass (Bondari and Sheppard, 1987). Different materials with different nutrient compositions can be used as feed substrates to produce larvae with varying nutritional values (St-Hilaire *et al*., 2007; Diener *et al*., 2009; Spranghers *et al*., 2017; Shumo *et al*., 2019, Table 5). In the production of BSFL, the selection of feed substrates is generally based on the supply, ease of collection, water quality and cost of the materials (Bava *et al*., 2019). Therefore, most of the work has focused on food waste and manure, as these materials are always available and abundant, contributing to environmental pollution (Diener *et al*., 2011; Lalander *et al*., 2013). Brewers' spent grain (Shumo *et al*., 2019), vegetable waste and tofu waste (Nguyen *et al*., 2015; Kinasih *et al*., 2018), and cassava peel (Rahmi *et al*., 2020) were some of the plant-based organic wastes used for BSFL rearing, and animal-based materials included animal manure (Myer *et al*., 2008; Banks *et al*., 2014), animal remains (Tomberlin *et al*., 2005) and fish offal (St-Hilairie *et al*., 2007a). By-products from agro-industry are commonly used to rear BSFL due to the larvae's ability to convert various types of organic waste into valuable larvae biomass and nutrient-rich organic fertilizer (Beesigamukama *et al*., 2020). Agro-industrial by-products (AIBPs) are obtained mainly from the plant processing industries such as grain and oilseed milling, breweries, fruit and vegetable canneries and animal-based industries including whey, fishmeal, meat, and bone meal. The residues or by-products from these processing plants contain a high nutrient composition and constitute a vast potential supply of animal feed, some of which are currently underutilised.

Enhancing the substrate's absorption capacity and fortifying dietary components by the combination of two or three substrates is currently used in the production of BSFL (Rehman *et al*., 2017; Julita *et al*., 2018; Lim *et al*., 2019). Dietary changes can increase the nutrient constituent of larvae and produce high-quality larvae (Liland *et al*., 2017; Barragan-Fonseca *et al*., 2018; Wong *et al*., 2019) because the nutritional composition of BSFL is influenced by the substrate used (Tschirner and Simon, 2015). For instance, increasing the proportion of high-protein waste in the feed substrate may improve larvae protein content (Lim *et al*., 2019). High-quality feed substrates improve larvae development, feed conversion ratios, and insect survival (de Haas *et al*., 2006). In order to manage surplus by-products and compensate for nutrient deficiencies in any substrates, mixing two ingredients in a mixed feed substrate may provide an alternative method (Bonelli *et al*., 2020; Chia *et al*., 2020; Li *et al*., 2022). For example, combining soybean curd residue and kitchen waste, for instance, significantly increased larval yield, bioconversion rate, and bioaccumulation of lipids (Julita *et al*., 2018). Similarly, adding vegetable waste to horse manure improves the performance, development, and nutritional content of the black soldier fly (Li *et al*., 2022). The time larvae transform into prepupae is faster than on a single feed substrate. Lalander *et al*. (2019) reported that it took nearly 30 days for larvae raised on fruits and vegetables to reach the pupal stage. The larvae reached the prepupal stage in 12 days when the feed substrate was combined with abattoir waste. Combining substrates rather than a single feed substrate is better because the larvae perform better, whereby they can be collected faster, with less volume of feed substrate used, which makes the process cheaper.

Table 5: The impacts of different feed substrates on the crude protein and fat contents of black soldier fly larvae or pre-pupae.

Feed Substrates	Stage	Crude Protein (%)	Crude Fat (%)	References
Liver	Larvae	62.7	25.1	Nguyen *et al*., 2015
Fruit and vegetable		38.5	6.63	
Fish		57.9	34.6	
Food waste	Prepupae	43.7	31.8	Surendra et al., 2016
Reutealis trisperma fruit	Prepupae	37.6	33.2	Manurung *et al*, 2016
Mixture of middlings	Larvae	37.2	13.5	Tschirner and Simon, 2015
Dried distillers' grains		44.6	4.8	
Dried sugar beet pulp		52.3	22.9	
Chicken feed	Larvae	41.2	33.6	Spranghers *et al*., 2017
Fruit waste	Prepupae	30.8	40.7	Meneguz *et al*., 2018
70% vegetable and 30% fruit waste		41.9	26.3	
Brewery by-product		53.0	29.9	
Winery by-product		34.4	32.2	
Brewer waste	Larvae	49.9	33.7	Liu *et al*., 2018
Pig manure		45.9	17.5	
Semidigested grass		46.4	5.51	
Chicken manure	Larvae	41.1	30.1	Shumo *et al*., 2019
Kitchen waste		33.0	34.3	
Spent grain		41.3	31.0	
Fermented coconut waste	Larvae	18.6	42.7	Mohd Noor *et al*., 2018
Horse manure and vegetable	Larvae	46.6	13.0	Julita *et al*., 2018
Maize straw	Larvae	41.76	-	Gao *et al*., 2019
Wheat bran		42.74	-	
Putrified *Sesbania grandiflora* leaves	Prepupae	43.50	16.70	Kuppusamy *et al*., 2020

1.5.7 Factors Affecting Black Soldier Fly Larvae Performance and Nutrition

Various studies have shown variability in the performance and nutritional composition of BSFL, with some reportedly doing better than others. For example, BSFL fed on agricultural by-products (Nguyen *et al*., 2013; Manurung *et al*., 2016; Kinasih *et al*., 2018; Mohd Noor *et al*., 2018), faecal materials (Li *et al*., 2011; Banks *et al*., 2014), and chicken feed (Diener *et al*. 2009; Gobbi *et al*., 2013) showed superior nutritional composition. Researchers and breeders who worked with BSFL used all the available materials in the local area without precise guidelines for managing this insect. Raising BSF on biowaste and organic side-streams with different nutritional qualities produced larvae with various growth efficiencies and nutrient composition (Tschirner and Simon, 2015; Liland *et al*., 2017; Tinder *et al*., 2017). Proteins and carbohydrates are essential nutrients for insect growth, survival, and reproduction (Aguila *et al*., 2013). Crude fats are also essential since the BSFL store lipids during their development for metamorphosis and adult stages (Arrese and Soulages, 2010). Feed substrates influence the nutrient composition, namely protein, fat, ash, and fatty acids of BSFL (Oonincx *et al*., 2015a; Liu *et al*., 2017; Müller *et al*., 2017; Chia *et al*., 2020; Ewald *et al*., 2020). The larvae have been shown to imitate the nutrient composition of feed substrates for various macro- and micro-nutrients (Barroso *et al*., 2017; Barragan-Fonseca *et al*., 2018; Wong *et al*., 2019). Larvae fed on various substrates had variations in body CP and fat contents ranging from 37.0 to 62.7% and 6.6 to 39.2% on a DM basis, respectively (Barragan-Fonseca *et al*., 2017). These variations indicate that the nutrient quality of the substrates influences the protein and fat content of the larvae. Feed substrates containing low protein and energy levels impeded larval development, while optimal growth was observed with a more balanced diet (Galassi *et al*., 2021). These results indicate that the protein content of the larvae's diet is very crucial for its development.

On the other hand, life phases also influence the nutritional content of BSF. There are a considerable number of variations in the nutrient composition of the larvae during the development period. For example, the CP decreased with age, with the highest concentration of 61% recorded from 5-day old larvae, while the lowest content of 44% and 42%, respectively, were registered from 15- and 20-day old larvae (Rachmawati *et al*., 2010; Nguyen *et al*., 2015; Oonincx *et al*., 2015b). The DM of larvae is highly dependent on the types of feed substrate increased with larval age and which is reported to be higher in later instars due to the formation of chitin (Rachmawati *et al*., 2010; Nguyen *et al*., 2015). The nutrient composition of feed substrates also affects the development time of larvae. Larvae took less time to harvest on high-protein than on low-protein diets (Oonincx *et al*., 2015b). On the contrary, Gobbi *et al*. (2013) discovered that larvae reared on meat meals containing approximately 50% CP took longer to complete their developmental stages than those raised on poultry diets (14.5% CP). This outcome could be attributed to the texture of the meat meal, which is quite dense and thus reduces larval food intake. Feeding BSFL with rice straw takes a longer time to develop into prepupae from days 38 to 54 (Manurung *et al*., 2016a) due to high amounts of cellulose, hemicellulose, and lignin (Sarnklong *et al*., 2010). Mixing the substrate with bacteria accelerates the breaking down of cellulose and hemicellulose. More nutrients can be added to the substrate with the aid of microbes, thus further improving the BSFL conversion performance (Zheng *et al*., 2012).

The performance of larvae also depends on feeding rates. Diener *et al*. (2009) concluded that a daily rate of 100 mg chicken feed per larva was optimal for the development, whereby larvae transformed into to prepupae within a short time. Larvae performance on the feed substrate varies with the optimal amount of food required. The difference in nutrient content in feed substrates causes the optimal feeding rate for larvae growth and development to differ for most substrates. In low-nutrient feed substrate, larvae consume more to meet their nutritional requirements. Environmental

conditions such as temperature, humidity and light intensity affecting the BSFL growth (Barragan-Fonseca *et al*., 2017; Shumo *et al*., 2019). Temperature and humidity significantly impact the maturation, reproduction, and spawning of BSF (Tomberlin *et al*., 2009). The moisture level of the feed substrate is an important factor that affects larval development. Moisture contents between 50 and 80% are ideal for larval development, final weight, feed conversion efficiency, and yield (Cammack and Tomberlin, 2017; Cheng *et al*., 2017; Abduh *et al*., 2018). Substrates with moisture contents of 90–97.5% cause lower survival rates and body weights of larvae (Lalander *et al*., 2020). However, the protein content of the BSFL diet is crucial for larval development and survival (Song *et al*., 2021; Seyedalmoosavi, *et al*., 2022). It appears that BSFL on substrates with higher protein content (22% of DM) achieves higher larval weight, greater bioconversion and feed conversion ratio, increased larval protein and lipid content, and shorter developmental time (Nguyen *et al*., 2013; Oonincx *et al*., 2015a; Tinder *et al*., 2017). However, high protein levels, such as 37%, appear to be detrimental, as survival and adult emergence are hampered.

1.5.8 Bioconversion Technology of Black Soldier Fly

Bioconversion refers to the ability of living organisms such as earthworms, insect larvae or microorganisms to convert organic waste into biomass, an energy such as methane and inorganic compounds (Barry, 2004; Newton *et al*., 2005). Insects can break down vast quantities of organic waste and excrement into various commodities, such as insect biomass of proteins, lipids, pharmaceutical products, biofuels, lubricants, and fertilisers (Fowles and Nansen, 2019). As a result, insect-based bioconversion services provide marketable solutions for reducing food waste that is modest in space and energy requirements, environmentally sustainability, and generates higher feed conversion ratios than conventional animals such as cattle, pigs and poultry (van Huis

and Oonincx, 2017). Developing an insect-based bioconversion technique is an excellent strategy for reducing food waste (Nyakeri *et al*., 2017; Wang and Shelomi, 2017). The larvae of BSF (*Hermetia illucens*) have been suggested as one of the best insects for the bioconversion of organic waste into valuable biomass (Surendra *et al*., 2016; Wang and Shelomi, 2017). BSFL is a saprophagous and polyphagous species with numerous enzyme activities, including amylase, lipase, and protease, which allow it to utilise a range of feeding materials (Kim *et al*., 2011). These enable a wide range of organic waste to be converted from low-value organic resources into high-quality nutrients for animal feed, such as protein and fat sources (Veldkamp *et al*., 2012; Nguyen *et al*., 2013; Bosch *et al*., 2019). Bioconversion has been observed to reduce municipal and household waste materials by about 50 to 79% (Diener *et al*., 2011; Lalander *et al*., 2013; Banks *et al*., 2014). Salomone *et al*. (2017) reported that food-waste materials of about 10 tonnes could produce 300 kg of dried larvae and 3,346 kg of compost. Although the accumulation and bioconversion of nutrients from substrates to BSFL are not well determined and documented, it is thought to depend on the types, quantity, and quality of waste materials (Diener *et al*., 2011) as well as environmental conditions (Paz *et al*., 2015).

The bioconversion process by *H. illucens* is a very appealing choice as it provides a possible solution to problems such as food waste management and the rising worldwide demand for alternative ingredients such as dried larvae for aquaculture production (Salomone *et al*., 2017). Organic waste conversion using BSFL as a nutrient recycling technology can produce animal feed and even biofuel on a large scale (Surendra *et al*., 2016). This technology makes the rearing of BSF a promising activity for smallholders who may grow the larvae as a feed source for animal proteins at a low investment cost by recycling organic waste in a surrounding area (Diener *et al*., 2011). In addition to producing larval biomass, producing frass fertiliser or residue from bioconverting as a secondary value-added product from BSF rearing would increase

insect farmers' profit margins. Feeding the BSFL with waste streams could become more valuable than soya meal and FM because this insect can generate organic compost and energy (biogas) for food, whereas biogas has the potential to replace fossil fuels (Makkar *et al*., 2014; Smetana *et al*., 2019; Wong *et al*., 2019; Hasnol *et al*., 2020). Li *et al*. (2011) reported that 1 kg of cattle manure produced about 127.6 g (DM) of BSFL with a lipid content of 38.2 g. From the amount of lipids, 35.6 g of biodiesel, equivalent to a 93% yield, can be extracted. Using BSF larvae to bio-convert feed might prove environmentally friendly and economically viable when feeding grains to animals becomes very expensive.

BSFL are voracious feeders that can convert carbohydrates and protein-rich substrates very rapidly. It can effectively convert tonnes of organic food waste into tonnes of larvae protein and organic fertiliser (Nguyen *et al*., 2015; Beesigamukama *et al*., 2020). The bioconversion process depends on the type of organic waste, environmental factors, and the microorganisms present in the substrates (Gao *et al*., 2019). The nutrient composition of feed substrates changed after the BSFL bioconversion. The proportion of crude protein (CP) and crude fat (Cfat) decreases, whilst nitrogen-free extracts (NFE) increase significantly in the organic substrate at the end of the bioconversion process. For instance, the initial amount of CP of 11.10% in maize straw dropped to 3.16%, a decrease in Cfat content from 1.97 to 0.30%, and an increase in NFE from 56.34 to 62.48% after the completion of the bioconversion process (Gao *et al*., 2019). The considerable decrease in CP and Cfat of the substrates during bioconversion indicates that larvae have fully used these nutrients due to the low nutrients of this material, increasing NFE. In another study, bioconversion yielded nitrogen and phosphorus residues in 30 – 50% and 61 – 70%, respectively (Diener *et al*., 2009; van Huis *et al*., 2013), implying that organic waste was transformed into high-quality organic fertilisers. Furthermore, bioconversion removes the bad odours of

organic waste and produces methane gas by anaerobic decomposition during the conversion of waste materials (Perednia *et al*., 2017).

1.5.9 Black Soldier Fly in Aquafeed

Interest in using insects as animal feed to replace conventional protein sources, either FM or SBM, has increased in the last decade (FAO, 2013). Although insects are yet to be used in commercial fish feed processing, they show tremendous potential in aquaculture feed (Makkar *et al*., 2014; Tran *et al.*, 2015). However, most experiments have shown that BSFL meals can partially substitute FM in aquafeed (Fawole *et al*., 2020; Madibana *et al*., 2020; and Rawski *et al*., 2021). Only a few studies demonstrated that using BSFL meals as a full substitute for FM is possible. BSFL can be incorporated as a fish feed ingredient in various forms, such as live, chopped, dried, or ground (Bondari and Sheppard, 1987; Makkar *et al*., 2014). The feeding trials have demonstrated that FM replacement in fish was potential at low to moderate inclusion levels of BSFL. At these levels of BSFLM, no adverse effects of BSFLM on fish performance or feed conversion ratio are observed (Magalhães *et al*., 2017; Renna *et al*., 2017; Zhou *et al*., 2017). It is also possible to state that the average amount of BSFLM in the reported feed trials was less than 200 g/kg of the fish diet. In African catfish (*Clarias gariepinus*), slower growth is seen when the amount of BSFLM rises above 50%, or 115 g/kg of BSFLM in the diet (Fawole *et al*., 2020). Higher inclusion levels ranging from 120 to 300 g/kg significantly slowed growth in channel catfish, rainbow trout, and turbot (Newton *et al*., 2005; St-Hilaire *et al*., 2007; Kroeckel *et al*., 2012). A higher level of BSFL has been linked to problems with the health of the intestines and the occurrence of stress in fish (Li *et al*., 2017; Xu *et al*., 2020). Different fish species performed differently in the feeding trials using BSFL because of gut microbiota in fish (Terova *et al*., 2019; Weththasinghe *et al*., 2022). Diet shapes gut

microbiota which can improve fish nutrient utilisation, growth, and health (David *et al*., 2014; Carmody *et al*., 2015). Fish-fed diets containing full-fat insect meals had a unique gut microbiota composition dominated by beneficial lactic acid bacteria and *Actinomyces*, demonstrating a predicted increase in mucin degradation (Weththasinghe *et al*., 2022). Replacing FM with *Hermetia* prepupae meal (HM) in juvenile turbot (*Psetta maxima*) diets showed impaired feed consumption, which decreased with an increasing amount of HM in the diet due to poor palatability (Kroeckel *et al*., 2012). On the other hand, it was also due to lower protein digestibility when the inclusion level of HM in the feed approached 330 g/kg, affecting the growth rates of fish. Low digestibility of nutrients in fish fed with BSFL due to harvesting age of larvae. Late harvesting of larvae exhibiting the presence of chitin, the major substance forming exoskeleton in the cuticle of insects (Siddiqui *et al*., 2022), that inhibits nutrient digestibility and feed utilisation by fish, although the chitin has other benefits that cannot be denied (Surendra *et al*., 2020; Purkayastha and Sarkar, 2021). However, Belghit *et al*. (2019) discovered that BSFLM completely replaced 100% FM at a 150 g/kg diet with no adverse effects on market-size salmon's growth performance or sensory quality. Likewise, substituting FM with 100% BSFLM in Jian carp diets equivalent to 140 g BSFLM/kg of diet had no detrimental effects on the fish growth rate, as observed by Zhou *et al*. (2017). One of the reasons that allow 100% FM replacement is because the larvae used are harvested at a very young age, eight days, when the presence of chitin is very low and may be absent (Belghit *et al*., 2019).

Renna *et al*. (2017) reported replacing FM with *Hermetia illucens* (HI) meal at 40% in rainbow trout (*Oncorhynchus mykiss Walbaum*) did not have any adverse effects on fish physiology or fillet physical appearance but lowered the levels of valuable polyunsaturated fatty acids (PUFAs) in fish. Most of the fatty acids (FAs) in BSFL fat are saturated fatty acids, especially lauric acid (C12:0), which makes up to 52% of all fatty acids (Spranghers *et al*., 2017; Ewald *et al.*, 2020). Lauric acid has a beneficial regulatory

function against breast, colon, and endometrial cancer cells despite being considered unfavourable for human consumption due to its effect on low-density lipoprotein (LDL) cholesterol levels and, consequently, cardiovascular disease (Briggs *et al*., 2017; Lappano *et al*., 2017). As a result, much research into the use of BSFL in aquafeed is required to ensure that the quality of the fish produced is safe and free of side effects. The low concentration of PUFAs needed by fish which are supposed to supply through diet is lacking. Additionally, an analysis of the FAs indicated lower omega-3 fatty acids in the whole body or fish muscle in groups substituted with 50% and 100% BSFLM (Zhou *et al*., 2017). Therefore, providing larvae with feed substrate containing high PUFA is necessary to produce larvae with high PUFA value and to the value of BSFL-fed fish. The growth of rainbow trout (*Oncorhyncuss mykiss*) fed with fish offal-enriched black soldier fly diets were not significantly different from fish fed with a fishmeal-based control diet (Sealey *et al*., 2011) due to the BSFL being enriched with omega-3 fatty acids (EPA, DHA, and ALA), which are required for fish growth (St-Hillaire *et al*., 2007a). The reports demonstrate that FM can be replaced with BSFLM, but the larvae quality is crucial to lessen the drawbacks of feeding fish with BSFLM and in order to increase the proportion of BSFLM in the diet.

1.5.10 Protein Conversion Factor in Insect

Recent studies have raised the issue of overestimating insect protein content (Janssen *et al*., 2017; Jonas-Levi and Martinez, 2017; Nery *et al*., 2018). The amount of protein was calculated by multiplying the amounts of free NH_2 groups (Schasteen *et al*., 2007) in the feedstuffs with a specific nitrogen-to-protein (N:P) conversion factor (N-factor), by methods such as the Kjeldahl or Dumas (Saez-Plaza *et al*., 2013; Chromý *et al*., 2015). The Kjeldahl method is widely used to quantify crude protein content in insects which ranges from 8 to 70% of dry mass (Jonas-Levi and Martinez, 2017). The

conventional conversion factor of 6.25 is generally used to estimate the protein content in insects (Yi *et al*., 2013; Spranghers *et al*., 2017) as well as in other foodstuffs (Mariotti *et al*., 2008). The value of 6.25 was used, considering that 1 kg of plant or animal protein contains 160 g N (1000/160 = 6.25) (Sosulski and Imafidon, 1990). However, this factor has been considered high due to the presence of non-protein nitrogen (NPN), particularly in insects (Janssen *et al*., 2017; Nery *et al*., 2018). Hall and Schönfeldt (2013) suggested that the protein content as dictated by total nitrogen is not precise because of the chemical and compositional changes between proteins and the presence of NPN. The new nitrogen-to-protein conversion factor has been determined in three edible insects, namely *Tenebrio molitor, Alphitobius diaperinus* and *Hermetia illucens,* using amino acid analysis. The NPN has been reported to be around 16 – 26% *in Hermetia illucens* (BSFL) and 12 – 23% in *Tenebrio molitor*. Hence, the new conversion factors, of 4.76 and 5.60 for whole larvae and insect, respectively has been introduced and it was thought to be more accurate for protein estimation by taking into consideration the presence of NPN. The fraction of non-protein compounds such as urea, ammonia, nitrate and nitrite, amines, nucleic acids, phospholipids and chitin are considered in the calculation (Mariotti *et al*., 2008; Janssen *et al*., 2017).

Using the new conversion factor, the protein value seemed to be lower by more than 20% compared to the amount of protein reported based on the conversion factor of 6.25 (Janssen *et al*., 2017). The total amino acids in the protein can be used as the actual protein in the samples (Sriperm *et al*., 2011). The value of protein in BSFL was about 36% compared to the range of 37 – 56% in previous reports (Bosch *et al*., 2014; Sanchez-Muros *et al*., 2014). The use of the appropriate nitrogen conversion factor is vital in formulating a diet as it allows feedstuffs to be better reflected on a protein basis (Nery *et al*., 2018). The deviation in CP and actual protein was related to the presence of nitrogen in chitin compounds in the larvae, giving an overestimated crude protein value. Liland *et al*. (2018) and Spranghers *et al*. (2017) estimated the protein

content of prepupae by deducting the presence of chitin in its body of about 2 – 2.5%. The presence of chitin overestimates the amount of protein and has been reported to affect the digestibility of insects even at low inclusion levels in animal feed (Finke, 2007; Jonas-Levi and Martinez, 2017).

CHAPTER 2

SCREENING OF THE NUTRIENT COMPOSITION OF POTENTIAL FEEDSTUFFS FOR FEEDING BLACK SOLDIER FLY LARVAE

2.1 Introduction

Food components suitable for direct human consumption, such as soybeans and fish, are expensive due to food competition (Munguti *et al*., 2014). Furthermore, when aquaculture has increased, overfishing has caused the population of marine fish to decrease over time (Worm, 2016), causing the price of fishmeal (FM) to become expensive (Olsen and Hassan, 2012). In addition, increased soybean cultivation contributes to land grabbing, deforestation, and environmental issues. Usually, the FM used derived from small trash fish is unsustainable, depleting wild fish stocks with dwindling supplies. Therefore, the increasing cost of FM and soybean meal, as well as the need to improve the sustainability of the aquaculture industry, has prompted the search for alternative protein sources for inclusion in fish feed. As consequences, there is an urgent need to replace conventional ingredients with a novel, cost-effective, and ecologically acceptable alternatives. Currently, fish feed producers worldwide are interested in incorporating insect meals into fish diets. Furthermore, insects are valuable repositories of proteins, amino acids, fatty acids, minerals, and energy (Nowak *et al*., 2016). Each insect-based meal to complement conventional ingredients in a fish diet should have a high nutrient value and be available at low prices. The black soldier fly (BSF) is the preferred insect because it can convert low-quality organic waste into protein- and fat-dense biomass. A wide range of feeding substrates has been shown to produce nutritious black soldier fly larvae (BSFL) with high biomass (Veldkamp *et al*., 2012; Surendra *et al*., 2016; Wang and Shelomi, 2017). BSFL can survive and thrive on

decaying organic materials (Diener *et al*., 2011; van Huis *et al*., 2013), and food industry waste streams with highly diet-dependent parameters such as development time, mortality, larval weight, and nutrient composition (Zhou *et al*., 2013; Shumo *et al*., 2019).

Nonetheless, not all organic materials are suitable for BSF production in a controlled environment. Therefore, the quality and quantity of the product must be determined by appropriate production substrates (St-Hilaire *et al*., 2007a). The choice of feed substrates for BSFL rearing is critical and is determined by a country's regulatory structure to address feed and food safety concerns (van Huis and Oonincx, 2017) and avoid unfavourable public perceptions. Since BSFL are considered to be "farmed animals" (Reg. (EC) 1069/2009), they are only permitted to be reared on feed ingredients that are approved for use in farmed animals, exclusively plant-based substrates (Lalander *et al*., 2020). Presently, there is no legislation in Asian countries, including Malaysia, governing insects as animal feed or feedstock substrates for insect rearing. Hence, numerous organic wastes have been utilised and studied to evaluate their capacity to produce larvae with high nutritious value (Shumo *et al*., 2019; Holeh *et al*., 2022). Thus, underutilised plant species *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG), as well as agro-industrial by-products (AIBPs) such as soybean waste (SBW), wheat pollard (WP), rice bran (RB), and milk-extracted coconut meat (MECM) were chosen to assess their potential as feed substrates for rearing BSFL. Previously, the insect farming sector has opted for vegetable based BSFL feedstuffs such as macroalgae (Biancarosa *et al*., 2015; Liland *et al*., 2017), rice straw (Manurung *et al*., 2016), semi-digested grass (Liu *et al*., 2018), seaweed (Swinscoe *et al*., 2019), and *Sesbania grandiflora* (Kuppusamy *et al*., 2020). However, larval performance varies according to the nutrient content of these materials. The underutilised plant species were chosen for their nutritional availability and quality, particularly proteins and minerals (Bhat and Karim, 2009). In comparison, AIBPs are preferred as BSFL feed

substrates due to their acceptability, low cost, and availability in the local market. Thus, the Phase 1 of the objective to analyse the nutrient content of six feedstuffs for use as potential feed substrates for rearing BSFL was addressed in this chapter.

2.2 Materials and Methods

2.2.1 Description of the Raw Materials

Two underutilised plant species, *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG) (Figure 12), together with four AIBPs, namely, i) soybean waste (SBW) ii) wheat pollard (WP) (Figure 13) iii) rice bran (RB) and iv) milk-extracted coconut meat (MECM) (Figure 14), were used in the present study to determine their potential as feed substrates for the production of BSFL. SBW consists of the insoluble parts of soybeans collected from the tofu production plants following the water extraction process. SBW is a by-product of soymilk production and is generally white or yellowish. It commonly dumps directly into the water sewer and creates environmental pollution. SBW contains 28% crude protein, 23% hemicellulose, and 16% cellulose (Ratnadewi *et al*., 2016). While MECM is the coconut (*Cocos nucifera* L.) endosperm tissue residue following milk extraction (Yong *et al*., 2009). The nutrients in MECM were very low, most of which were lost during milk extraction but were still high in lipids. The waste is usually fed to animals such as chickens or livestock or dumped in a landfill.

WP is a by-product of the wheat flour milling of grain plants containing bran, a mixture of the seed coat or outer layer of the cereal grain and wheat middlings. WP contains with substantial protein levels and are high in crude fibre (Mwesigwa *et al*., 2013). RB is a by-product of the white rice milling process (Gul *et al*., 2015). In processing polished rice, two steps involve removing the husk to produce brown rice and subsequently milling and polishing to remove the bran (yellowish-brown layer) and

germ to produce white or polished rice. The milling of paddy generates 70% rice (endosperm) as the primary product and 20% husk, 8% bran, and 2% germ as by-products (Van Hoed *et al*., 2006). RB contains high fat and lipase, which destroy rice bran's oil, rendering it rancid and inedible (Gul *et al*., 2015). WP and RB are commonly used as raw materials in the production of animal feeds, whereby they act as natural binders in the pelleting of feed (Saunders, 1986).

The MO and SG leaves were harvested from the Crops For the Future Research Centre (CFFRC) farm at Semenyih, Selangor. The leaves were placed in a cool room and processed 24 hours after harvesting. The MO leaves were separated from the stem and twigs manually. The MO leaves, together with rachis, were used in this study, while for SG, only leaves were used for rearing BSFL.

2.2.2 Sample Preparation

The leaves from three sources of different plants (MO and SG) from the same location (CFFRC) were collected for the analysis. The fresh MO leaves, complete with rachis and SG leaves and AIBP samples such as SBW, WP, RB, and MECM, were dried in an oven at 60°C (Binder, Germany) for two days until they reached constant weight. The dried plant samples were ground in a lab blender (Sumeet, India) and kept at 4°C in a refrigerator before chemical analysis was performed. All analyses were carried out in triplicate by standard laboratory methods according to the Association of Official Analytical Chemists (AOAC).

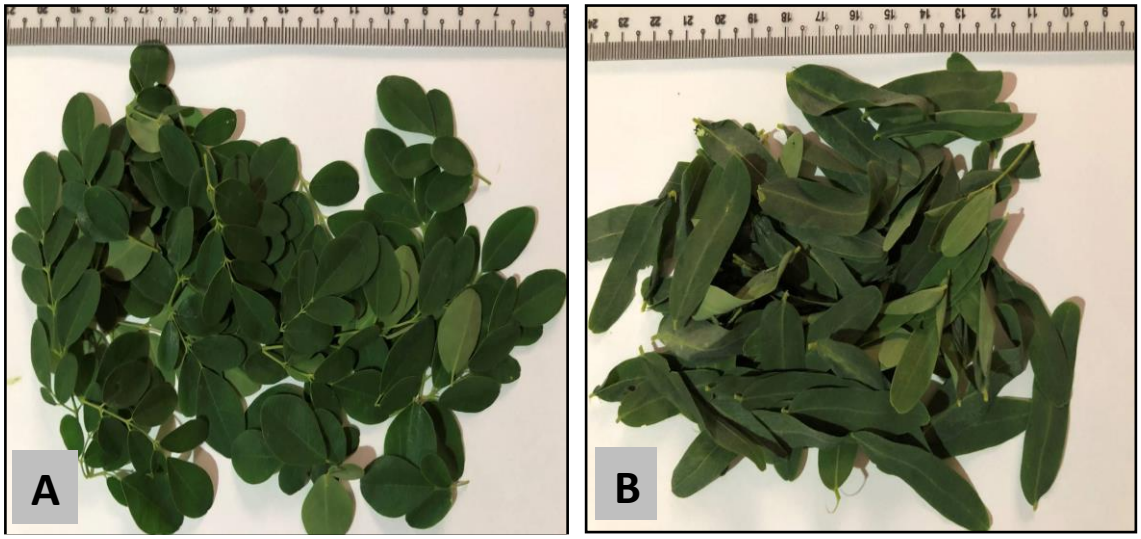


Figure 12: *Moringa oleifera* leaves and rachis (A), *Sesbania grandiflora* leaves (B).

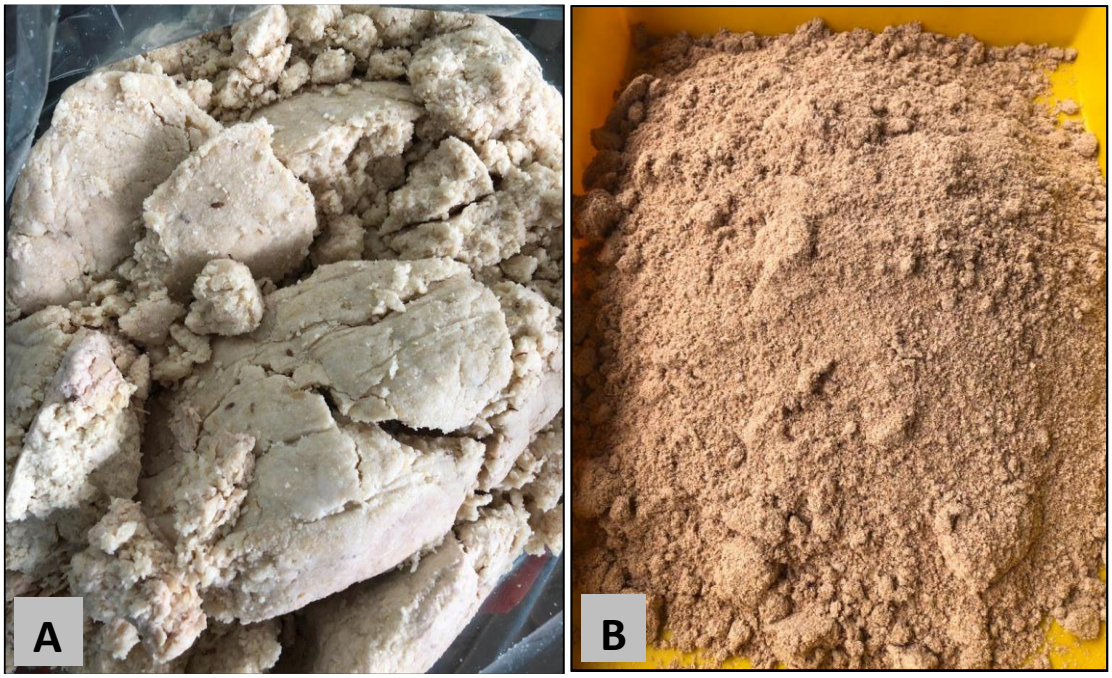


Figure 13: Soybean waste (A) and wheat pollard (B).



Figure 14: Rice bran powder (A) and milk-extracted coconut meat (B).

2.2.3 Proximate Analysis

2.2.3.1 Dry Matter and Ash Content

The procedure for dry matter (DM) was performed following Method 967.03 (AOAC, 1990) with slight modifications, while for ash, according to Method 942.05 (AOAC, 1990). Exactly 2 g of sample (MO and SG leaves, SBW, WP, RB, and MECM) ($W_1 g$) was placed in a crucible with a known predetermined empty crucible weight ($W_2 g$). Crucibles with samples were dried at 105°C for overnight until a constant weight was achieved. Sample-containing crucibles were cooled in a desiccator and the weight recorded ($W_3 g$). The DM was calculated using Formula 1. The crucibles were incinerated in a furnace (Nabertherm, Germany) at 550°C for 5 h until the formation of

carbon-free ash. After ashing, the crucibles were removed from the furnace and placed in a desiccator for further cooling. Samples were reweighed to determine the weight of crucibles and ash (W_4g). The ash content was calculated according to formula (2). All analyses were carried out in triplicate.

Formula (1)

$$Dry\ matter\ (DM, g/100g) = \frac{W_3g - W_2g}{W_1g} \times 100$$

$$Moisture\ \left(\frac{g}{100g}\right) = 100 - DM$$

Formula (2)

$$Ash\ \left(\frac{g}{100g}\right) = \frac{W_4g - W_2g}{W_3g - W_2g} \times 100$$

where W_1g = fresh sample, W_2g = empty crucible, W_3g = dried sample plus crucible after heating at 105°C, W_4g = weight of the crucible + sample after heating at 550°C.

2.2.3.2 Crude Protein

The Kjeldahl method (Sáez-Plaza *et al*., 2013) was used to measure the crude protein (CP) content of samples (Method 984.13; AOAC, 1990). The method has three stages: digestion, distillation and titration, following the outlined procedure by the manufacturer (Gerhardt GmbH, Germany). Duplicates of dried samples (0.5 g) were placed in Kjeldahl digestion flasks. Two flasks were left blank as controls. Two catalyst tablets (Kjeltabs Cu/3,5, Kjeltabs^{TM}) were placed in each flask together with the empty flasks. A volume (20 ml) of 96% (v/v) sulphuric acid (H_2SO_4, Fisher, USA) was added to

each flask, and the samples were transferred into an acid hydrolyser. The flasks were placed on electric coil heaters (Gerhardt GmbH, Germany) in a fume chamber until the colour of the content turned black (200°C for 40 min). The extraction was continued at 400°C for another 2 h. The digestion process lasted for 2 h and 40 min, until a clear pale blue solution without black particles was obtained, indicating that the digestion process was completed. At the end of the digestion process, the flasks were left open to release the acid vapour. Samples were cooled for 1 h and then transferred to a Kjeldahl Distillation apparatus (Vapodest 45s, Gerhardt GmbH, Germany) for the subsequent distillation process.

One digested sample-containing flask and a 200-ml empty Erlenmeyer flask were placed in the distillation apparatus (Vapodest, Gerhardt, Germany). A volume (59 ml) of 2% (w/v) boric acid (R & M Chemicals, UK) was automatically added to the flask from a tank attached to the Kjeldahl apparatus. This was followed by 77 ml of 32% (w/v) sodium hydroxide (NaOH, Fisher Scientific, USA) and 45 ml of distilled water. A stream of water was passed through the flasks until approximately 180 ml of distillate was recovered in the Erlenmeyer flask. The Erlenmeyer flask was taken out of the distillation apparatus for titration. Four drops each of bromocresol green (Sigma-Aldrich, USA) and methyl red (Sigma-Alrich, USA) were added to the distillate as an indicator, followed by titration with the standard 0.1 N hydrochloric acid solution, HCl (Titrisol®, Merck, Germany). The HCl was automatically titrated until the green colour of the distillate changed to violet. The volume of HCl used was recorded to calculate the protein content based on formula (3). The total nitrogen of feed substrates was multiplied with different conversion factors for different substrates to obtain the value of CP. The specific conversion factors used were 6.25 for SG and MO (Kumar *et al*., 2017), 5.71 for SBW, 6.31 for WP, 5.95 for RB, and 5.30 for MECM (FAO, 1986). In addition, the control samples in triplicate of known nitrogen and protein values, ammonium ion (II) sulphate ($(NH_4)_2Fe(SO_4)_2.6H_20$, Chem*AR*®, Systerm®) were carried out

simultaneously to ensure the efficiency of the distillation. A 0.2 g of $(NH_4)_2Fe(SO_4)_2.6H_20$ was placed in a Kjeldahl digestion flask and directly distilled, and the distillate was then titrated with 0.1 N HCl, a conversion factor was used to calculate the CP content.

Formula (3)

$$Nitrogen\left(\frac{g}{100g}\right) = \frac{\left((T - B) \times 14.007\right)}{Weight\ of\ sample\ (g)} \times 100$$

$$Crude\ protein\left(CP, \frac{g}{100g}\right) = Nitrogen \times F$$

$$CP\left(\frac{g}{100g}\ DM\ basis\right) = \frac{CP}{DM} \times 100$$

where T = titration volume of sample (ml), B = titration volume of blank (ml), N = normality of NaOH, F = conversion factor of nitrogen to protein, DM = total dry matter.

2.2.3.3 Crude Fat

The total crude fat (Cfat) of samples was determined by ether extract analysis (Method 920.29; AOAC, 1990), following the manufacturer's procedure by Soxtec™ 8000 Extraction Unit (FOSS GmbH, Germany). Two grams of oven-dried sample (W_1g) was transferred to cellulose thimbles (33 mm × 80 mm, CT3380 Favorit®). The thimbles were covered with cotton wool and placed in the condenser of the Soxhlet extractor flask. One hundred millilitres (100 ml) of petroleum ether (J.T. Baker, USA) were pipetted into each pre-weighed aluminium extraction cup (W_2g). The digestion and

extraction processes lasted approximately 70 min. After the extraction process, the Cfat in the cup was oven dried at 60°C for 30 min and cooled in a desiccator before weighing the net weight of the extract (W_3g). All samples were measured in duplicate. The Cfat content was calculated using the formula (4):

Formula (4)

$$Crude\ Fat\ \left(Cfat, \frac{g}{100g}\right) = \frac{W_3g\ -\ W_2g}{W_1g}\ \times\ 100$$

$$Cfat\ \left(\frac{g}{100g}DM\ basis\right)\ = \frac{Cfat}{DM}\ \times\ 100$$

where W_1g = weight of the sample, W_2g = weight of the empty cup, W_3g = weight of cup after the oven, DM = total dry matter.

2.2.3.4 Crude Fibre

The crude fibre (CF) of samples was measured using a fully automatic crude fibre analyser, Fibretec™ 8000 (FOSS GmbH, Germany), according to Method 978.10 (AOAC, 1996). Cellite (1 g) and 1 g of dried sample (W_1g) was weighed and placed in the pre-weighed crucible (W_2g). Crucibles were transferred to the Cold Extraction Tecator System. Acetone (25ml) was added to cover the top of the sample, and the valve was placed in a "vacuum" position to extract and filter the solvent. The sample-containing crucible was left for 5 min to dissolve the fat in the samples. Crucibles were removed from the acetone and transferred to a crucible stand, left at room temperature until the acetone evaporated from the sample.

The crucibles were inserted into the Hot Extraction Tecator System platform and locked into position. The manufacturer's instructions were followed. There are two buttons in the system, namely R1 (Reagent 1) containing 96% (v/v) H_2SO_4 (Fisher Scientific, USA) and R2 (Reagent 2) containing 1.25% NaOH (Fisher Scientific, USA). Initially, the R1 button was pressed to allow 150 ml of H_2SO_4 to fill the flasks. When the reagent started to boil, the heater was adjusted to moderate boiling and boiled for 30 min. Subsequently, the flasks were washed with hot water three times using the water spray device. This step was followed by pressing the R2 button to allow 150 ml of preheated 1.25% NaOH (Fisher Scientific, USA) into the flasks. The same procedure was repeated as above when R1 was inserted into the flasks. The crucibles were removed from the system and heated in an oven at 105°C for 3 h. The crucibles were transferred to a desiccator and weighed after cooling (W_3g). The crucibles were placed in a furnace at 550°C for 5 h (Nabertherm, Germany), and reweighed after cooling in a desiccator (W_4g). The CF was calculated according to formula (5):

Formula (5)

$$Crude\ Fibre\ \left(CF, \frac{g}{100g}\right) = \frac{(W_3g - W_4g - W_2g)}{W_1g} \times 100$$

$$CF\ \left(\frac{g}{100g}\ DM\ basis\right) = \frac{CF}{DM} \times 100$$

where W_1g = weight of the sample, W_2g = weight of the crucible, W_3g = weight of the crucible + sample after heating at 105°C, W_4g = weight of the crucible + sample after heating at 550°C, DM = total dry matter.

2.2.3.5 Neutral Detergent Fibre

The neutral detergent fibre (NDF) content of samples was measured using the FiberCap 2023 analyser (FOSS GmbH, Germany) according to the manufacturer's procedure (Method 2002:04, AOAC 2006). All samples were measured in duplicate. One gram of ground sample ($W_1 g$) was placed in a known predetermined empty capsule and cap weight ($W_2 g$). The sample-containing capsules were inserted into a short holder with eighteen holes and placed in a beaker filled with acetone until the sample was levelled and soaked for 5 min. Meanwhile, 1000 ml (1 L) of NDS solution (Appendix 1) was added to a 2-L beaker and heated on a hot plate 2022 (FOSS GmbH, Germany) until boiling. The holder with eighteen capsules was transferred into an NDS beaker and heated for 30 min. Subsequently, approximately 525 ml of the hot NDS was poured out and a new NDS solution was added to a final volume of 1000 ml and reboiled. The holder and capsules were transferred again to a boiled NDS and heated for another 30 min. The holder and capsules were taken out from the beaker and washed with hot water three times, soaked in acetone for 5 min and air-dried for 5 min to remove the acetone. The capsules were heated in an oven for 3 h at 105°C and cooled in a desiccator before weighing ($W_3 g$). The capsule was placed in an empty crucible with a known predetermined weight ($W_4 g$). The crucible containing the capsule and a sample was placed in a furnace at 550°C for 5 h (Nabertherm, Germany) and reweighed after cooling in a desiccator that contained ash ($W_5 g$). The NDF was calculated according to formula (6):

Formula (6)

$$Neutral\ Detergent\ Fibre\ \left(NDF, \frac{g}{100g}\right) = \frac{W_3 g - (W_2 g \times C) - (W_5 g - W_4 g - D)}{W_1 g} \times 100$$

$$NDF\left(\frac{g}{100g}DM\ basis\right) = \frac{NDF}{DM} \times 100$$

where W_1g = Weight of sample, W_2g = Weight of capsule and cap, W_3g = weight of capsule and cap + sample after heating at 105°C, W_4g = weight of empty crucible, W_5g = Weight of crucible + capsule and cap + sample after heating at 550°C, D = average ash of capsule and cap, 0.001, C = empty capsule and cap correction, 0.992 (running average of final oven-dried weight/original blank capsule and cap weight), DM = total dry matter.

2.2.3.6 Acid Detergent Fibre

The acid detergent fibre (ADF) of samples was measured using the FiberCap 2023 analyser (FOSS GmbH, Germany) according to the manufacturer's procedure based on Method 973.18 (AOAC, 1990). All samples were measured in duplicate. A 1 g sample of feed substrate (W_1g) was placed in a pre-weighed empty capsule and cap weight (W_2g). The capsules were inserted into a short holder containing eighteen holes. The holder with eighteen capsules was put in a beaker, filled with acetone to submerge the samples, and soaked for 5 min. Meanwhile, 1000 ml (1 L) of ADS solution (Appendix 2) was added to a 2-L beaker and boiled on a hot plate 2022 (FOSS GmbH, Germany). When the solution reached boiling point, the holder with eighteen capsules was transferred into a beaker and heated for 30 min. Subsequently, approximately 525 ml of the hot ADS was poured out and replaced with a similar amount of new ADS solution to reach 1000 ml and boiled. The holder and capsules were removed from the ADS beaker and washed three times with hot water. The holder and capsules were transferred again in a boiled ADS and heated for another 30 min. The holder and capsules were removed from the beaker and washed with hot water three times, then

soaked in acetone for 5 min and air-dried for 5 min to remove acetone. The capsules were heated in an oven for 3 h at 105°C and cooled in a desiccator before weighing (W_3g). The capsule was placed in an empty crucible with a known predetermined weight (W_4g). The crucible containing the capsule and a sample was placed in a furnace at 550°C for 5 h (Nabertherm, Germany) and reweighed after cooling in a desiccator that contained ash (W_5g). The ADF was calculated according to the formula (7):

Formula (7)

$$Acid\ Detergent\ Fibre\ \left(ADF, \frac{g}{100g}\right) = \frac{W_3g\ -\ (W_2g\ \times C)\ -(W_5g\ -W_4g\ -D)}{W_1g}\ \times\ 100$$

$$ADF\ \left(\frac{g}{100g}DM\ basis\right)\ =\ \frac{ADF}{DM}\ \times\ 100$$

where W_1g = weight of the sample, W_2g = weight of the capsule and cap, W_3g = weight of the capsule and cap + sample after heating at 105°C, W_4g = weight of empty crucible, W_5g = weight of crucible + capsule and cap + sample after heating at 550°C, D = average ash of capsule and cap, 0.001, C = empty capsule and cap correction, 0.992 (running average of final oven-dried weight/original blank capsule and cap weight), DM = total dry matter.

2.2.3.7 Gross Energy

The gross energy (GE) of all samples was determined using a bomb calorimeter (IKA C6000, Germany). All samples were measured in duplicate. A total of 0.5 g of the dried sample was placed in a combustion crucible. A cotton thread was prepared for the

ignition wire and placed on the sample. This is critical for successful contact with the sample. The crucible was then closed tightly with a decomposition vessel before being transferred to the machine for automatic detection using an RFID reader. The result was displayed on the screen.

2.2.4 Amino Acid

2.2.4.1 Acid Hydrolysis Method

All samples were screened for the presence of seventeen amino acids (AAs) (alanine, arginine, aspartic acid, cysteine, histidine, isoleucine, glutamic acid, glycine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, proline, and valine). Amino acid concentrations were determined using an amino acid analyser (Biochrom 30, UK) according to the acid hydrolysis procedure described in 1998/64/EC (Anon, 1998). All samples were analysed in duplicate. Powdered samples (0.05 g) of feed substrates were placed in test tubes, and 15 ml of 6N HCl (Fisher Scientific, USA) added to each sample. The tubes were placed on a heater block for 23 h at 110°C. The cap of each tube was loosened for the first hour and tightened for the next 22 h. The hydrolysate was poured into a beaker and titrated by adding 2 N NaOH and 3 N HCl until the pH reached 2.2. The volume of NaOH and HCl added to each sample was recorded. The hydrolysate was stirred and filtered through a PVDF filter (Econofltr 13m 0.2 µm, Agilent Technologies, USA) into an amino acid vial (12 x 32 mm with 9 mm screw, Cronus) using a 5-ml syringe. Vials containing 1 ml of filtrate and amino acid standard (200 µl amino acid standard, (AAS18-5ML, Sigma-Aldrich, USA) diluted with 800 µl of AA buffer) were run through an amino acid analyser. The peaks of each AA were read and analysed by comparing them with the amino acid standard using the Ezchrome Elite software programme.

2.2.4.2 Alkaline Hydrolysis Method

The acid hydrolysis procedure destroys tryptophan, hence was measured using the alkaline hydrolysis method according to the Waters AccQTag Ultra chemistry method (Waters Corporation, Totowa, NJ, USA) and analysed using Waters Acquity Ultra Performance Liquid Chromatography (UPLC; Waters Corporation, APC™, Milford, MA, USA). Analyses were carried out in duplicate. A sample (0.100 g) was placed in a screw-capped tube containing 5 ml of 6 N HCl. The reaction tubes were nitrogen flushed for 15 min and sealed by a screw cap. The tubes were incubated for 22 h at 112°C on a heater block and left at room temperature for cooling. The internal standard (10 ml of 2.5 mM α-aminobutyric acid) was added to the cooled hydrolysate mixture, transferred to a 250 ml volumetric flask, and filled the meniscus with MilliQ® water. Approximately 0.5 ml of the mixture was filtered with a 0.45 µm acid-compatible filter. An aliquot (10 µl) of the filtrate was mixed with 70 µl of AccQ•Fluor™ derivatisation buffer and 20 µl of AccQ•Fluor reagent to derivatise. The sample was inserted into an autosampler vial and heated at 55°C for 10 min in a heating block. A sample of 5 µl was injected into the HPLC machine (Waters, USA) to determine the tryptophan content. HPLC System: 626 LC System with heater 717plus Autosampler with heater/cooler 474 Scanning Fluorescence Detector Millennium® Chromatography Manager. The AccQTag column with a Nova-Pak® C18 Sentry™ Guard column was used to separate the AA.

2.2.5 Fatty Acid

The fatty acid (FA) analysis was performed using the direct methylation method by Wang *et al*. (2000). A lipid sample (0.1 g) was placed in a 15 ml test tube, and 1 ml of methanol was added. The mixture was neutralised by adding 3 ml of 3N methanolic-HCl and vortexed. An internal standard consisting of 2 mg/ml (w/v) of heneicosanoic acid

(C21:0, Sigma-Aldrich, USA) dissolved in methanol (Fisher Scientific, USA) was used to determine the amount of FA. A total of 200 µl of C21:0 was added to the mixture, and the tubes were heated in a water bath for one hour at 95°C (Isotemp, Fisher Scientific, USA) to complete the methylation process before being cooled at room temperature. Subsequently, 8 ml of 0.88% (w/v) sodium chloride (NaCl, Fisher Scientific, USA) and 3 ml of n-hexane (Fisher Scientific, USA) were added to the fatty methyl esters (FAME) and mixed well by vortexing.

The FAME samples were separated by centrifugation at 3,000 g for 5 min in a Kubota 2000 centrifuge (Interscience, Germany), forming three layers. One ml of the hexane containing FAME in the top layer was pipetted out and transferred into a vial (P/N: 226-54111-41, Shimadzu, Japan) to analyse FAs. For this purpose, 1 µl of FAME was injected into the injection port of gas chromatography, GC Clarus 500 (Perkin Elmer, USA) equipped with an autosampler. The FAME was analysed using GC software fitted with a flame ionisation detector. Helium was the carrier gas with a flow rate of 0.5 ml/min. The oven was programmed to have an initial temperature of 140°C for 5 min, the first ramping rate at 4°C /min at 240^{o}C and hold for 10 min. The injector temperature was at 225^{o}C while the detector temperature was set at 250°C. The gas flow mixture consisted of 450 ml of compressed air and 45 ml of hydrogen. The total run time for each sample was 40 min. A fused silica capillary column (30 m long x 0.25 mm internal diameter, 0.25 µm thickness, Crossbond® polyethylene glycol, FAMEWAX column; Restek Corporation) was used to separate the esters. A split injection type was used at a split ratio of 100:1. The peaks were identified by comparing the relative retention times of FAME peaks in the samples with those of the standard FAME (Supelco, Sigma-Aldrich, USA). The percentage of each FA was calculated by dividing the retention area of the identified peak by the total retention area of all peaks present in the sample.

2.2.6 Statistical Analysis

Analysis of variance (ANOVA) using general linear model (GLM) procedure of the Statistical Analysis System Inc. Version 9.4 (2013) was carried out to determine the significant differences between the nutrient composition of six different feedstuffs including moisture, ash, crude protein (CP), crude fat (Cfat), crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF), gross energy (GE), and amino acid (AA) profile. Due to differences in the way the data were distributed, the fatty acid (FA) profile could not be processed for statistical analysis. The Tukey's honest significant difference (HSD) test on SAS software was used when a significant treatment effect was observed to compare means between the feed substrates. Differences were statistically significant when $p < 0.05$. All data were expressed as means ± standard error of the mean (SEM) in tables.

2.3 Results

2.3.1 Nutrient Composition of Feedstuffs

The nutrient composition of all potential substrates (SG, MO, SBW, WP, RB, and MECM) was determined before being used to rear BSFL. The most important condition for BSFL is moisture content and other nutrient contents, including protein and fat (Ma *et al*., 2018; Meneguz *et al*., 2018; Lim *et al*., 2019; Nyakeri *et al*., 2019), besides the environmental condition. These factors influence the development rate and the nutritional value of the BFSL.

2.3.1.2 Moisture and Ash

The moisture content of the six substrates ranged from 4.38 to 76.57 g/100g (Table 6) and differed significantly ($p < 0.0001$) according to substrate type. The highest moisture was found in SG, MO, and SBW, followed by MECM, WP and RB. The differences in moisture content of the substrates are due to the inherent difference in the type of substrate and the pre-processing of the agro-industrial by-products. WP and RB contained less than 6 g/100g moisture since these materials were processed in the dried form. While SG, and MO were collected and processed directly from fresh leaves.

The ash in MO was observed to be significantly the highest, and MECM was the lowest ($p < 0.0001$). SG, SBW, and RB had a similar ash content of about 6.5 g/100g DM. The lowest range of ash in MECM at 1.27 g/100g DM might be due to the milk

extraction process because the nutrients were mostly found in the endosperm tissue where the coconut milk was extracted.

2.3.1.3 Crude Protein and Amino Acid Profiles

Table 6 shows the CP contents varied significantly amongst the feedstuffs. The CP content was significantly high ($p < 0.0001$) in MO, SG, and SBW, ranging between 27 – 31 g/100g DM as high-protein feedstuffs, moderate in WP and RB at around 12 g/100 DM, while MECM had the least amount of CP at less than 4 g/100g DM (low-protein). Table 7 shows that the amino acid profiles in leaf meals and AIBPs differed considerably ($p < 0.05$). Feedstuffs can be divided into three groups depending on their AA content. Amongst the feedstuffs evaluated, MO, SG, and SBW had the highest total AA content, average in WP and RB, and lowest in MECM. The total essential amino acids (EAAs) in MO were 12.26 g/100g, and SG was 11.19 g/100g DM, while SBW, WP, RB, and MECM had 7.86 g/100g, 3.90 g/100g, 4.40 g/100g, and 0.92 g/100g DM, respectively. Among the non-EAAs, glutamic acid was prevalent in all feedstuffs except MECM, with SBW containing the highest concentration of around 4.26 g/100g DM. Leucine levels were more significant among the EAA, particularly in MO and SG ($p < 0.0001$). Tyrosine, an EAA, was abundant in leaf meals, SG, and MO but deficient in AIBPs, particularly MECM.

2.3.1.4 Crude Fat and Fatty Acid Profiles

Based on crude fat content, there were significantly different ($p < 0.0001$) among feedstuffs. The crude fat (Cfat) of feedstuffs can also be divided into three types;

high-fat content in MECM and RB was significantly higher compared to the other feedstuffs with values of more than 11 g/100g, while leaf meals, MO and SG, were moderate fat content (3.2 – 3.9 g/100g DM), SBW and WP were considered to be low in Cfat with values of less than 3 g/100g. The FA profiles (Table 8) indicated that polyunsaturated FA (PUFA), such as linolenic acid, was found at a high proportion in SG (46.9% of total FA), MO (43.9% of the total FA), and WP (36% of the total FA). SBW contained the highest linoleic acid, 37.1%, among the feedstuffs, followed by WP at 30.7%. The next most abundant FA was palmitic acid recorded in SG, MO, and SBW at 23.9%, 31.9%, and 21.4%, respectively. Linoleic acid was higher in SG than MO; however, trans-oleic acid was not detected in these feedstuffs. MECM exhibited the highest lauric acid, myristic acid, and pentadecanoic acid among feedstuffs at 43.8%, 22.6%, and 12.2%, respectively. Nevertheless, MECM lacked polyunsaturated fatty acids such as linoleic and linolenic acid. While WP revealed an excellent level of these polyunsaturated FAs, which accounted for 67.7% of the total FAs in this feed. Meanwhile, RB has the most saturated fatty acid, lauric acid (26.2%), and palmitic acid (18.3%). It was high in linoleic acid (11.2%) and low in linolenic acid (1.31%).

2.3.1.5 Crude Fibre and Components

The result showed that MECM had the highest ($p < 0.0001$) CF, NDF, and ADF content, which were recorded at 26.2 g/100g, 82.9 g/100g and 58.3 g/100g DM basis, respectively. Other substrates, such as RB and MO had significantly lower ($p < 0.0001$) content of CF of less than 10 g/100g DM. SG, SBW, and WP were moderate in CF content ranging from 11.2 to 16.1 g/100g DM. SG contained the second highest ADF of 31 g/100g DM, followed by SBW (27.6 g/100g DM), and the lowest was in MO leaves of around 12.2 g/100g DM.

2.3.1.6 Gross Energy

The gross energy (GE) of six feed substrates is considerably different ($p < 0.0001$), with the MECM, SBW and SG containing considerable amounts of GE of around 19 MJ/kg DM, followed by MO at 17.8 MJ/kg DM. In contrast, WP and RB were the least around 15.5 MJ/kg DM.

Table 6: The nutrient composition of selected substrates used for rearing black soldier fly larvae.

Substrate/ Nutrient Composition	***Sesbania Grandiflora***	***Moringa oleifera***	**Soybean Waste**	**Wheat Pollard**	**Rice Bran**	**Milk-extracted Coconut Meat**	***p* -value**
Moisture	76.57 ± 0.23^{a}	76.03 ± 0.06^{a}	73.91 ± 0.43^{b}	5.70 ± 0.13^{d}	4.38 ± 0.13^{e}	40.08 ± 0.43	< 0.0001
Ash	6.53 ± 0.05^{b}	11.96 ± 0.87^{a}	6.48 ± 0.89^{b}	5.01 ± 0.03^{c}	6.48 ± 0.85^{b}	1.27 ± 0.08^{d}	< 0.0001
Crude Protein	27.15 ± 0.09^{b}	30.82 ± 0.04^{a}	27.89 ± 0.04^{b}	12.61 ± 1.53^{c}	12.15 ± 0.61^{c}	3.73 ± 0.23^{d}	< 0.0001
Crude Fat	3.22 ± 0.05^{c}	3.85 ± 0.12^{c}	2.02 ± 0.10^{e}	2.85 ± 0.10^{d}	11.86 ± 0.26^{b}	14.83 ± 0.04^{a}	< 0.0001
Crude Fibre	12.33 ± 0.32^{c}	8.06 ± 0.36^{d}	16.07 ± 1.18^{b}	11.22 ± 0.10^{c}	6.58 ± 0.73^{d}	26.18 ± 0.46^{a}	< 0.0001
Neutral Detergent Fibre	47.11 ± 0.45^{bc}	43.84 ± 1.22^{c}	50.13 ± 1.50^{b}	49.65 ± 0.67^{b}	30.03 ± 0.49^{d}	82.87 ± 0.21^{a}	< 0.0001
Acid Detergent Fibre	30.99 ± 2.91^{c}	12.21 ± 1.17^{c}	27.57 ± 1.29^{b}	16.67 ± 0.87^{c}	13.38 ± 1.25^{c}	58.27 ± 1.94^{a}	< 0.0001
Gross Energy	18.56 ± 0.05^{b}	17.84 ± 0.09^{c}	19.00 ± 0.09^{b}	15.51 ± 0.02^{d}	15.63 ± 0.11^{d}	19.70 ± 0.09^{a}	< 0.0001

^{abcde}Means ± standard error of the mean (g/100g DM) (n = 3) except for moisture in g/100g and gross energy is expressed in MJ/kg DM. The same row followed by different superscripts are significantly different at $p < 0.05$.

Table 7: The amino acid profiles of selected substrates.

Amino Acid (g/100g DM)	SG	MO	SBW	WP	RB	MECM	*p* – value
Alanine	1.55 ± 0.07^{ab}	2.25 ± 0.24^{a}	1.66 ± 0.31^{ab}	0.66 ± 0.00b^{c}	0.83 ± 0.02^{c}	0.13 ± 0.01^{c}	0.0014
Aspatic acid	2.68 ± 0.08^{ab}	2.83 ± 0.02^{a}	2.56 ± 0.07^{b}	0.94 ± 0.00^{c}	1.16 ± 0.01^{c}	0.22 ± 0.00^{d}	<0.0001
Glutamic acid	3.19 ± 0.19^{b}	3.64 ± 0.05^{ab}	4.26 ± 0.18^{a}	2.34 ± 0.02^{c}	1.97 ± 0.02^{c}	0.62 ± 0.03^{d}	<0.0001
Glycine	1.31 ± 0.02^{ab}	1.53 ± 0.06^{a}	1.18 ± 0.06^{b}	0.71 ± 0.01^{c}	0.66 ± 0.02^{c}	0.12 ± 0.01^{d}	<0.0001
Proline	1.32 ± 0.08^{ab}	1.51 ± 0.06^{a}	1.26 ± 0.00^{b}	0.77 ± 0.01^{c}	0.64 ± 0.00^{c}	0.11 ± 0.01^{d}	<0.0001
Serine	0.89 ± 0.00^{b}	1.02 ± 0.04^{a}	1.01 ± 0.01^{ab}	0.47 ± 0.00^{c}	0.52 ± 0.01^{c}	0.09 ± 0.01^{d}	<0.0001
Tyrosine	1.22 ± 0.04^{a}	1.24 ± 0.01^{a}	0.87 ± 0.04^{b}	0.48 ± 0.01^{c}	0.53 ± 0.01^{c}	0.15 ± 0.00^{d}	<0.0001
Arginine*	1.92 ± 0.34^{ab}	2.33 ± 0.23^{a}	1.81 ± 0.01^{ab}	1.31 ± 0.01^{bc}	1.42 ± 0.03^{ab}	0.43 ± 0.02^{c}	0.0009
Histidine*	0.84 ± 0.18^{a}	0.82 ± 0.13^{a}	0.65 ± 0.01^{a}	0.44 ± 0.00^{ab}	0.43 ±0.01^{ab}	0.08 ± 0.00^{b}	0.005
Isoleusine*	0.93 ± 0.03^{b}	1.13 ± 0.02^{a}	0.80 ± 0.06^{b}	0.30 ± 0.03	0.33 ± 0.01^{c}	0.09 ± 0.00^{d}	<0.0001
Leucine*	2.25 ± 0.11^{a}	2.48 ± 0.02^{a}	1.76 ± 0.06^{b}	0.71 ± 0.01^{c}	0.85 ± 0.01^{c}	0.15 ± 0.01^{d}	<0.0001
Lysine*	1.60 ± 0.04^{a}	1.56 ± 0.05^{a}	1.13 ± 0.02^{b}	0.52 ± 0.00^{c}	0.58 ± 0.01^{c}	0.13 ± 0.01^{d}	<0.0001
Methionine*	0.36 ± 0.02^{ab}	0.42 ± 0.01^{a}	0.24 ± 0.11^{abc}	0.04 ± 0.01b^{c}	0.05 ± 0.01^{bc}	0.01 ± 0.00^{c}	0.0047
Phenylalanine*	1.35 ± 0.04^{b}	1.63 ± 0.01^{a}	1.06 ± 0.02^{c}	0.40 ± 0.00^{e}	0.51 ± 0.00^{d}	0.10 ± 0.00^{f}	<0.0001
Threonine*	1.13 ± 0.31^{a}	1.02 ± 0.02^{a}	0.61 ± 0.02^{ab}	0.30 ± 0.00^{b}	0.33 ± 0.00^{b}	0.07 ± 0.00^{b}	0.0006
Tryptophan*	0.13 ± 0.00^{c}	0.38 ± 0.00^{a}	0.04 ± 0.00^{d}	0.24 ± 0.00^{b}	0.22 ± 0.00^{b}	<0.01^{d}	<0.0001
Valine*	1.38 ± 0.06^{ab}	1.58 ± 0.16^{a}	0.97 ± 0.05^{bc}	0.48 ± 0.00^{cd}	0.56 ± 0.02^{cd}	0.15 ± 0.01^{d}	<0.0001

^{abcdef} Means ± standard error in the same row followed by different superscripts is significantly different at $p < 0.05$. *Essential amino acids.

Table 8: The fatty acid profiles of leaf meals and agro-industrial by-products used to feed black soldier fly larvae.

Substrate/ Composition (% of total FA)	**Type of FA**	**SG**	**MO**	**SBW**	**WP**	**RB**	**MECM**
Capric acid (C10:0)	SFA	-	-	-	-	0.16 ± 0.06	2.03 ± 0.11
Lauric acid (C12:0)	SFA	0.68 ± 0.03	-	0.29 ± 0.01	0.38 ± 0.03	26.19 ± 0.31	43.82 ± 0.22
Myristic acid (C14:0)	SFA	-	-	-	0.25 ± 0.02	8.81 ± 0.34	22.58 ± 0.17
Methyl tetradecanoate (C14:1)	MUFA	-	-	-	-	0.3 ± 0.03	0.82 ± 0.02
Pentadecanoic acid (C15:0)	SFA	-	-	0.93 ± 0.05	0.03 ± 0.01	0.74 ± 0.03	
Pentadecanoic acid (C15:1)	MUFA	-	-	-	-	0.52 ± 0.03	12.16 ± 0.03
Palmitic acid (C16:0)	SFA	23.91 ± 0.12	31.87 ± 0.63	21.44 ± 1.36	23.15 ± 0.25	18.27 ± 0.28	0.34 ± 0.00
Palmitoleic acid (C16:1)	MUFA	1.68 ± 0.11	3.67 ± 0.22	-	-	3.88 ± 0.08	5.57 ± 0.04
Heptadecanoic acid methyl ester (C17:0)	SFA	-	-	-	-	1.15 ± 0.08	-
cis-10-heptadecanoic acid methyl ester (C17:1)	MUFA	-	-	-	-	2.52 ± 0.06	-
Stearic acid (C18:0)	SFA			17.39 ± 0.17	-	14.27 ± 0.10	
Trans-oleic acid (C18:1 trans)	MUFA	10.39 ± 0.02	7.06 ± 0.07	-	5.3 ± 0.10	-	9.23 ± 0.00
cis-oleic acid (C18:1n9c)	MUFA	-	-	16.14 ± 0.75	-	9.84 ± 0.61	2.11 ± 0.01
Linoleic acid (C18:2n6c)	PUFA	15.83 ± 0.38	11.91 ± 0.10	37.07 ± 0.28	30.65 ± 0.38	11.19 ± 0.22	-
Linolenic acid (C18:3n3)	PUFA	46.58 ± 0.22	43.9 ± 0.15	2.21 ± 0.06	36.99 ± 0.99	1.31 ± 0.03	-
Methy linolenate (C18:3n6)	PUFA	-	-	-	-	0.41 ± 0.02	-

continue…

Composition (% of total FA)	Type of FA	SG	MO	SBW	WP	RB	MECM
Arachidic acid (C20:0)	SFA			0.49 ± 0.07			0.89 ± 0.09
Eicosenoic (C20:In9)	MUFA	-	-	-	2.2 ± 0.19	-	-
Methyl 11,14,17-eicosatrienoate (C20:3n3)	PUFA	0.34 ± 0.03	0.21 ± 0.06	-	-	-	0.37 ± 0.04
Eicosapentaenoic acid (C20:5n3)	PUFA	-	-	-	0.54 ± 0.03	-	-
Behenic acid(C22:0)	SFA	0.46 ± 0.05	0.89 ± 0.04	-	-	0.39 ± 0.01	-
Erucic acid methyl ester (C22:1)	MUFA	-	-	0.67 ± 0.02	0.5 ± 0.01	-	-
Tricosanoic (C23:0)	SFA	-	-	1.56 ± 0.10	0.02 ± 0.01	-	-
Methyl lignocerate (C24:0)	SFA	-	-	1.80 ± 0.42	-	0.07 ± 0.01	-
Docosahexaenoic acid (C22:6n3)	PUFA	-	0.9 ± 0.10	-	-	-	-

SFA=saturated fatty acid, MUFA=mono-unsaturated fatty acid, PUFA=polyunsaturated fatty acid.
SG=*Sesbania grandiflora*, MO=*Moringa grandiflora*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

2.4 Discussion

Due to a lack of food in many developing countries and the need to feed 9 billion people by 2050, people all over the world are becoming more interested in insects to get protein (FAO, 2013). Insects provide protein, amino acids (AA), lipids, carbohydrates, vitamins, and trace elements (Chen *et al*., 2009). The substrate that is supplied to insects and the method that is employed to prepare it both influence the nutritional content of the insects as well as the larvae (Makkar *et al*., 2014). As a consequence of this, insect larvae can contain essential nutrients from sources that are not particularly suitable for the nutrition of humans or animals. The Black soldier fly larvae (BSFL) was selected in this study since this insect can be reared on diverse organic waste with varying nutritional composition. However, the BSFL dietary requirements for growth are unclear, but it was reported that the nutritional values of the larvae, including CP, Cfat, minerals, ADF, and NDF, are altered by the rearing substrates. Vitamins are the only nutrients that are not impacted by the substrates (Shumo *et al*., 2019). Thus, it is vital to screen the nutrient composition of the selected materials before they are used as feed substrate for rearing BSFL. In this study, six feedstuffs from leaf meals (SG and MO) and agro-industrial by-products (SBW, WP, RB, and MECM) have been selected and used as feeding substrates, whereby all of them were significantly different in terms of nutrient composition ($p < 0.0001$).

The moisture content of the feed substrate is one of the factors that are important for rearing larvae. The moisture content of the substrate is known to influence the development of BSFL (Cammack and Tomberlin, 2017; Ma *et al*., 2018; Meneguz *et al*., 2018). Larvae choose substrates with a water content between 60 and 80% (Bortolini *et al*., 2020). When larvae are fed a substrate containing more than 90%, they grow, but their performance is reduced (Lalander *et al*., 2020). Moisture lower than 40% caused the performance of larvae slow and stunt (Bortolini *et al*., 2020). A volume of water may be added to feedstuffs such as WP, RB and MECM to prepare for comfortable conditions for larval growth. The high moisture content in

SBW was due to the nature of soymilk processing which used a water extraction process (Redondo-Cuenca et al., 2008). The amount of ash in a sample refers to the residue that remains after all moisture has really been eliminated and all organic components such as fat, protein, carbons, vitamins, organic acid have been burned. The ash content measures the overall quantity of minerals present in biomass (Liu, 2019). Minerals are essential for insect nutrition even though they require minute levels. However, their absence or shortage significantly impacts insect biology such as metamorphosis and behaviour, even when the major nutrient groups are in appropriate amounts and ratios (Ortiz *et al*., 2016).

Most insects have nutritional needs that are qualitatively similar in terms of water, proteins, lipids, carbohydrates, minerals, vitamins, and trace elements (Kraus *et al*., 2019). These substances can be u85tilised8585 by the insects, supplied by symbiotic relationships, or obtained through food (Chapman, 2012). Protein and amino acid intake are critical for proper growth and reproduction. Numerous insects feeding on natural foods have a positive correlation between growth and reproduction and the nitrogen concentration of the food. However, it is inaccurate to presume that more nitrogen is better for the growth and development of the insect (Chapman, 2012). The CP content in SBW displayed the highest CP amongst the AIBPs. The amount of protein in SBW showed that about 30% of the protein in soybean seed, which was 40% (Banaszkiewicz, 2011), was extracted during the tofu processing. The amount of CP in SBW and RB in the present study was similar to that reported by Redondo-Cuenca *et al*. (2008) and Moongngarm *et al*. (2012). In Japan, soybean waste, called okara is used as feedstock, 85fertilise85r or dumped in landfills. Besides that, it has also been used in emulsification, water and fat binding, and foaming properties (Li *et al*., 2013). On the contrary, MECM had a considerably lower CP than the self-fermentation for four weeks of coconut waste in a study reported by Lim *et al*. (2019), which was used as a rearing substrate in their study. On the other hand, the CP content in MO was like that reported by Moyo *et al*. (2011) and Gopalakrishnan *et al*. (2016). Still, it differed from other studies, which found about 22.23 g/100g (Aye and Adegun, 2013) and 24.1 g/100g

(Mendieta-Araica *et al*., 2011). However, the CP content of SG leaves in this study was significantly lower than that of Duke (1983), at about 36.3 g/100g but higher than the values reported by Kumar *et al*. (2017). The variations of this nutrient could be attributed to the genetic background of the plants, environmental conditions including climate, temperature, altitude, soil types and fertility, stage of plant development and time of harvest (Sanchez-Machado *et al*., 2009; Moyo *et al*., 2011; Valdez-Solana *et al*., 2015; Sahay *et al*., 2017). MO has been known to have a high concentration of nutrients, and every part of the tree is edible or commercially viable (Gopalakrishnan *et al*., 2016). MO contains high-quality protein, easily digested, and quality amino acids (Foidl *et al*., 2001). MO and SG have been used as alternative feed in livestock such as goats and sheep (Nhan, 1998; Reyes-Sánchez *et al*., 2006; Ben Salem and Makkar, 2009; Mohammed *et al*., 2012).

Proteins have been the most critical macronutrient affecting BSFL performance (Nguyen *et al*., 2013; Tinder *et al*., 2017). Proteins play many functions in the development of insects, such as hormones, structure, enzymes, and haemoglobin and protein, which are crucial to completing insects' life cycles (Hoffman and Falvo, 2004; Kola *et al*., 2015; Ortiz *et al*., 2016). In addition, proteins can be used as energy, although they are not the primary source (Hoffman and Falvo, 2004). Protein quality is critical in achieving optimal insect biomass output (Purschke *et al*., 2017). High-quality protein contains all the essential AAs generally found in animal protein. High-quality protein is shown by its amino acid profiles and its digestibility, which relate to how well the protein is u86tilised (Hoffman and Falvo, 2004). Bonelli *et al.* (2020) showed that BSFL reared on substrates with high protein and fat content weighed heavier and achieved that weight within a shorter period than those fed on a lesser quality substrate. The high amount of CP in MO, SG, and SBW substrates may produce BSFL with better performance and nutrient composition, particularly in protein content. While the moderate amounts of protein in WP and RB may help larvae to grow.

Analyses of chemical elements such as AA are essential before the feed substrate is used for rearing BSFL to determine the effects of the nutrient composition on harvested BSFL. The amino acid profiles are classified into two groups, namely essential amino acids (EAAs) and non-essential amino acids (non-EAAs). Animals cannot synthesise EAAs and, therefore, must be provided in their diet to ensure the normal functions of the animals, such as physiological activities of cells, tissues, and the entire body, are not compromised (Baker, 2009; Wu, 2014). Most insects need nine to ten EAAs such as arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Kraus *et al*., 2019), and at the same time, deficiency of non-EAAs in diets allowed insects to survive but resulted in delayed larval growth. In adults lacking some AAs, there were no effects on survivorship, sexual maturity, or egg hatch, but the fecundity was significantly reduced (Chang, 2004). The leucine content in MO was higher than that reported in previous studies (Sanchez-Machado *et al*., 2009; Moyo *et al*., 2011). While tyrosine, an EAA, was abundant in leaf meals, SG, and MO but deficient in AIBPs, it is a significant component of sclerotin and is required in considerable amounts throughout the moulting process of insects (Hopkins, 1992). On the contrary, non-EAAs are those that an animal can synthesise on its own, generally from other AAs or other substances, and do not require supplementation in the diet (Li *et al*., 2011). It has been reported that the amount of AAs present in the feed substrate does not influence the concentrations of AAs in BSFL (Shumo *et al*., 2019; El-Dakar *et al*., 2021). Although MECM had the lowest AA concentration, it was feasible to produce larvae containing AA levels equivalent to those fed with MO, SG, and other feedstuffs.

Crude fat (Cfat) in leaf meals, SBW and WP were comparable among the six feedstuffs tested. The Cfat content of SG was much lower than in a study by Duke (1983), whereby their Cfat was 7.5 g/100g, as well as for MO, as reported by Aye and Adegun (2013). SBW contained the least Cfat, while MECM showed the highest. This is due to the fact that the extraction methods have a significant impact on the nutrient composition of the feedstuffs. The greater the amount of

nutrients that are extracted, the fewer nutrients are left in the samples. BSFL retained fat throughout their larval life until they metamorphosed into prepupae and achieved the adult stage (Sheppard *et al*., 1994; Sheppard *et al*., 2002). Prepupae and adults did not feed due to the transformation of their mouthparts into hook-like organs required for movement (Diener *et al*., 2011; Li *et al.*, 2011). As a result, they rely on only the fat stored in their bodies as a source of metabolic energy (Ortiz *et al*., 2016) and save enough protein for growth and development (Sang-Min and Tae-Jun, 2005). On the other hand, fats stored act as a water-conserving barrier in arthropod cuticles (Ortiz *et al*., 2016), generate prolonged periods of flight (Beenakkers *et al*., 1984), and for embryo development (Ziegler and van Antwerpen, 2006). Besides that, it acts as a nutrient transporter, defensive compound, and hormone synthesis (Kraus *et al*., 2019). The Cfat content in the feed substrate influences the fat content of BSFL, according to Nguyen *et al*. (2015). However, there was no discernible influence of the feed substrates on the Cfat content of BSFL (Shumo *et al*. 2019). The high-fat content in the BSFL complicates the feed manufacturing processes, particularly during grinding and pelleting (Briggs *et al*., 1999). A lower fat content would facilitate the commercialisation of BSFL meals (Tschirner and Simon, 2015).

SG and MO were shown to contain a high concentration of polyunsaturated fatty acid (PUFA), such as linoleic and linolenic acid, and a low or absence of lauric acid. A high level of linoleic acid in MO was also reported by Moyo *et al*. (2011), which contradicted Nadeen and Imran (2016), who reported that trans oleic acid was the highest in MO. The linolenic acid content in RB was similarly shown to be low as in Latha and Nasirullah (2014), accounting for only 1.25% of total FAs in RB. MECM, however, contained a high percentage of lauric acid (saturated fatty acid), while other important FA such as PUFA were low. Insects can synthesise most FAs; however, PUFAs, including linoleic and linolenic acids, are required in the diet (Morales-Ramos *et al*., 2014). The FAs content in feed substrate influences the FA composition of insects (van Broekhoven *et al*., 2015). Therefore, BSFL breeders must provide feed with high FA values to tailor the FA quality in BSFL. Insect diets

with more n-3 PUFAs would have higher n-3 PUFA concentrations and lower n-6/n-3 ratios. Even if fed with the same diet, species would differ in their accumulation efficiency and de novo synthesis of FAs, including omega 3 (n-3) and omega 6 (n-6) (Oonincx *et al*., 2020). The availability of high content of linoleic and linolenic acids in all feedstuffs except MECM indicates the usefulness of these materials as feed resources for rearing BSFL. However, the low nutrient density levels in feedstuffs should not be discounted as it might have the potential as feed substrate since BSFL are capable of surviving and producing biomass with high nutrient content compared to their diets (Danieli *et al.,* 2019).

The amount of fibre in the meal influences the larvae's body. Crude fibre (CF) is composed of cellulose with trace amounts of hemicellulose and lignin, as well as fibre fractions such as NDF and ADF, which show the extent and rate of digestion of feedstuffs (Rubanza *et al*., 2005). Archer *et al*. (1997) discovered a negative relationship between cellulose, lignin levels, and feed digestibility which resulting less biomass (Supriyatna *et al*., 2016). Consuming a lot of fibre and ash produced larvae with high fibre and ash content, but it's not necessarily proportionate (Rahmi *et al*., 2020). The energy in diets is also necessary to maintain insect growth, tissue maintenance, and reproduction (Kraus *et al*., 2019). This is due to the difficulty of digesting certain components, which reduces the efficacy of nutritional use (Rehman *et al*., 2017a). High levels of CF can induce intestinal discomfort, poor digestibility, and impaired nutritional utilisation in animals and humans (Jimoh and Oladiji, 2005), which impacts the larvae in this investigation. The digestion of larvae on RB substrate may be more efficient than other NDF-rich feedstuffs, notably MECM. Larvae that fed MECM are probably small because the nutritional profile of this substrate, like the amount of CF, ADF, and NDF, was high. This made larvae fed less DM, and the low amount and quality of protein in this substrate could affect either (Bencini *et al*., 2010). Compared to two leaf meals, larvae that fed MO may perform better and get more nutrients than those that fed SG. However, it is possible that the larvae fed SBW show better performance due to the high protein content in addition to the low fibre (Redondo-Cuenca *et al*., 2008). Although there

are other factors that will affect the development of the larvae apart from the nutrient composition of the feed substrates, environmental conditions also play an equally important role.

2.5 Conclusions

The nutrient analysis of underutilised plants, MO and SG, demonstrated that these leaf meals had excellent nutrient content and had the potential to be used as feed substrates for the development of highly nutritious BSFL. The AIBPs, RB, and WP had a moderate nutrient level compared to the MECM, which had a very low nutrient density. The variations in the nutrient composition of feedstuffs, such as protein, fat, amino acid, and fatty acid profiles, will influence the development and accumulation of nutrients in BSFL, affecting the nutrient composition of the harvested BSFL. The study has focused entirely on the primary macronutrients, in addition to water and energy. Although other factors such as the presence of anti-nutritional factors (ANF) in each feedstuff could potentially play a role in determining the overall quality of the substrate, this factor was not measured. ANFs influence the growth of the larvae, and the effects should be measured in any future research. Thus, the effect of feed substrate composition variation on the performance and nutritional value of BSFL was determined through a series of feeding trials, as discussed in Chapters 3 and 4.

CHAPTER 3

THE GROWTH PERFORMANCE AND NUTRIENT COMPOSITION OF BLACK SOLDIER FLY LARVAE REARED ON UNDERUTILISED PLANTS AND AGRO-INDUSTRIAL BY-PRODUCTS

3.1 Introduction

The larvae of the Black Soldier Fly (BSF), scientifically known as *Hermetia illucens* can be grown on various organic wastes containing varying amounts of nutrients. The potential of organic waste from agro-industrial by-products (AIBPs) has been widely researched, including food and vegetable waste (Nguyen *et al.*, 2015; Spranghers *et al.*, 2017; Bonelli *et al.*, 2020) and animal manure (Li *et al.*, 2011; Zhou *et al.*, 2013; Miranda *et al.*, 2019; Parodi *et al.*, 2021) as feed substrates for rearing BSFL. However, there are still limited studies on the bioconversion of leaf meals by BSFL, especially those involving underutilised crops. At the same time, larvae have different abilities in transforming various organic matters and accumulating nutrients leading to variable nutritional values (Bosch *et al*., 2019, Parodi *et al*., 2021). The nutrient compositions of the rearing substrates were reported to influence larvae development time significantly, nutritional values of harvested biomass and bioconversion efficiencies (Wang *et al.*, 2017; Gold *et al.*, 2018; English *et al.*, 2021; Siddiqui *et al.*, 2022). However, there is a lack of detailed knowledge of BSFL nutrient requirements for larval production.

Understanding and enhancing bioconversion efficiencies are critical to ensure the sustainability of long-term BSFL production. The bioconversion efficiency is characterised by the amount of nutrients available in the substrate that are assimilated into the larvae biomass (Boschert *et al.*, 2019). The present study reports on the potential of six feedstuffs comprising plant meals, namely *Moringa oleifera* and *Sesbania grandiflora*, and AIBPs including soybean waste, wheat

pollard, rice bran and milk-extracted coconut meat with considerably varying nutrient compositions for BSFL rearing. This chapter addresses the Phase 2 of the objective to evaluate the growth performance and nutrient composition of BSFL reared on selected substrates. The best time for harvesting larvae and the efficiency of conversion of substrate into larval biomass were also determined in order to answer the Research Question 1 and 2;

1. What is the impact of different feed substrates on the performance and nutrient composition of black soldier fly larvae (BSFL)? Is there an impact of feed substrate on the ability of BSFL to convert substrate meal into larval biomass, considering factors such as waste reduction, bioconversion, and protein conversion ratio?
2. Which feed substrate is optimum for larval production? When is the best time to harvest larvae, considering the development and nutrient composition factors, such as protein, fat and total larval weight?

3.2 Materials and Methods

3.2.1 Preparation of Feed Substrates

Moringa oleifera (MO) and *Sesbania grandiflora* (SG) leaves were harvested from the Crops for the Future Research Centre (CFFRC) farm at Semenyih, Selangor, Malaysia. Fresh MO and SG leaves were collected, ground without water (Figure 15), using a laboratory grinder (The Baker, Taiwan) to acquire a size of about 1 mm and were placed in clean barrels and stored in a cold room at 4°C for future use. The moisture contents of ground MO and SG leaves were determined and were used as the basis for preparing all substrate meals. Four AIBPs, namely soybean waste (SBW), wheat pollard (WP), rice bran (RB), and milk-extracted coconut meat (MECM), were obtained from local suppliers. Wet SBW, with a particle size of about 1.18 mm and moisture content of approximately 74 g/100g, was purchased from a tofu processing plant in Seri Kembangan, Selangor, while MECM, with a particle size

of about 2 mm and 40.8 g/100g moisture content, was acquired from a coconut milk processing shop in Bandar Baru Bangi, Selangor. Usually, these feedstuffs (SBW and MECM) were purchased one or two days before use and stored at 4°C. The WP and RB, with a particle size of about 1.0 mm and 850 µm and moisture of 5.70 and 4.4 g/100g, respectively, were purchased from a local supplier (Zal Agro Sdn. Bhd.).

In order to standardise the moisture content of the substrates, a measured volume of water was added to dry or semi-moist feed substrates, including SBW, WP, RB and MECM, to obtain a moisture content of about 76 g/100g, which is similar to the moisture content of ground MO and SG. The amount of water needed to achieve 76 g/100g moisture content (wt/wt) of dry weight basis was calculated using formula (8) below as suggested by Fathurochim *et al.* (1989).

Formula (8)

$$X\ (ml) = \frac{Y\ (D)}{1 - Y} - C$$

where X is the required amount of distilled water to be added, Y is the expected proportion of water in the medium, D is the oven-dried weight of 100 g feed substrate, and C is the grams of water in 100 g of the oven-dried feedstuff.

3.2.2 Preparation of Stock Colony

The stock colony preparation was carried out in an outdoor insectarium at the CFFRC Facility, Semenyih, Selangor. The BSFL eggs were obtained from Satoyama Farm in Kuching, Sarawak, and brought to CFFRC for incubation. The eggs were hatched after 3 – 4 days, and after hatched the larvae were reared, and adult BSFs (flies) were allowed to mate naturally according to the methods described previously (Kuppusamy *et al*., 2020). MECM substrate was used to lure the female BSFs to oviposit eggs between the wooden gaps (Figure 16). An ovipositor was

created by stacking three wooden planks (23 x 3 x 1 cm) on top of each other, using screws between planks to create a 2 mm gap between the planks. The stacked plank was placed on a tray containing MECM substrate. As shown in Figure 17, the BSF eggs were collected by scraping eggs from the wooden plank using thick paper.

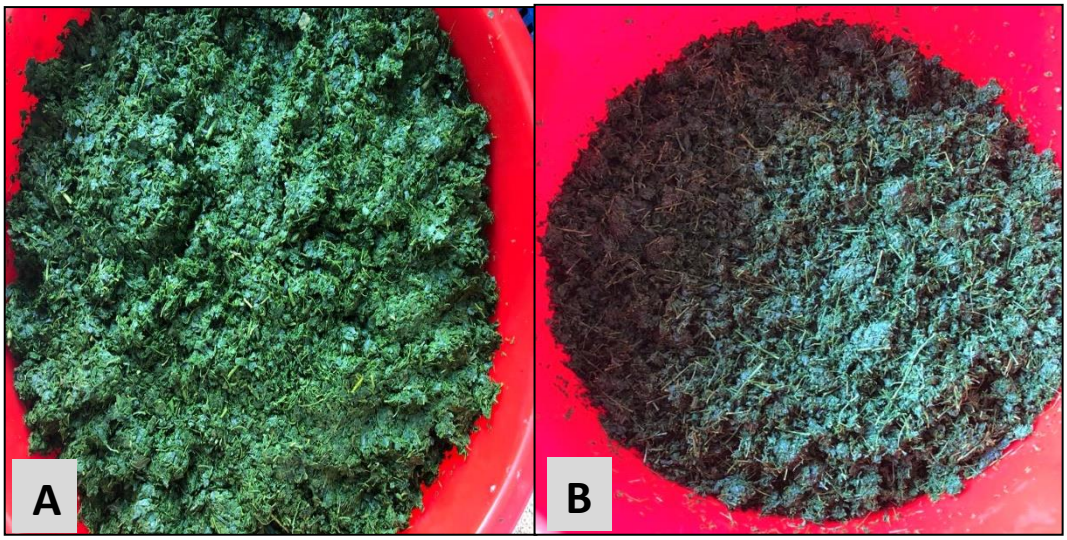


Figure 15: Ground *Sesbania grandiflora* (A) and *Moringa oleifera* (B) leaves placed in plastic barrel for storage.



Figure 16: Females lay eggs between the gap of the wooden planks



Figure 17: BSF eggs sticking to a wooden plank.

3.2.3 Rearing of the Black Soldier Fly Larvae

The substrate feeding trial was carried out in an indoor insectarium at the CFFRC Facility, Semenyih, Selangor. Fresh BSF eggs of about 0.25 g (Kuppusamy *et al.*, 2020) were placed on 150 g of each feed substrate in small plastic containers (14 x 9 x 5.5 cm) with aeration slits on the lid until the eggs hatched (Figure 18). On day 5, the substrates and five-day-old tiny larvae were gently transferred to larger incubation trays measuring 50 x 36 x 7.5 cm and containing the respective feed substrates. The feeding trial was conducted for three weeks at 27 ± 1.5°C and 75 ± 5% relative humidity (HOBO U23 Pro v2 Temperature/Relative Humidity Data Logger, USA). During the growth trial, larvae were not exposed to direct light except during feeding and sampling. Substrates were added to each incubation tray every three days at the optimum feeding rate of 100 mg/larva/day (Diener *et al.*, 2009; Abduh *et al.*, 2017). The total feed substrates were added to each incubation tray on days 5, 8, 11, 14, and 17 based on the total number of larvae in the tray left after sampling. Water was sprayed on the top of the substrate whenever the feed substrate looked dry to ensure the substrates were sufficiently moist and conducive for larvae growth. The feed in the tray was gently mixed by hand to release heat from the bottom of the tray to minimise heat built up in the substrates bed. Daily addition of substrate at a rate of 100 mg/larva/day was calculated according to the formula below:

Weight of substrates added = 3,750 larvae X 100 mg/larva/day

= 375,000 mg/day (375 g/day)

with the following assumptions:

i. 1 g of eggs contains 15,000 eggs
ii. 0.25 g of eggs used per tray, which contains 3,750 eggs

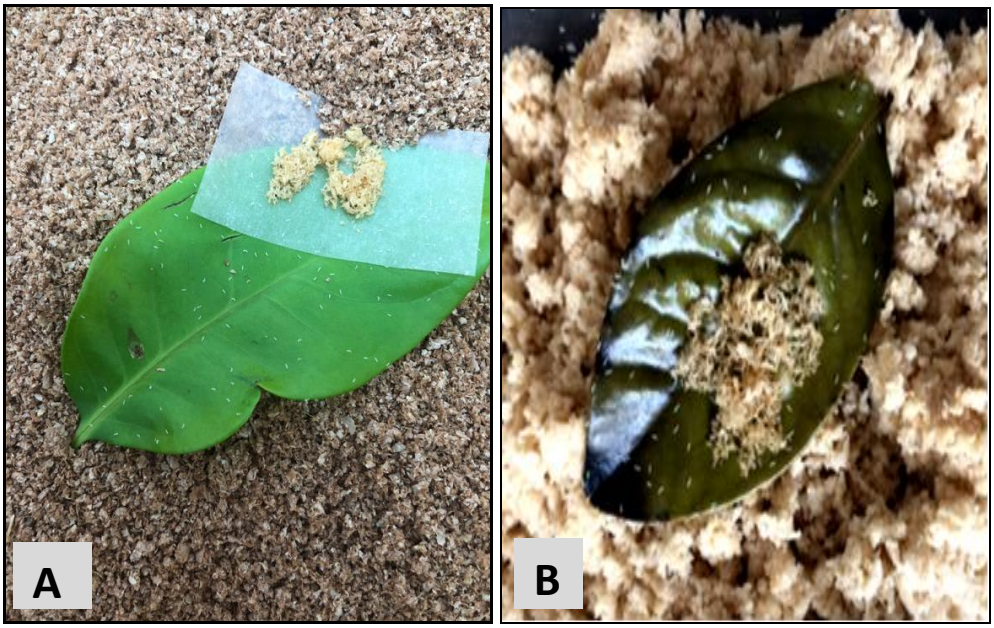


Figure 18: Freshly hatched BSFL actively crawled towards SBW (A) and MECM (B) substrates, where they vigorously feed during this life phase.



Figure 19: Trays containing BSFL placed on a customised portable steel rack stacker.

3.2.4 Experimental Design and Data Collection

Trays containing six dietary treatments with five-day-old tiny larvae, namely MO, SG, SBW, WP, RB, and MECM, with four replicates for each treatment (four trays per treatment) were randomly placed on a customised portable steel rack tray stacker (Figure 19). Since the BSFL were only visible but too small for sampling on day five after the egg hatched, larvae sampling and data recording only started on day 7 when the larvae were large enough to be handpicked. The sampling was performed every two days on days 7, 9, 11, 13, 15, 17, 19, and 21 to determine the larvae's growth performance and chemical compositions. Further sampling was not done after day 21, when half of the larvae in the trays transformed into prepupae.

In every sampling, about 470 larvae were randomly handpicked from each of the 24 incubation trays, as shown in the schematic diagram below (Figure 20). From those 470 larvae, 50 larvae were separated and cleaned for larval weight determination. Any substrate material attached to the larvae was removed by thoroughly washing them under tap water and wiped with tissue papers. The cleaned BSF larvae were weighed and measured for length and width. The combined weight of 50 larvae was recorded for each tray, and the average weight of individual larva was determined, while the length and width of 30 larvae were individually measured. The length was recorded when larvae were fully extended using a ruler, as suggested by Nguyen *et al.* (2015), while the width of larvae was measured at the middle of the abdomen, and the measurements were made in triplicates. The samples of 50 larvae were dried at 60°C until constant weight achieved, and weight of dried larvae were recorded. The weight of one individual larva was determined by dividing it by 50.

In the present study, the development time is defined as the time between the eggs hatched, designated Day 1, and the first observation of prepupae (Harnden and Tomberlin, 2016). The transformation can be observed in the colour change of the integument from white/cream (larvae) to dark/brown (prepupae), as reported

by Tomberlin *et al*. (2009) and Diener *et al*. (2009). Day 1 was the day of the first prepupae observed in each treatment tray. Larval development parameters, which included the measurement of larval dry weight (Formula 9), larval weight gain (Formula 10), and average rate of weight gain (Formula 11) for each dietary treatment, were determined according to the formulae below as described by Liu *et al.* (2018).

Formula (9)

$$Larval\ dry\ weight\ (g) = \frac{Fresh\ larval\ weight\ (g) \times Larval\ dry\ matter\ \left(\frac{g}{100g}\right)}{Number\ of\ larvae\ (50\ larvae)}$$

Formula (10)

$$Larval\ weight\ gain\ (mg) = Dry\ weight\ (day\ 21) - Initial\ dry\ weight\ (day\ 7)$$

Formula (11)

$$Average\ rate\ of\ weight\ gain\ \left(\frac{mg}{d}\right) = \frac{Larval\ weight\ gain\ (mg)}{Development\ time\ (days)}$$

The rest of the BSFL sampled on day 7 through day 21 were also dried at 60°C until constant weight (Figure 21). The dried larvae were ground in a laboratory blender (Sumeet, India) and kept in a refrigerator at 4°C before further analysis. The chemical analyses were carried out, including dry matter (DM), ash, crude protein (CP) and crude fat (Cfat), according to the protocols for nutrient composition analysis of feedstuffs as outlined in Chapter 2. CP content of BSFL was calculated by multiplying the total nitrogen by 6.25 (Finke, 2007; Kuppusamy *et al.*, 2020; Nyangena *et al.*, 2020; Oonincx and Finke, 2020).

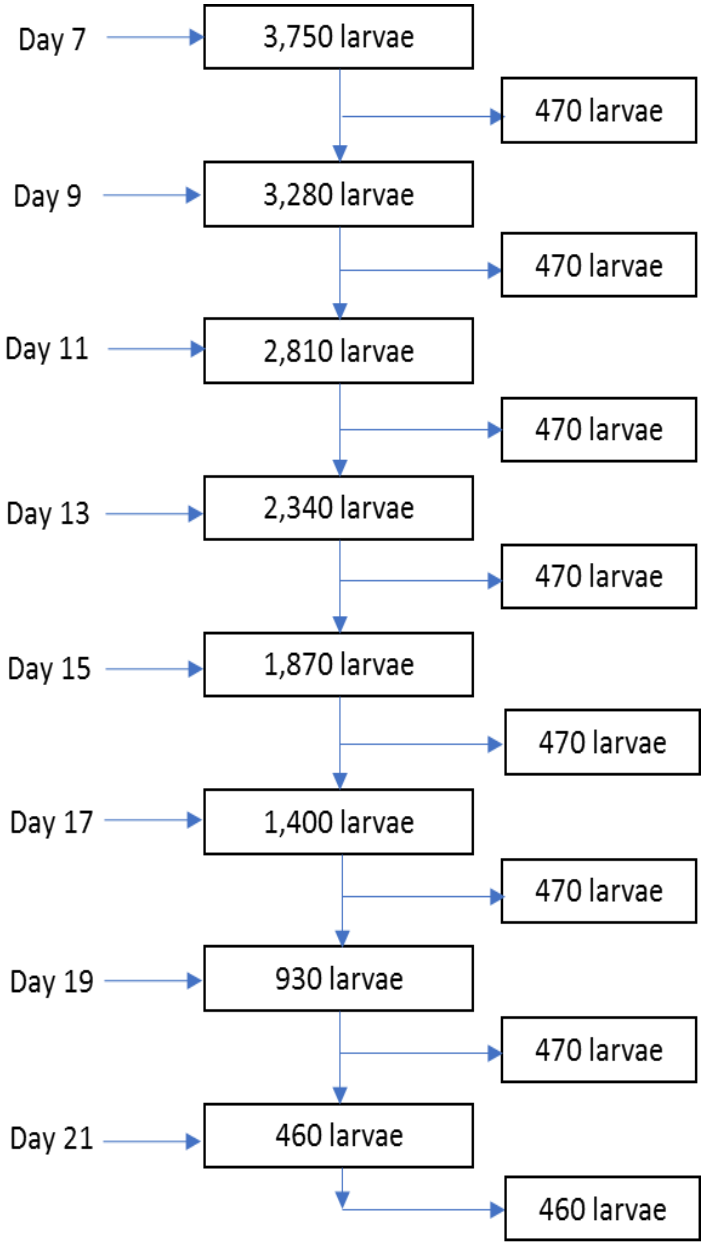


Figure 20: Schematic diagram of sampling BSFL for analysing performance and nutrient composition



Figure 21: Larvae were dried at 60°C for the chemical analysis

3.2.5 Black Soldier Fly Larvae Yield and Rearing Performance

For the determination of the growth performance of BSFL on each substrate (MO, SG, SBW, WP, RB, and MECM), the second set of BSFL was reared according to the protocol described in Section 3.2.3 without periodic sampling activities. All the larvae were harvested on day 17 based on previous feeding trial data (Section 3.2.3), whereby the developmental growth and chemical compositions mainly CP content, were optimal on this day. The rearing of BSF was conducted using 0.25 g egg on all six feed substrates in triplicate trays. The nutrient contents of feed substrates were determined (Table 9) as described in Chapter 2 prior to the second feeding trial. Substrates with a moisture content of about 76 g/100g were added to each incubation tray every three days. After harvest, the larvae were cleaned from residues containing secretory products and uneaten feed substrates (Figure 22), as defined by Meneguz *et al.* (2018). The cleaned larvae and residues were weighed and dried at 60°C.

The rearing performance was calculated based on the efficiency of larvae in converting the dry matter in the feed substrate into biomass. Larval performance parameters were measured, including total larvae biomass, individual larva weight,

number of larvae, feed substrate reduction, feed-to-biomass conversion, and protein conversion efficiency. The weight of 50 larvae was recorded in triplicates from each tray with a precision scale (AND GF-6100, Japan). The average weight of individual larvae was calculated by dividing the total weight by the number of larvae. Meanwhile, the total number of larvae in each tray was calculated by dividing the total yield of larvae harvested by the average weight of individual larva. The nutrient compositions of dried larvae were determined, including 5 constituents of proximate analysis (DM, ash, CP, Cfat, CF), detergent fibre (NDF, ADF), amino acids and fatty acids as described in Chapter 2.

Table 9: The nutrient composition of feed substrates and the feed on offer at the start of the BSFL performance trial.

Substrate	Ash	CF	CP	Cfat	Ratio CP:Cfat	Fresh Feed Offered (g)	Dried Feed Offered (g, DM)
SG	6.77 ± 0.26	12.87 ± 0.20	32.74 ± 0.12	3.22 ± 0.05	10.2:1	5050.38 ± 0.01	1231.70 ± 4.37
MO	8.41 ± 0.21	8.38 ± 0.22	32.90 ± 0.58	3.05 ± 0.09	10.8:1	4950.69 ± 67.72	1116.60 ± 18.74
SBW	3.95 ± 0.13	16.71 ± 0.76	22.98 ± 0.78	5.19 ± 0.71	4.4:1	5250.74 ± 0.24	1269.50 ± 24.16
WP	5.08 ± 0.07	12.38 ± 0.24	17.30 ± 0.08	3.06 ± 0.02	5.7:1	5125.38 ± 0.10	1265.10 ± 6.68
RB	8.67 ± 0.05	7.10 ± 0.47	13.71 ± 0.02	11.54 ± 0.16	1.2:1	5153.48 ± 0.20	1280.70 ± 13.94
MECM	1.61 ± 0.04	28.44 ± 0.32	3.45 ± 0.15	18.23 ± 0.15	0.2:1	4950.50 ± 0.01	1115.10 ± 3.69

Means ± standard error of the mean in g/100g DM. CF=crude fibre, CP=crude protein, Cfat=crude fat. SG=*Sesbanaia grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

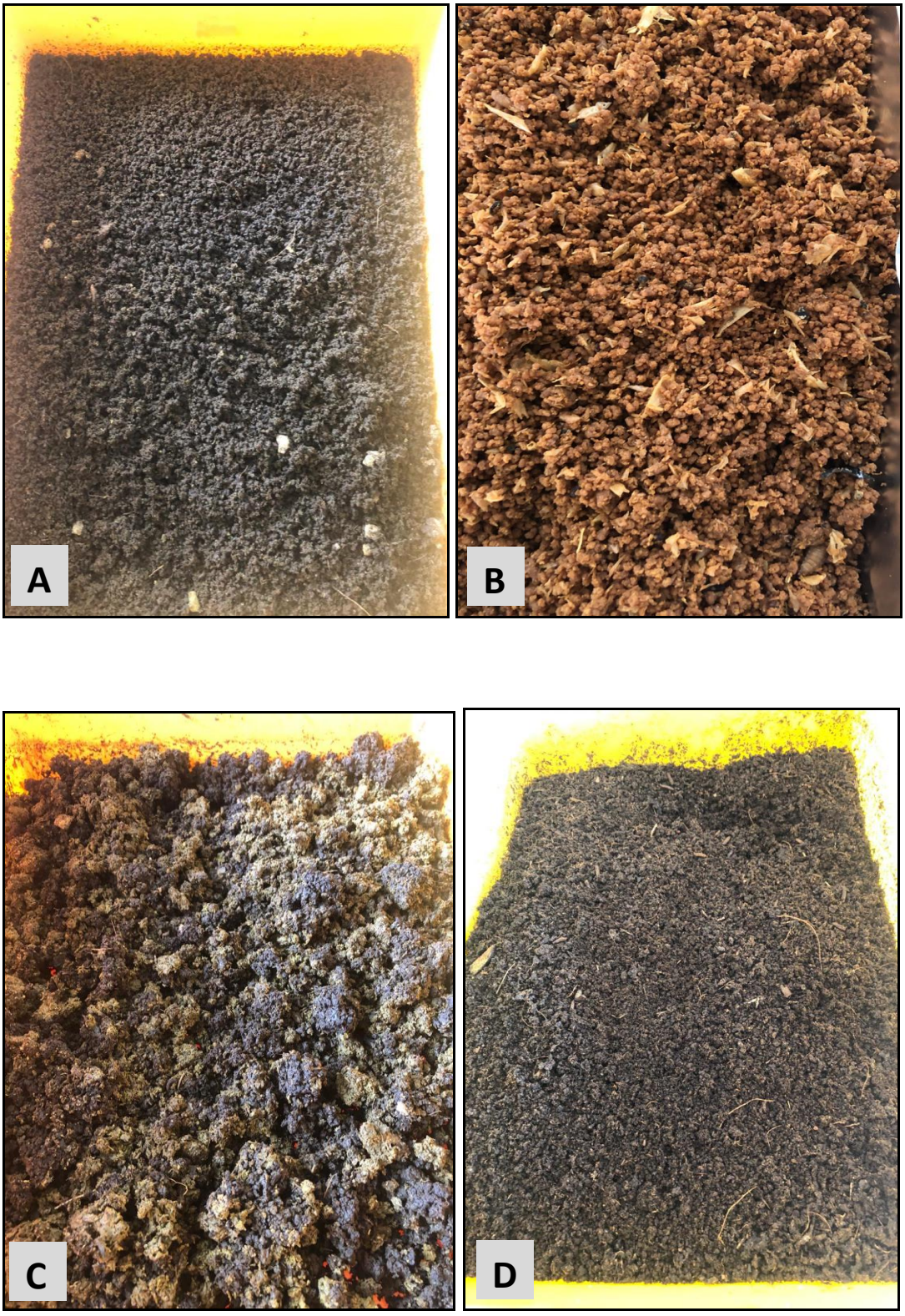


Figure 22: Residues from MO-fed larvae (A) and SBW-fed larvae (B), SG-fed larvae (C), and MECM-fed larvae (D).

3.2.6 Biomass Conversion of Black Soldier Fly Larvae

Conversion efficiency was determined by calculating the waste reduction efficiency and feed conversion into increased larval body mass. Protein conversion efficiency is a measure of the efficiency of larvae in converting protein substrates into protein larvae. The following formulae (12-14) were used to calculate the conversion efficiencies (Liu *et al*., 2018; Lalander *et al.*, 2019). Due to the different number of larvae in each feed substrate, the calculation is based on the capability of 1000 larvae in order to standardise the calculation.

a. Feed Substrate Reduction

The percentage of substrate reduction ($Sub.redDM$) on a DM basis is calculated using the following formula (12);

Formula (12)

$$Sub.redDM\ (\%) = \frac{sub.inDM - sub.outDM}{sub.inDM} \times 100$$

where $sub.inDM$ and $sub.outDM$ are the total feed substrate given and the residue left in dry matter DM in the tray after BSF bioconversion, respectively.

b. Feed-to-Biomass Conversion Efficiency of BSFL

The bioconversion rate is used to measure the quantity of feed substrate that is converted to larval biomass. The percentage of feed to the biomass conversion efficiency of BSFL ($BCEDM$) is calculated using the following formula (13). A high bioconversion rate indicates good bioconversion efficiency. The result presented based on the 1000 larvae.

Formula (13)

$$BSFLDM\ (g)\ =\ Weight\ of\ larvae/(\frac{No.\ of\ larvae}{1000\ larvae})$$

$$sub.fedDM\ (g)\ =\ Total\ substrate\ fed/(\frac{No.of\ larvae}{1000\ larvae})$$

$$BCEDM\ (\%)\ =\ \frac{BSFLDM(g)}{sub.fedDM(g)}\ \times\ 100$$

where $BSFLDM$ and $sub.fedDM$ are the total BSFL biomass and the total substrate fed in DM fed throughout the experiment, respectively.

c. Protein Conversion Efficiency of BSFL

The percentage of protein conversion ($PrCEDM$) is calculated based on DM basis using the following formula (14). The result presented based on the 1000 larvae.

Formula (14)

$$PrCEDM\ (\%)\ =\ \frac{BSFLDM\ (g)\ \times CP.BSFL\left(\frac{g}{100g}DM\right)}{sub.fedDM\ (g)\ \times\ CP.sub(\frac{g}{100g}DM)}\ \times\ 100$$

where $BSFLDM$ and $sub.fedDM$ are the total BSFL biomass and total substrate in DM fed by 1000 larvae throughout the experiment, respectively. The $CP.BSFL$ and $CP.sub$ are the concentration of CP in larvae and substrate, respectively.

3.2.7 Price of Black Soldier Fly Larvae

It is essential to consider the purchasing price of each feed substrate when estimating the production cost of BSFL, although in the present study, some feedstuffs were provided free of charge. Feed prices substantially impact which feed product is chosen over another. MO, SG, SBW, WP, RB, and MECM substrates were priced at RM5/kg, RM3/kg, RM0.50/kg, RM1.45/kg, RM1.30/kg, and RM0.20/kg, respectively. The substrate cost (RM) to produce larvae was compared between the six substrates using Formula 15. The larval price per kg was determined using Formula 16 below, based on a DM basis.

Formula (15)

$$Cost\ of\ substrate\ used\ (RM) = Price\ of\ substrate\ \left(\frac{RM}{kg}\right) \times sub.inDM\ (kg)$$

where $sub.inDM$ is a total feed substrate given to larvae.

Formula (16)

a. $$The\ total\ cost\ of\ larvae\ (RM/kg) = \frac{Cost\ of\ substrate\ used\ (RM)}{Protein\ produced\ by\ larvae} \times 100$$

b. $$The\ total\ cost\ of\ larvae\ (RM/kg) = \frac{Cost\ of\ substrate\ used\ (RM)}{Larvae\ yield\ (kg, DM) \times CP.BSFL\ (\frac{g}{kg}DM)} \times 100$$

where $CP.BSFL$ is the concentration of CP in larvae.

3.2.7 Statistical Analysis

All data were analysed for normal distribution using Kolmogorov-Smirnov for samples more than 50 (n > 50) and the Shapiro-Wilk test for samples less than 50 (n < 50). The analysis of variance (two-way ANOVA) using the general linear model (GLM) procedure of the Statistical Analysis System Inc. version 9.4 (2013) was performed on the data that were normally distributed to assess the influence of the feed substrates on BSFL growth performance and nutrient composition. Post-hoc multiple comparisons, Tukey's honest significant difference (HSD) test was used when a significant treatment effect was observed to compare the differences between means within and between substrates throughout larvae development. The analysis was also done to determine the statistical differences in growth performance, and nutrient composition between the group means of each treatment on the respective sampling days on days 7, 9, 11, 13, 15, 17, 19, and 21. Differences were statistically significant when $p < 0.05$. All data were expressed as means ± standard error of the mean (SEM) in the tables and graphs. In addition, Pearson's correlation was performed using the PROC CORR procedure of SAS to examine the correlation between the nutrient contents in the feed substrate and BSFL. A *p*-value < 0.05 was considered statistically significant. Microsoft Excel was used for creating graphical representations of the data.

3.3 Results

3.3.1 Developmental Study of Black Soldier Fly Larvae

3.3.1.1 Larval Wet and Dry Weight

Mean wet larval weights differed significantly among the six feed substrates ($p < 0.0001$, Table 10). Larvae reared on SG, MO, SBW, WP, RB, and MECM recorded an average weight gain of 52.1 mg, 67.7 mg, 75.9 mg, 53.6 mg, 113.9 mg, and 62.9 mg, respectively over the 14-day development period (from days 7 to 21). During the early stage of development, the larva reared on SG, MO, and WP grew faster and weighed 50 mg more than those fed on SBW, RB, and MECM, which were approximately 30 mg on day 7. The weight of the larva doubled on day 9 and progressively increased until day 15 (Figure 23). The RB, WP, and MECM substrates were observed to dry quickly during the first nine days of the experiment. These substrates showed poor water holding capacity, so the water was sprayed on these substrates almost every day to prevent dehydration. In addition, RB substrate was observed to be infected with a thick layer of fungus, covering the top surface of the substrate, particularly during the first week of larval development, whereby the conversion of substrates was slow based on changes in texture and the colour of the substrate. The high increment in weight could be associated with vigorous substrate consumption after day 7 when the texture of substrates became porous and resembled compost in appearance and colour. Depending on the feed substrates, the BSFL grew at variable rates from 15 – 17 days to achieve their maximum weight. RB-fed larvae grew rapidly and gained the highest weight (159.2 ± 2.72 mg) on day 15 compared to other feed substrates. The lowest weight-gain was observed in MECM-fed larvae. The weight of WP-fed larvae was much lower than those fed with RB, although the protein levels in both substrates were similar (Table 6, as explained in Chapter 2). MO-fed larvae gained more weight than SG-fed larvae, with the greatest gain seen on day 17 at 136.40 ± 1.96 mg. The larvae reared on SBW reached a weight of 105.00 ± 3.31 mg on day 13, which remained

stable for a few days afterwards. Meanwhile, the MECM-fed larvae weight increased considerably and remained constant from day 17 onwards. The larvae reared on MECM showed the most nominal growth during the whole lifecycle, which reached a maximum weight of only 92.42 ± 2.67 mg. Interestingly, the high dry matter content of MECM-fed larvae resulted in a higher dry weight, despite having the lowest wet weight. Figure 24 shows that the dry weight of MECM-fed larvae is the second highest after RB-fed larvae. The RB-fed larvae gained the most weight, 51 mg DM, followed by MECM at 34 mg, and the least at 22.5 mg of the WP-fed larva.

3.3.1.2 Larval Size

Significant differences were observed in the length of larvae reared on the six feed substrates ($p \leq 0.0256$) and between sampling intervals ($p < 0.0001$) throughout their development. Larvae showed a significant increase in length during the 21-day rearing period. On day 7, the length of BSFL reared on SG, MO, RB, and MECM were approximately 10 mm compared to those reared on SBW and WP, which were 7.11 ± 0.53 mm and 8.83 ± 0.29 mm, respectively (Table 10). The larvae continued to grow and reached maximum length at different times, where larvae reared on SBW reached maximum length by day 13, followed by WP (day 15), MO (day 17), RB (day 19) and both SG and MECM on day 21. Larvae reared on RB showed the highest mean length over the 21 days of development at 16.5 mm, and the shortest was the MECM-fed larvae at 14.9 mm. The larvae reared on RB attained the longest at 19.37 ± 0.06 mm on day 19, while the SBW-fed larvae were the shortest at 16.06 ± 0.36 mm on day 13. The width of larvae almost doubled during growth for all substrates ($p \leq 0.0008$) over the 14 days of measurement (Table 10). Overall, the mean for larval width showed that RB-fed larvae were the widest width at 4.5 mm while the smallest width was the SBW-fed larvae at 3.9 mm.

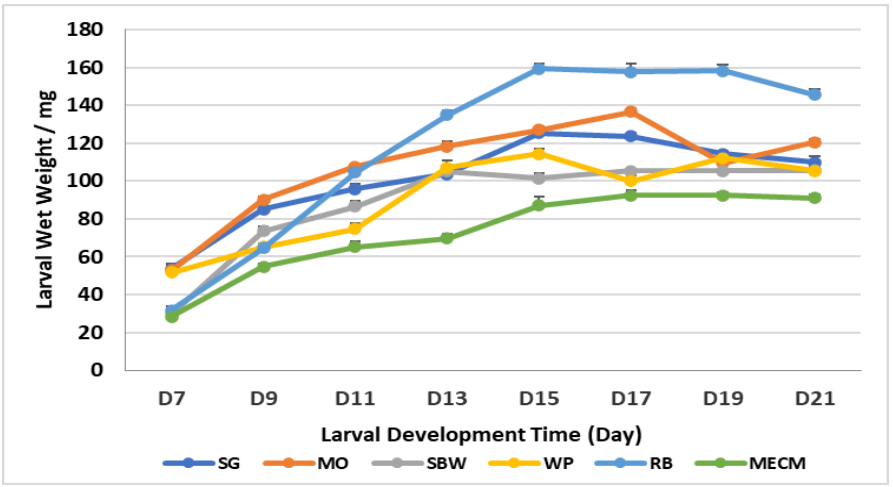


Figure 23: Mean wet weight (mg) of individual BSF larva reared on *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG) leaf meals and AIBPs: soybean waste (SBW), wheat pollard (WP), rice bran (RB), and milk-extracted coconut meat (MECM) over development time. Error bars represent the standard errors of the mean larval wet weight of quadruplicate trays. D7-D21= Sampling of larvae between days 7 and 21.

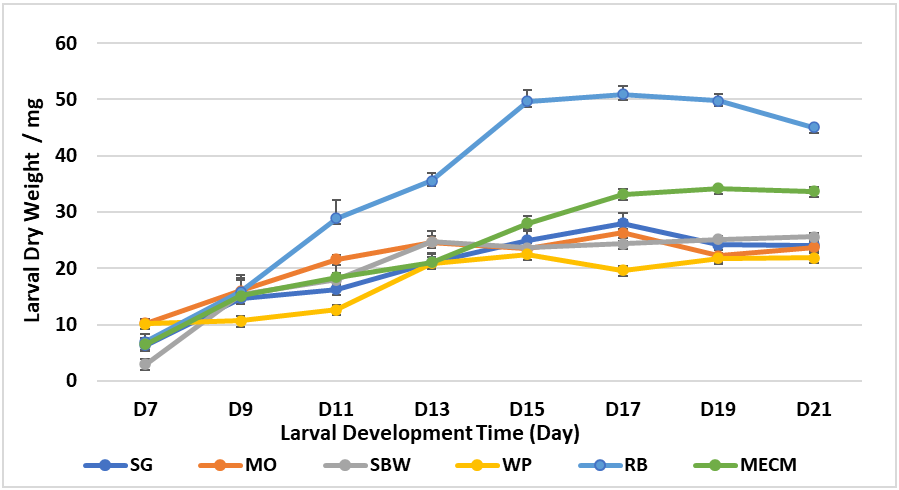


Figure 24: Mean dry weight (mg) of individual BSF larva reared on *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG) leaf meals and AIBPs: soybean waste (SBW), wheat pollard (WP), rice bran (RB), and milk-extracted coconut meat (MECM) over development time. Error bars represent the standard errors of the mean larval dry weight of quadruplicate trays. D7-D21= Sampling of larvae between days 7 and 21.

Table 10: Growth performance of BSFL on different feed substrates at different larval ages.

Substrate	SG	MO	SBW	WP	RB	MECM	*p-value*
Day			Wet Weight (mg)				
7	$53.75 \pm 2.77^{z,a}$	$52.72 \pm 2.97^{z,a}$	$29.79 \pm 1.61^{z,b}$	$51.71 \pm 2.53^{z,a}$	$31.68 \pm 2.15^{z,b}$	$28.23 \pm 1.69^{z,b}$	<0.0001
9	$85.05 \pm 3.45^{y,a}$	$90.10 \pm 1.63^{y,a}$	$73.50 \pm 2.65^{y,b}$	$65.10 \pm 2.14^{y,bc}$	$64.60 \pm 1.50^{y,bc}$	$54.85 \pm 1.70^{y,c}$	<0.0001
11	$95.85 \pm 2.70^{xy,ab}$	$107.50 \pm 0.94^{x,a}$	$86.40 \pm 3.31^{x,bc}$	$74.68 \pm 3.05^{y,cd}$	$104.37 \pm 2.24^{x,a}$	$65.02 \pm 2.87^{xy,d}$	<0.0001
13	$103.70 \pm 2.93^{wx,c}$	$118.20 \pm 2.68^{vw,b}$	$105.00 \pm 3.31^{w,bc}$	$107.10 \pm 3.77^{wx,bc}$	$134.93 \pm 2.18^{w,a}$	$69.62 \pm 2.608^{x,d}$	<0.0001
15	$125.20 \pm 2.26^{v,b}$	$127.00 \pm 1.80^{uv,b}$	$101.40 \pm 2.68^{w,c}$	$114.35 \pm 2.62^{w,bc}$	$159.20 \pm 2.72^{u,a}$	$86.97 \pm 4.74^{w,d}$	<0.0001
17	$123.50 \pm 1.97^{v,c}$	$136.40 \pm 1.96^{u,b}$	$105.42 \pm 1.04^{w,d}$	$100.13 \pm 2.76^{x,de}$	$157.68 \pm 4.29^{uv,a}$	$92.42 \pm 2.67^{w,e}$	<0.0001
19	$114.25 \pm 1.36^{vx,b}$	$109.50 \pm 2.35^{wx,b}$	$105.44 \pm 0.73^{w,b}$	$112.14 \pm 2.55^{wx,b}$	$158.18 \pm 3.35^{uv,a}$	$92.28 \pm 1.48^{w,c}$	<0.0001
21	$109.90 \pm 3.25^{w,bc}$	$120.41 \pm 1.92^{v,b}$	$105.64 \pm 1.89^{w,c}$	$105.26 \pm 2.36^{wx,c}$	$145.58 \pm 3.11^{vw,a}$	$91.13 \pm 1.77^{w,d}$	<0.0001
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
			Larval Length (mm)				
7	$9.36 \pm 0.46^{z,ab}$	$10.48 \pm 1.03^{z,a}$	$7.11 \pm 0.53^{z,b}$	$8.83 \pm 0.29^{z,ab}$	$9.59 \pm 0.57^{z,ab}$	$9.80 \pm 0.37^{z,ab}$	0.0256
9	13.93 ± 0.21^{y}	14.02 ± 0.48^{y}	13.73 ± 0.24^{y}	12.55 ± 0.49^{y}	12.18 ± 0.69^{y}	13.44 ± 0.19^{y}	0.0545
11	14.26 ± 0.39^{y}	14.80 ± 0.30^{xy}	13.83 ± 0.68^{y}	13.77 ± 0.25^{y}	15.90 ± 0.64^{x}	15.04 ± 0.15^{wx}	0.0585
13	$15.46 \pm 0.28^{xy,bc}$	$15.78 \pm 0.45^{wxy,abc}$	$16.06 \pm 0.36^{w,ab}$	$15.69 \pm 0.28^{x,bc}$	$17.31 \pm 0.44^{wx,a}$	$14.32 \pm 0.29^{xy,c}$	0.0007

continue…

Substrate	SG	MO	SBW	WP	RB	MECM	*p-value*
15	16.55 ± 0.26^{x,bcd}	16.61 ± 0.46^{wx,bc}	15.53 ± 0.11^{w,d}	17.35 ± 0.09^{x,b}	19.03 ± 0.17^{vw,a}	15.71 ± 0.14^{w,cd}	<0.0001
17	16.18 ± 0.48^{x,c}	17.63 ± 0.16^{w,b}	14.75 ± 0.04^{wx,d}	16.84 ± 0.38^{x,bc}	19.32 ± 0.17^{vw,a}	17.00 ± 0.26^{v,bc}	<0.0001
19	15.95 ± 0.40^{x,c}	17.09 ± 0.25^{w,b}	14.93 ± 0.19^{wx,c}	17.29 ± 0.32^{x,b}	19.37 ± 0.06^{v,a}	17.26 ± 0.12^{v,b}	<0.0001
21	16.61 ± 0.02^{x,c}	17.45 ± 0.14^{w,b}	14.98 ± 0.05^{wx,d}	16.77 ± 0.46^{x,bc}	19.36 ± 0.19^{v,a}	17.27 ± 0.10^{v,bc}	<0.0001
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
				Larval Width (mm)			
7	3.21 ± 0.13^{x,ab}	3.44 ± 0.26^{z,a}	2.49 ± 0.20^{z,ab}	2.47 ± 0.47^{z,b}	2.81 ± 0.18^{z,ab}	3.01 ± 0.08^{z,ab}	0.0246
9	3.91 ± 0.06^{y}	4.01 ± 0.15^{yz}	3.91 ± 0.04^{y}	3.37 ± 0.26^{yz}	3.80 ± 0.23^{y}	3.89 ± 0.03^{y}	0.1828
11	4.06 ± 0.06^{y}	4.14 ± 0.10^{y}	3.86 ± 0.21^{y}	3.86 ± 0.09^{yz}	4.26 ± 0.10^{xy}	4.12 ± 0.06^{xy}	0.2234
13	4.08 ± 0.05^{y,b}	4.18 ± 0.10^{y,b}	4.17 ± 0.10^{y,b}	4.32 ± 0.09^{xy,ab}	4.61 ± 0.06^{wx,b}	3.98 ± 0.08^{y,b}	0.0008
15	4.05 ± 0.05^{y,c}	4.19 ± 0.06^{y,c}	4.08 ± 0.06^{y,c}	4.59 ± 0.08^{x,b}	4.99 ± 0.03^{w,a}	4.66 ± 0.07^{v,b}	<0.0001
17	4.00 ± 0.10^{y,c}	4.58 ± 0.05^{y,b}	4.15 ± 0.03^{y,bc}	4.46 ± 0.25^{x,bc}	5.08 ± 0.03^{w,a}	4.49 ± 0.01^{vw,bc}	<0.0001
19	4.17 ± 0.09^{y,b}	4.29 ± 0.15^{y,b}	4.19 ± 0.03^{y,b}	4.51 ± 0.16^{x,b}	5.08 ± 0.02^{w,a}	4.38 ± 0.04^{wx,b}	<0.0001
21	4.17 ± 0.03^{y,b}	4.25 ± 0.11^{y,b}	4.17 ± 0.03^{y,b}	4.34 ± 0.22^{xy,b}	5.09 ± 0.04^{w,a}	4.32 ± 0.03^{wx,b}	<0.0001
p-value	<0.0001	0.0008	<0.0001	<0.0001	<0.0001	<0.0001	

^{abcde}Means ± standard error of the mean within row showed that the comparison within treatment by day followed with different superscripts are significantly different ($p < 0.05$).

^{uvwxyz}Means ± standard error of the mean within the column showed that the comparison between treatments by day followed with different superscripts are significantly different ($p < 0.05$). SG=*Sesbania grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

3.3.2 Nutrient Composition of Black Soldier Fly Larvae

3.3.2.1 Dry Matter Content of BSFL

Highly significant changes in dry matter larvae were observed between treatments throughout larvae development ($p \leq 0.0007$, Table 11). The dry matter (DM) content of larvae was low in all substrates in the early days of development but increased gradually when the age of larvae increased ($p \leq 0.0109$). Overall, the DM content of larvae on the six feed substrates ranged from 14.34 ± 0.46 to 37.05 ± 0.39 g/100g throughout their development. Larvae reared on MECM had the highest DM (at the end of the 21-day rearing period), followed by RB-fed larvae, which reached more than 30 g/100g whilst the lowest dry matter was WP-fed larvae.

3.3.2.2 Crude Protein Content of Black Soldier Fly Larvae

There were marked variations in the crude protein (CP) composition of BSFL throughout the development time, both within ($p \leq 0.0153$) and between feed substrates ($p < 0.0001$). The CP level of BSFL showed a reducing trend from day 7 to day 21 (Figure 25). In the early stage of development, day 7, larvae contained the highest protein level, which was more than 50 g/100g DM, except for MECM-fed larvae, but decreased to lower values by day 21. The CP content of BSFL reared on SG, MO, SBW, WP, and MECM reduced by about 3.3 g/100g, 8 g/100g, 9.4 g/100g, 6.8 g/100g, and 7.1 g/100g, respectively on a DM basis throughout 14 days of observation (Table 11). Although the CP level of BSFL decreased to a lower value by day 21, however, the total protein increased due to the increase in weight gain (biomass). The CP content of RB-fed larvae did not differ during the rearing period ($p = 0.2576$), ranging from 47.27 ± 1.84 to 50.77 ± 0.61 g/100g DM. The CP content in larvae reared on WP was the highest among the substrates, whereas the lowest level was observed in MECM-fed larvae.

The CP content of larvae reared on WP decreased from day 7 to day 15 (56.97 ± 0.51 to 50.20 ± 0.76 g/100g DM). An upsurge of CP level to 54.07 ± 0.30 g/100g was observed on day 17 but reduced again from day 19 onwards. The CP of SG-fed larvae reduced from 53.69 ± 0.56 to 47.90 ± 1.10 g/100g DM in 8 days (days 7 to 15) but increased to 49.89 ± 3.25 g/100g DM on day 17 before dropping on day 19 but rose back to 50.43 ± 0.80 g/100g DM on day 21. In the present study, the protein level in the substrates significantly affected the protein content of BSFL, although it was not strongly correlated (r = 0.40343, *p* = 0.0147, Figure 26). Larvae reared on protein-rich substrates of around 27 – 31 g/100g DM in SG, MO, and SBW produced BSFL with a higher protein content of 42.97 ± 1.10 – 55.49 ± 2.79 g/100g DM. However, the larval protein was comparable to moderate protein substrates such as WP and RB (12 g/100g DM), which produced BSFL with a higher protein concentration of around 47.36 ± 0.33 – 56.97 ± 0.51 g/100g DM.

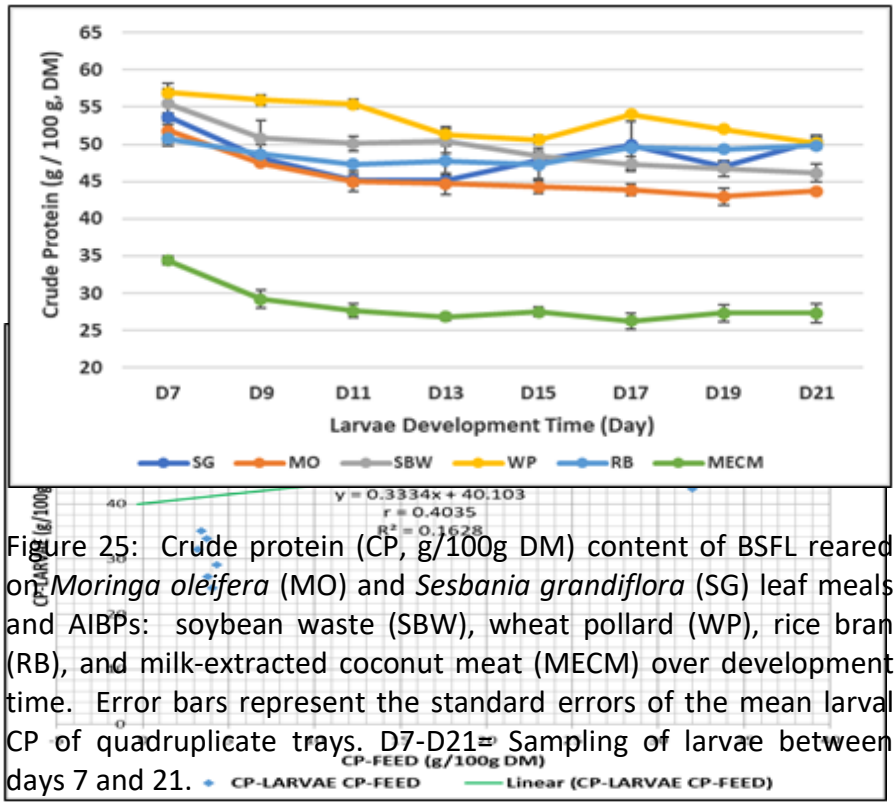


Figure 25: Crude protein (CP, g/100g DM) content of BSFL reared on *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG) leaf meals and AIBPs: soybean waste (SBW), wheat pollard (WP), rice bran (RB), and milk-extracted coconut meat (MECM) over development time. Error bars represent the standard errors of the mean larval CP of quadruplicate trays. D7-D21= Sampling of larvae between days 7 and 21.

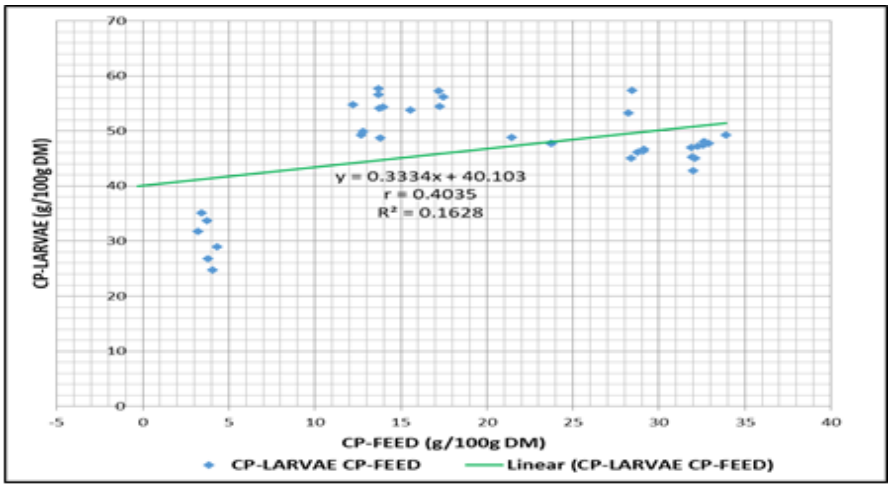


Figure 26: Linear relationship between the crude protein of BSFL produced from six feed substrates with varying crude proteins.

Table 11: Nutrient composition of BSFL reared on different feed substrates at various larval ages.

Substrate/Day	SG	MO	SBW	WP	RB	MECM	*p - value*
			Dry Matter (g/100g wet weight)				
7	14.34 ± 0.46^{z,d}	15.18 ± 1.03^{cd}	16.10 ± 1.46^{z,bcd}	20.30 ± 2.48^{abc}	21.79 ± 0.42^{z,ab}	23.16 ± 0.56^{z,a}	0.0005
9	17.21 ± 0.48^{yz,c}	17.70 ± 2.02^{bc}	25.41 ± 3.57^{y,ab}	16.31 ± 0.75^{c}	24.34 ± 0.99^{yz,abc}	27.67 ± 1.09^{y,a}	0.0007
11	18.19 ± 0.52^{xyz,c}	19.66 ± 2.02^{c}	21.90 ± 1.48^{yz,bc}	17.07 ± 1.27^{c}	27.18 ± 1.20^{xy,ab}	28.30 ± 0.85^{y,a}	<0.0001
13	20.32 ± 1.05^{wxy,bc}	18.78 ± 1.68^{c}	23.51 ± 0.61^{y,b}	19.55 ± 0.50^{bc}	29.80 ± 0.91^{wx,a}	30.30 ± 0.69^{xy,a}	<0.0001
15	21.31 ± 1.18^{wx,bc}	18.19 ± 2.26^{c}	23.46 ± 0.64^{y,b}	19.714 ± 0.87^{bc}	31.18 ± 0.34^{w,a}	32.22 ± 0.52^{x,a}	<0.0001
17	22.62 ± 1.14^{w,b}	19.34 ± 1.88^{b}	23.13 ± 0.53^{yz,b}	19.65 ± 0.51^{b}	32.31 ± 0.21^{w,a}	35.84 ± 0.30^{w,a}	<0.0001
19	19.59 ± 1.04^{wxy,cd}	18.8 ± 2.26^{d}	23.91 ± 0.65^{y,c}	19.47 ± 0.35^{cd}	31.45 ± 0.28^{w,b}	37.05 ± 0.39^{w,a}	<0.0001
21	21.95 ± 0.32^{wx,c}	19.94 ± 1.71^{c}	24.25 ± 0.58^{y,c}	20.77 ± 0.81^{c}	31.46 ± 0.75^{w,b}	36.94 ± 0.19^{w,a}	<0.0001
p-value	<0.0001	0.7198	0.0109	0.1148	<0.0001	<0.0001	
			Crude Protein (g/100g DM)				
7	53.69 ± 0.56^{y,b}	51.81 ± 1.94^{y,b}	55.49 ± 2.79^{y,b}	56.97 ± 0.51^{x,a}	50.77 ± 0.61^{b}	34.40 ± 0.56^{y,c}	<0.0001
9	48.23 ± 0.74^{yz,b}	47.45 ± 0.41^{yz,b}	50.82 ± 2.41^{yz,ab}	55.94 ± 0.71^{x,a}	48.67 ± 1.29^{b}	29.17 ± 1.24^{z,c}	<0.0001
11	45.21 ± 0.78^{z,c}	45.01 ± 1.31^{z,c}	50.11 ± 1.00^{yz,b}	55.38 ± 0.66^{x,a}	47.36 ± 0.33^{bc}	27.64 ± 0.89^{z,d}	<0.0001
13	45.11 ± 0.46^{z,bc}	44.72 ± 1.42^{z,c}	50.47 ± 1.62^{yz,ab}	51.32 ± 0.99^{yz,a}	47.77 ± 1.99^{abc}	26.82 ± 0.51^{z,d}	<0.0001

continue….

Substrate/ Day	SG	MO	SBW	WP	RB	MECM	*p - value*
15	$47.90 \pm 1.10^{z,ab}$	$44.27 \pm 0.87^{z,b}$	$48.42 \pm 1.10^{z,ab}$	$50.63 \pm 0.54^{yz,a}$	47.27 ± 1.84^{ab}	$27.47 \pm 0.63^{z,c}$	<0.0001
17	$49.89 \pm 3.25^{yz,ab}$	$43.88 \pm 0.72^{z,b}$	$47.36 \pm 1.00^{z,ab}$	$54.07 \pm 0.30^{xy,a}$	49.54 ± 0.35^{ab}	$26.24 \pm 1.06^{z,c}$	<0.0001
19	$47.05 \pm 0.65^{z,b}$	$42.97 \pm 1.10^{z,c}$	$46.80 \pm 1.07^{z,bc}$	$52.05 \pm 0.32^{yz,a}$	49.37 ± 0.31^{ab}	$27.33 \pm 1.15^{z,d}$	<0.0001
21	$50.43 \pm 0.80^{yz,a}$	$43.69 \pm 0.32^{z,c}$	$46.13 \pm 1.21^{z,bc}$	$50.20 \pm 0.76^{z,ab}$	49.85 ± 0.19^{ab}	$27.33 \pm 1.26^{z,d}$	<0.0001
p - value	0.0025	0.0002	0.0135	<0.0001	0.2576	<0.0001	
				Crude Fat (g/100g DM)			
7	$6.19 \pm 0.22^{z,e}$	$6.59 \pm 0.58^{z,de}$	14.24 ± 1.29^{c}	$9.47 \pm 0.47^{z,d}$	$20.00 \pm 0.42^{z,b}$	$43.18 \pm 0.67^{z,a}$	<0.0001
9	$8.67 \pm 0.50^{xy,b}$	$14.10 \pm 0.67^{y,b}$	21.32 ± 2.99^{a}	$9.29 \pm 0.49^{z,b}$	$25.52 \pm 0.34^{y,a}$	$51.48 \pm 0.61^{y,a}$	<0.0001
11	$7.96 \pm 0.60^{xyz,e}$	$16.29 \pm 0.89^{xy,cd}$	21.05 ± 3.11^{bc}	$10.99 \pm 0.67^{z,de}$	$25.79 \pm 0.91^{y,b}$	$53.93 \pm 0.86^{y,a}$	<0.0001
13	$10.69 \pm 0.46^{w,e}$	$16.05 \pm 1.07^{y,d}$	22.25 ± 1.97^{bc}	$17.41 \pm 0.31^{xy,cd}$	$26.20 \pm 0.91^{y,b}$	$54.02 \pm 0.91^{y,a}$	<0.0001
15	$8.72 \pm 0.59^{xy,e}$	$15.07 \pm 0.99^{y,d}$	19.99 ± 1.44^{c}	$19.86 \pm 1.06^{x,c}$	$30.76 \pm 0.67^{x,b}$	$54.56 \pm 0.46^{y,a}$	<0.0001
17	$9.92 \pm 0.36^{wx,d}$	$16.86 \pm 0.52^{y,c}$	19.62 ± 0.93^{c}	$16.08 \pm 0.20^{y,c}$	$31.76 \pm 0.84^{x,b}$	$54.98 \pm 2.14^{y,a}$	<0.0001
19	$7.25 \pm 0.28^{yz,e}$	$13.90 \pm 0.59^{xy,d}$	20.34 ± 1.71^{c}	$18.29 \pm 0.64^{xy,c}$	$31.58 \pm 0.49^{x,b}$	$54.63 \pm 0.51^{y,a}$	<0.0001
21	$17.63 \pm 0.06^{v,c}$	$19.76 \pm 0.32^{x,c}$	21.52 ± 1.83^{c}	$18.14 \pm 0.35^{xy,c}$	$30.75 \pm 0.63^{x,b}$	$54.12 \pm 0.54^{y,a}$	<0.0001
p - value	<0.0001	<0.0001	0.2158	<0.0001	<0.0001	<0.0001	

continue….

Substrate/ Day	SG	MO	SBW	WP	RB	MECM	*p - value*
				Ash (g/100g DM)			
7	$19.45 \pm 0.21^{z,a}$	$18.37 \pm 0.57^{xy,ab}$	$10.34 \pm 0.65^{z,c}$	$16.34 \pm 0.50^{z,b}$	$10.34 \pm 0.52^{x,c}$	$5.18 \pm 0.11^{v,d}$	<0.0001
9	$20.14 \pm 0.43^{z,a}$	$18.81 \pm 0.50^{xy,ab}$	$11.56 \pm 1.09^{wx,c}$	$16.96 \pm 0.31^{yz,b}$	$7.34 \pm 0.49^{y,d}$	$4.25 \pm 0.06^{w,e}$	<0.0001
11	$20.89 \pm 0.91^{yz,a}$	$18.12 \pm 0.59^{y,b}$	$11.55 \pm 0.58^{wx,c}$	$16.99 \pm 0.42^{yz,b}$	$7.36 \pm 0.19^{y,d}$	$3.80 \pm 0.11^{wx,e}$	<0.0001
13	$21.72 \pm 0.58^{xyz,a}$	$18.82 \pm 0.40^{xy,b}$	$12.87 \pm 0.64^{vwx,c}$	$17.58 \pm 0.36^{xyz,b}$	$7.23 \pm 0.14^{y,d}$	$3.66 \pm 0.19^{yz,e}$	<0.0001
15	$22.46 \pm 0.44^{wx,a}$	$19.50 \pm 0.44^{xy,b}$	$13.46 \pm 0.31^{vw,d}$	$17.91 \pm 0.18^{wxyz,c}$	$5.96 \pm 0.24^{wx,e}$	$3.22 \pm 0.07^{yz,f}$	<0.0001
17	$23.25 \pm 0.38^{wx,a}$	$19.79 \pm 0.57^{xy,b}$	$13.96 \pm 0.20^{vw,d}$	$18.23 \pm 0.20^{wxy,c}$	$5.57 \pm 0.23^{z,e}$	$3.03 \pm 0.05^{z,f}$	<0.0001
19	$23.49 \pm 0.38^{wx,a}$	$20.67 \pm 0.75^{xy,b}$	$14.64 \pm 0.14^{v,c}$	$19.05 \pm 0.35^{wx,b}$	$6.86 \pm 0.39^{yz,d}$	$2.87 \pm 0.05^{z,e}$	<0.0001
21	$24.06 \pm 0.19^{w,a}$	$20.83 \pm 0.65^{x,b}$	$15.29 \pm 0.22^{v,c}$	$19.34 \pm 0.52^{w,b}$	$6.90 \pm 0.12^{yz,d}$	$2.98 \pm 0.09^{z,e}$	<0.0001
p-value	<0.0001	0.0154	<0.0001	<0.0001	<0.0001	<0.0001	

[abcde]Means ± standard error of the mean in a g/100g DM within row showed that the comparison between treatments by day followed with different superscripts are significantly different ($p < 0.05$).

[vwxyz]Means ± standard error of the mean in a g/100g of a DM basis within the column showed that the comparison within treatment by day followed with different superscripts are significantly different ($p < 0.05$).

SG=*Sesbania grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

3.3.2.3 Crude Fat Content of Black Soldier Fly Larvae

The fat accumulation increased over the 14 days of measurement (Figure 27). Four substrates (MO, SG, WP, SBW) achieved a final crude fat content of around 20 g/100gDM (17.63—21.52 g/100g DM). Larvae reared on RB and MECM accumulated significantly more fat (30.75 and 54.12 g/100g DM, respectively). There was a significant difference ($p < 0.0001$) in crude fat (Cfat) content between larvae reared on different substrates (Table 11). At the early stage of development, larvae reared on RB and MECM accumulated fat at the highest concentration of 20.00 ± 0.42 g/100g DM and 43.18 ± 0.67 g/100g DM, respectively. While SG-, MO-, or WP-fed larvae began fat accumulation at 6.19 ± 0.22, 6.59 ± 0.58 and 9.47 ± 0.47 g/100g DM, respectively, followed by a rapid increase at day 9 and a relatively modest increase after that. The average Cfat content of BSFL reared on MECM, RB, SBW, WP, MO, and SG was 52.6 g/100g, 27.8 g/100g, 20.0 g/100g, 14.9 g/100g, 14.8 g/100g, and 9.6 g/100g on a DM, respectively. Cfat in BSFL was low at the initial stage but rose significantly with age, particularly over the 14-day development period ($p < 0.0001$). The larvae reared on MECM, and RB had notably high-fat content, which reflects the high-fat content of these substrates (Table 6 in Chapter 2). The SG, which had the lowest Cfat content amongst the six substrates, justifiably produced larvae with the lowest Cfat that ranged from 6.19 ± 0.22 to 17.63 ± 0.06 g/100g. There was a strong correlation between the fat content of the substrate and the amount accumulated in the larvae ($r = 0.8176$, $p < 0.0001$, Figure 28). Larvae reared on SBW achieved the highest level of fat on day 13 (22.25 ± 1.97 g/100g DM), WP on day 15 (19.86 ± 1.06 g/100g DM), RB-fed larvae (31.76 ± 0.84 g/100g DM) and MECM-fed larvae (54.98 ± 2.14 g/100g DM) on day 17, while SG- and MO-fed larvae later on day 21.

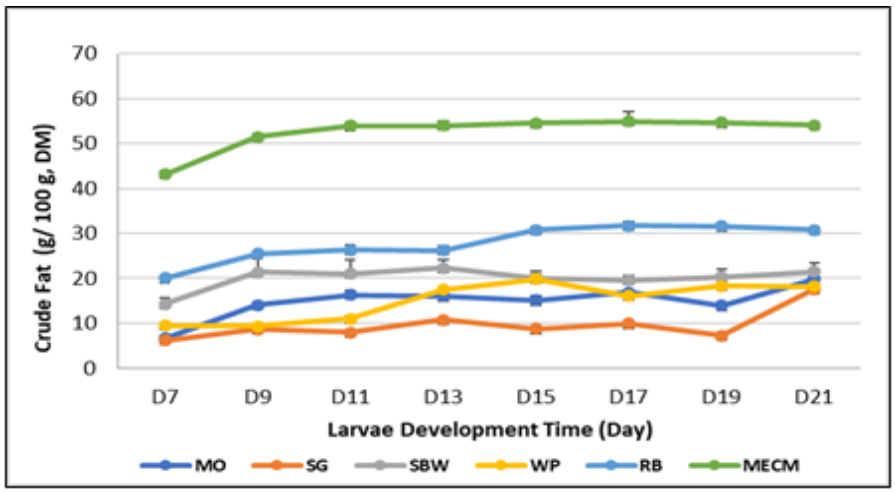


Figure 27: Crude fat (Cfat, g/100g DM) content of BSFL reared on *Moringa oleifera* (MO) and *Sesbania grandiflora* (SG) leaf meals and AIBPs: soybean waste (SBW), wheat pollard (WP), rice bran (RB), and milk-extracted coconut meat (MECM) over development time. Error bars represent the standard errors of the mean larval Cfat of quadruplicate trays. D7-D21=Sampling of larvae between days 17 and 21.

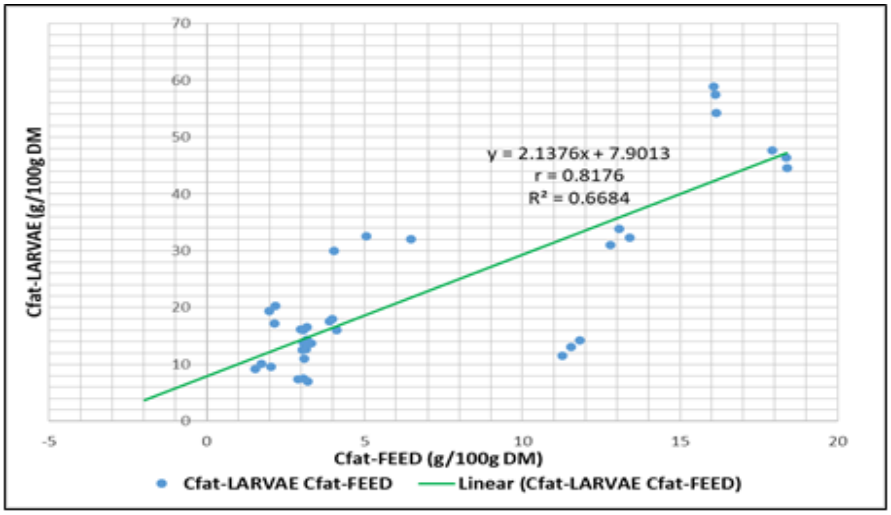


Figure 28: Linear relationship between the crude fat of BSFL produced from six feed substrates with varying crude fat contents.

3.3.2.4 Ash Content of Black Soldier Fly Larvae

The ash content of BSFL significantly increased during their development, especially in those reared-on SG, MO, SBW, and WP. SG- and MO-fed larvae contained ash ranging from 19.45 ± 0.21 to 24.06 ± 0.19 g/100g and from 18.12 ± 0.59 to 20.83 ± 0.65 g/100g DM, respectively (Table 11). The amount of larval ash was greater than those found in the substrates, except for RB-fed larvae (averaging 7.2 g/100g DM), which had ash levels similar to those found in the substrate (6.48 g/100g DM). Throughout the larval development phase, highly significant changes in ash content were observed within ($p \leq 0.0154$) and between treatments ($p < 0.0001$). Besides that, there was a positive correlation between the ash content of substrate and larvae (r = 0.57843, $p = 0.0002$, Figure 29). Larvae reared on MO, and SG leaf meals contained higher ash than larvae raised on AIBP. MECM-fed larvae had the lowest ash level, averaging 3.6 g/100g, since the ash content of this substrate was the lowest amongst the substrates, at only 1.27 g/100g DM.

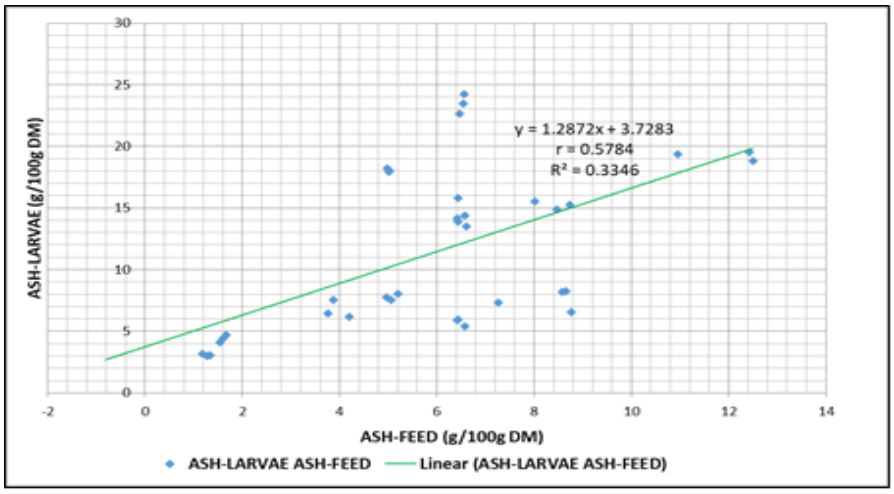


Figure 29: Linear relationship between ash of BSFL produced from six feed substrates with varying ash contents.

3.3.2.5 The Amino Acid Profiles of Black Soldier Fly Larvae

Both essential and non-essential AAs were measured in BSFL on day 17. Highly significant differences ($p < 0.0001$, Table 12) were obtained between the levels of AAs in BSFL reared on six feed substrates. The larvae contained the highest concentration of glutamic acid in comparison to the feeding substrates (Table 7 in Chapter 2). Glutamic acid levels were highest in RB-fed larvae at 5.19 ± 0.11 g/100g DM and lowest in larvae reared on MECM at 2.82 ± 0.02 g/100g DM. Leucine, an essential and its often-limiting AA in plants, was present at a higher concentration of about 3.70 g/100g DM in larvae reared on SBW, WP, and RB than in larvae reared on leaf meals. In comparison, tryptophan was the most abundant AA in SG-fed larvae (1.60 g/100g DM) but available in low concentrations in MECM-fed larvae. Methionine, often a limiting amino acid in plant foods, was present in all larvae at concentrations between 0.5 and 1.8 g/100g DM, with the highest levels in SBW-fed larvae. While other AAs, such as histidine, were abundant in RB, and tyrosine was abundant in SG, SBW, WP, and RB. In general, larvae reared on RB had higher amounts of essential and non-essential AAs than those fed other substrates. The total AAs of larvae on the six feed substrates, namely, SG, MO, SBW, WP, RB, and MECM, were around 43.33 ± 1.11, 43.26 ± 1.00, 45.09 ± 0.28, 50.76 ± 0.23, 49.75 ± 0.71, and 27.81 ± 0.10 g/100g DM, respectively, which was close to the total CP in the larvae.

Table 12: The amino acid profiles of BSFL on day 17 reared on the selected leaf meals and agro-industrial by-products.

Substrate/ Amino Acid	SG	MO	SBW	WP	RB	MECM	p-value
Aspartic Acid	3.74 ± 0.11^{a}	3.33 ± 0.05^{a}	3.48 ± 0.01^{a}	3.29 ± 0.12^{a}	3.59 ± 0.05^{a}	1.78 ± 0.15^{b}	< 0.0001
Arginine	2.07 ± 0.06^{d}	2.74 ± 0.06^{c}	3.03 ± 0.00^{b}	3.51 ± 0.04^{a}	2.85 ± 0.06^{bc}	1.74 ± 0.03^{e}	< 0.0001
Proline	2.12 ± 0.06^{d}	2.97 ± 0.06^{bc}	2.85 ± 0.05^{c}	3.42 ± 0.00^{a}	3.22 ± 0.03^{ab}	2.23 ± 0.11^{d}	< 0.0001
Serine	1.52 ±0.04^{e}	2.51 ± 0.06^{b}	2.23 ± 0.01^{c}	3.05 ± 0.04^{a}	2.83 ± 0.05^{a}	1.83 ± 0.03^{d}	< 0.0001
Glutamic Acid	5.79 ±0.16^{a}	4.78 ± 0.11^{b}	4.08 ± 0.08^{c}	4.65 ± 0.00^{b}	5.19 ± 0.11^{b}	2.82 ± 0.02^{d}	< 0.0001
Glycine	2.74 ± 0.08^{c}	3.08 ± 0.10^{c}	2.82 ± 0.00^{c}	3.99 ± 0.04^{a}	3.51 ± 0.06^{b}	1.94 ± 0.04^{d}	< 0.0001
Alanine	3.14 ± 0.09^{b}	2.82 ± 0.10^{b}	3.05 ± 0.07^{b}	3.18 ± 0.04^{b}	3.81 ± 0.05^{a}	2.08 ± 0.03^{c}	< 0.0001
Cysteine	2.38 ± 0.05^{a}	1.46 ± 0.01^{d}	1.66 ± 0.02^{c}	2.46 ± 0.02^{a}	1.93 ± 0.01^{b}	1.25 ± 0.03^{e}	< 0.0001
Valine*	2.47 ± 0.07^{b}	2.44 ± 0.05^{b}	2.81 ± 0.08^{a}	2.99 ± 0.04^{a}	2.85 ± 0.04^{a}	1.68 ± 0.01^{c}	< 0.0001
Methio-nine*	0.46 ± 0.00^{f}	1.27 ± 0.01^{d}	1.77 ± 0.01^{a}	1.49 ± 0.00^{c}	1.63 ± 0.01^{b}	1.01 ± 0.01^{e}	< 0.0001
Isoleucine*	1.48 ± 0.04^{c}	1.99 ± 0.04^{b}	2.22 ± 0.07^{ab}	2.29 ± 0.03^{a}	2.20 ± 0.05^{ab}	1.34 ± 0.01^{c}	< 0.0001
Leucine*	2.63 ± 0.07^{c}	3.22 ± 0.09^{b}	3.67 ± 0.13^{a}	3.68 ± 0.05^{a}	3.70 ± 0.07^{a}	2.26 ± 0.00^{c}	< 0.0001
Tyrosine*	4.65 ± 0.13^{a}	2.37 ± 0.03^{c}	2.80 ± 0.05^{b}	2.99 ± 0.03^{b}	2.66 ± 0.03^{bc}	1.30 ± 0.04^{d}	< 0.0001
Phenyl-alanine*	1.35 ± 0.04^{b}	2.04 ± 0.06^{a}	2.13 ± 0.06^{a}	2.19 ± 0.02^{a}	2.11 ± 0.04^{a}	0.95 ± 0.01^{c}	< 0.0001
Histidine*	1.49 ± 0.04^{b}	1.66 ± 0.04^{b}	1.52 ± 0.06^{b}	2.17 ± 0.01^{a}	2.20 ± 0.01^{a}	0.88 ± 0.02^{c}	< 0.0001
Trypto-phan*	1.60 ± 0.04^{a}	0.29 ± 0.00^{c}	0.68 ± 0.00^{b}	0.68 ± 0.00^{b}	0.73 ± 0.00^{b}	0.23 ± 0.00^{c}	< 0.0001
Lysine*	2.54 ± 0.07^{a}	2.11 ± 0.08^{b}	2.17 ± 0.05^{b}	2.12 ± 0.00^{b}	2.28 ± 0.03^{ab}	1.17 ± 0.00^{c}	< 0.0001
Threonine*	1.18 ± 0.03^{c}	2.19 ± 0.06^{b}	2.12 ± 0.01^{b}	2.62 ± 0.04^{a}	2.47 ± 0.05^{a}	1.34 ± 0.00^{c}	< 0.0001
ΣAA	43.33 ± 1.11	45.06 ± 1.00	45.09 ± 0.28	50.76 ± 0.23	49.75 ± 0.71	27.81 ±0.10	
Σnon-EAA	23.50 ± 0.65	23.68 ± 0.55	23.20 ± 0.23	27.54 ± 0.30	26.92 ± 0.41	15.67 ± 0.32	
ΣEAA	19.83 ± 0.55	19.38 ± 0.48	21.89 ± 0.50	23.22 ± 0.23	22.82 ± 0.32	12.14 ±0.07	

^{abcd}Means ± standard error of the mean is in g/100 g on a DM basis (n=3) in the same row followed by different superscripts are significantly different at $p < 0.0001$.
ΣAA=total amino acid, Σnon-EAA=total non-essential amino acid, ΣEAA=total essential amino acid. *Indicates essential amino acids.
SG=*Sesbania grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

3.3.2.6 The Fatty Acid Profiles of Black Soldier Fly Larvae

The fatty acid (FA) profiles of BSFL (Table 13) were shown to be significantly different among feed substrates, except for palmitic (C16:0, p = 0.1071) and stearic acid (C18:0, p = 0.1898). All larvae contained FAs with a carbon chain length of 12, 14, and 18 carbons regardless of the feed substrates. MECM-fed larvae exhibited the highest lauric acid content of 41.34 ± 0.40% of total FA, followed by SBW-fed larvae of 35.40 ± 4.79% of total FA, and the lowest was RB-fed larvae of only 9.84 ± 0.40% of total FA. Palmitic acid (C16:0) and stearic acid (C18:0) accounted for a large percentage of the FAs found in the larvae, ranging from 9.19 ± 1.57% to 26.51 ± 2.51% and 9.57 ± 0.02% to 21.19 ± 0.18% of total FA, respectively. Heptadecanoic acid (C17:0) was lacking in SBW- and MECM-fed larvae, although the substrates contained these FAs. MO-fed larvae were rich in linolenic acid (ALA, C18:3n3) at 7.57 ± 0.18% while, linoleic acid (C18:2n6) was identified as a significant percentage in larvae reared on WP (23.73 ± 2.10%) but was found in low parts in larvae fed with other substrates such as SBW, MECM, and MO ranging from 2.79 ± 0.27% to 4.98 ± 0.06%. Linoleic acid was higher in larvae reared on SG than MO larvae. Oleic and ALA were not found in SBW-fed larvae, possibly due to a small amount of this FA in the substrate. There were great similarities between the FA composition of larvae and the substrate, whereby palmitic and linoleic acid were some of the most common FAs found in the substrates (ranged 0.3 to 32% and 11.2 to 37% of total FA, respectively). RB-fed larvae contained eicosatrienoate (C20:3n3) at 12.43 ± 1.06%, whereas other larvae were not detected, apart from SBW-fed larvae containing only a small percentage. Eicosapentaenoic acid, EPA (C20:5n3) and docosahexaenoic acid, DHA (C22:6n3) were detected in trace amounts in RB- and WP-fed larvae, respectively. Most larvae had high percentages of saturated fatty acids (SFA) of up to 90%, followed by polyunsaturated fatty acids (PUFA) of up to 26% and monounsaturated fatty acids (MUFA) of up to 19%. SBW-fed larvae have the highest SFA (93.04± 0.66% of total FA), followed by MECM-fed larvae (85.04 ± 0.27% of total FA), while WP-fed larvae contained the lowest at 54.26 ± 4.65%. The highest MUFA was in WP-fed larvae, especially in cis-oleic acid (C18:1n9c), followed by larvae reared on RB, and was absent in MO- and SBW-fed larvae.

Table 13: The fatty acid profiles of BSFL on day 17 reared on selected leaf meals and agro-industrial by-products.

Substrate / Fatty acid (%, of total FA)	**SG**	**MO**	**SBW**	**WP**	**RB**	**MECM**	***p*-value**
Lauric Acid (C12:0)	28.08 ± 0.21^{b}	27.31 ± 0.19^{b}	35.40 ± 4.79^{b}	27.46 ± 2.40^{b}	9.84 ± 0.40^{c}	41.34 ± 0.40^{a}	0.0007
Myristic Acid (C14:0)	5.82 ± 0.01^{b}	5.72 ± 0.03^{b}	6.44 ± 0.63^{b}	6.27 ± 0.64^{b}	2.56 ± 0.04^{c}	16.28 ± 0.02^{a}	<0.0001
Myristoleic Acid (C14:1)	ND	ND	ND	ND	ND	1.06 ± 0.04	-
Pentadecanoic Acid (C15:0)	0.43 ± 0.02^{c}	1.25 ± 0.03^{b}	3.53 ± 0.19^{a}	0.09 ± 0.01^{c}	1.26 ± 0.05^{b}	ND	<0.0001
Palmitic Acid (C16:0)	17.98 ± 0.14	22.99 ± 0.13	26.51 ± 2.51	9.19 ± 1.57	21.44 ± 1.52	13.32 ± 0.02	0.1071
Palmitoleic Acid (C16:1)	4.71 ± 0.15^{b}	6.34 ± 0.00^{a}	3.29 ± 0.36^{c}	3.36 ± 0.25^{c}	3.79 ± 0.22^{bc}	3.55 ± 0.10^{bc}	0.0004
heptadecanoic Acid (C17:0)	0.93 ± 0.03^{b}	1.31 ± 0.01^{b}	ND	0.33 ± 0.01^{c}	2.10 ± 0.13^{a}	ND	0.0002
cis-10-heptadecanoic Acid (C17:1)	2.33 ± 0.06^{a}	1.34 ± 0.15^{c}	ND	ND	2.33 ± 0.14^{a}	ND	0.019
Stearic Acid (C18:0)	9.57 ± 0.02	21.19 ± 0.18	19.83 ± 8.10	10.74 ± 0.75	18.33 ± 1.67	12.64 ± 0.32	0.1898
cis-oleic Acid (C18:1n9c)	8.84 ± 0.17^{ab}	ND	ND	15.56 ± 1.97^{a}	10.99 ± 2.61^{ab}	5.96 ± 0.00^{b}	0.0536
Linoleic Acid (C18:2n6)	6.92 ± 0.05^{c}	4.98 ± 0.06^{c}	2.79 ± 0.27^{c}	23.73 ± 2.10^{a}	13.19 ± 0.28^{b}	4.67 ± 0.09^{c}	<0.0001
Linolenic Acid (C18:3n3)	2.22 ± 0.02^{b}	7.57 ± 0.18^{a}	ND	2.20 ± 0.24^{b}	0.47 ± 0.03^{c}	ND	<0.0001
Arachidic acid (C20:0)	12.17 ± 0.30^{a}	ND	1.34 ± 0.68^{b}	0.18 ± 0.12^{b}	0.92 ± 0.02^{b}	1.15 ± 0.05^{b}	<0.0001
Eicosenoic (C20:1n9)	ND	ND	0.87 ± 0.03^{a}	0.55 ± 0.06^{b}	0.15 ± 0.03^{c}	ND	0.0025

continue…

Substrate / Fatty Acid (%, of total FA)	SG	MO	SBW	WP	RB	MECM	*p*-value
Methyl 11,14,17-eicosatrienoate (C20:3n3)	ND	ND	0.18 ± 0.04[b]	ND	12.43 ± 1.06[a]	ND	0.0032
Eicosapentaenoic Acid (C20:5n3)	ND	ND	ND	ND	0.23 ± 0.03	ND	-
Docosahexaenoic Acid (C22:6n3)	ND	ND	ND	0.34 ± 0.13	ND	ND	-
ΣSFA	74.98 ± 0.32	79.77 ± 0.07	93.04± 0.66	54.26 ± 4.65	56.44 ± 3.61	85.04 ± 0.27	
ΣMUFA	15.88 ± 0.38[b]	7.69 ± 0.16	4.16 ± 0.39	19.47 ± 2.16	17.25 ± 2.28	10.57 ± 0.05	
ΣPUFA	9.14 ± 0.07	12.54 ± 0.23	2.79 ± 0.27	26.27 ± 2.48	26.32 ± 1.34	4.70 ± 0.09	

[abcd]Means ± standard error of the mean in percentage (n=3) within row followed with different superscripts are significantly different at $p < 0.05$. ΣSFA=Total Saturated Fatty Acid, ΣMUFA=Total Monounsaturated Fatty Acid, ΣPUFA=Total Polyunsaturated Fatty Acid. ND=not detected. SG=*Sesbania grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

3.3.3 Development Time of Black Soldier Fly Larvae

On average, the BSF eggs hatched after three days of incubation on feed substrates. Development time was significantly influenced ($p < 0.0001$) by feed substrates (Table 14). Larvae reared on AIBPs developed into prepupae earlier than those fed with leaf meals. The colour of the larvae varied according to the substrate colour. The SBW-fed larvae were brighter than RB-fed larvae, whereas MECM-fed larvae were lighter cream (Figure 30 and Figure 31). The SBW-fed larvae took slightly less time (12.75 ± 0.25 days) to develop into prepupae, whereas on WP and RB, the larvae took 14.25 ± 0.25 and 14.5 ± 0.29 days, respectively. The BSFL on MO and SG matured later at 16.5 ± 0.29 and 17.25 ± 0.48 days, respectively. MECM-fed larvae started developing into prepupae at 28.5 ± 0.29 days.

The weight gain of larvae varied significantly ($p < 0.0001$) across substrates, with the maximum weight gain of 38.13 ± 1.17 mg DM observed in RB followed by MECM (27.15 ± 0.51 mg DM), SBW (20.85 ± 0.78 mg DM), SG (16.42 ± 1.28 mg DM), MO (15.89 ± 2.19 mg DM), and WP (11.54 ± 1.59 mg DM). However, larvae reared on WP had the lowest DM average rate of weight gain of only 0.82 mg/day due to their soft body (high moisture). While larvae showed the highest average rate of weight gain reared on RB substrate at 2.72 ± 0.08 (mg/day DM). The larval weight gain and average rate of weight gain were not significantly different between leaf meals and SBW-fed larvae. The average weight gain of BSFL reared on MECM was much higher than SBW and leaf meals.

Table 14: Growth performance and development time of black soldier fly larvae reared on selected feed substrates.

Parameter/ Substrate	SG	MO	SBW	WP	RB	MECM	*p*-value
Development time (day)	17.25 ± 0.48^{b}	16.5 ± 0.29^{b}	12.75 ± 0.25^{d}	14.25 ± 0.25^{c}	14.5 ± 0.29^{c}	28.5 ± 0.29^{a}	<0.0001
Larval weight gain (during 14 days development, mg DM)	16.42 ± 1.28^{cd}	15.89 ± 2.19^{cd}	20.85 ± 0.78^{c}	11.54 ± 1.59^{d}	38.13 ± 1.17^{a}	27.15 ± 0.51^{b}	<0.0001
Rate of weight gain (mg/day DM)	1.17 ± 0.09^{cd}	1.14 ± 0.16^{cd}	1.49 ± 0.06^{c}	0.82 ± 0.11^{d}	2.72 ± 0.08^{a}	1.94 ± 0.04^{b}	<0.0001

^{abcd}Means ± standard error of the mean (n=4) within row followed with different superscripts are significantly different ($p < 0.0001$).
SG=*Sesbania grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

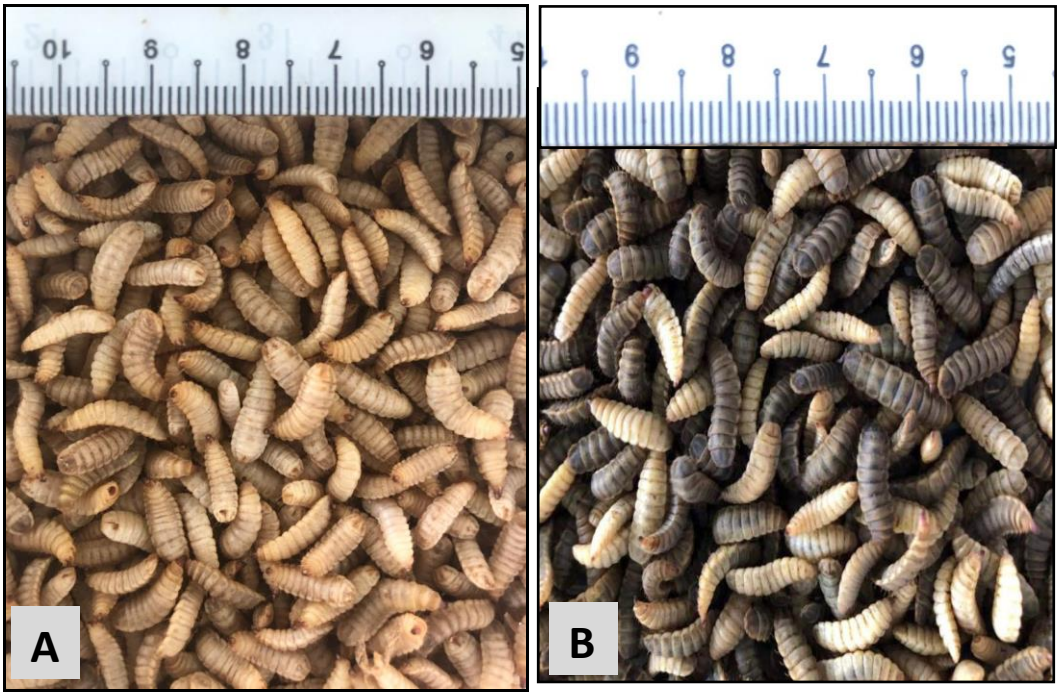


Figure 30: The BSFL reared on *Sesbania grandiflora* leaves on day 13 (A) turned darker and started to develop into prepupae (B).



Figure 31: The BSFL reared on milk-extracted coconut meat (A) are lighter coloured than the rice bran-fed larvae (B).

3.3.4 Harvesting Age of Black Soldier Fly Larvae

It is crucial to identify the optimum harvesting age of BSFL reared on the various feed substrates because age impacts the performance of the larvae. Several factors are considered to determine the best harvesting age of larvae, particularly their nutrient contents and growth performance, such as DM, larval wet weight (Figure 32), CP, and crude fat levels (Figure 33) on a given day. The highest mean wet weight of BSFL on six feed substrates of approximately 119 mg was observed on day 17, although it was not significantly different from the weight on days 15 and 19. On the other hand, the mean larval DM was at its peak on day 17 at 25.5 g/100g DM. The quantity of larvae DM is considered one of the key factors in determining the best age for harvesting larvae and the protein content. Early harvesting of the BSFL is not practical due to its smaller size, lighter weight, and high moisture content. The CP content is at its highest level during the early age of development. However, with the increase in total body weight, the total amount of CP produced increases concurrently. The total protein content is used to guide the decision-making process of the best time to harvest. There were no significant impacts of the substrate on protein content, but the highest mean level of 50.5 g/100 g occurred on day 7, compared to 46.7 g/100g DM on day 9, followed by 45.2 g/100g DM on day 17. The CP and DM contents of BSFL are inversely related because at high levels of CP during the early developmental stage, such as on day 7 and day 9, the DM content is at its lowest. Day 17 would be the best day for harvesting BSFL because the CP content of larvae was high in most individual substrates, such as SG, WP, and RB.

Additionally, the development-related increase in larval weight and dry matter larvae produced more protein on day 17 compared to days 15 or 19 and early development days. Besides, the mean fat content in larvae was 35.8 g/100g on day 21 and 24.9 g/100g on day 17. The mean Cfat of larvae on six feed substrates at day 17 was similar to days 13, 15, and 19. The fat content of the larvae is not a significant determinant in selecting the day for larvae harvesting because a high

content will impose restrictions on feed processing. Still, it is adequate to choose larvae with a moderate fat level. The CP levels showed a gradual decline during larval development, while the percentage of fat present in larvae increased over time. The best day to harvest the larvae was chosen carefully to ensure that the larvae had the optimal amount of nutrients. Thus, considering the above factors, day 17 was identified as the harvesting age of BSFL of the six feed substrates used in the study.

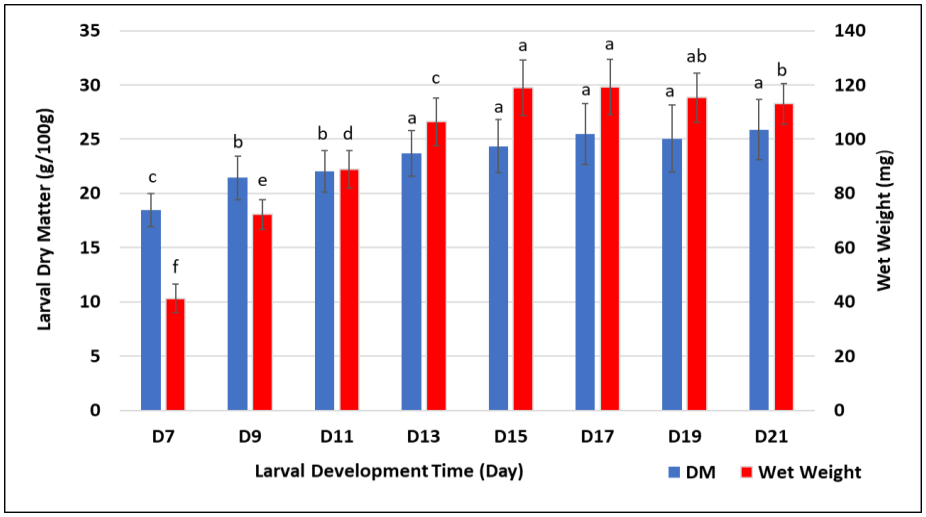


Figure 32: The overall mean of six feed substrates on dry matter (DM) and wet weight of BSFL at different sampling ages. D7-D21=Sampling of larvae between days 7 and 21.

[abc]The mean values indicated by different superscripts within the same parameter (DM, g/100g) are significantly different ($p < 0.05$). Error bars represent the standard errors of the mean of larval dry matter (n=24).

[abcdef]The mean values indicated by different superscripts within the same parameter (wet weight, mg) are significantly different ($p < 0.05$). Error bars represent the standard errors of the mean larval of wet weight from six feed substrates (n=24).

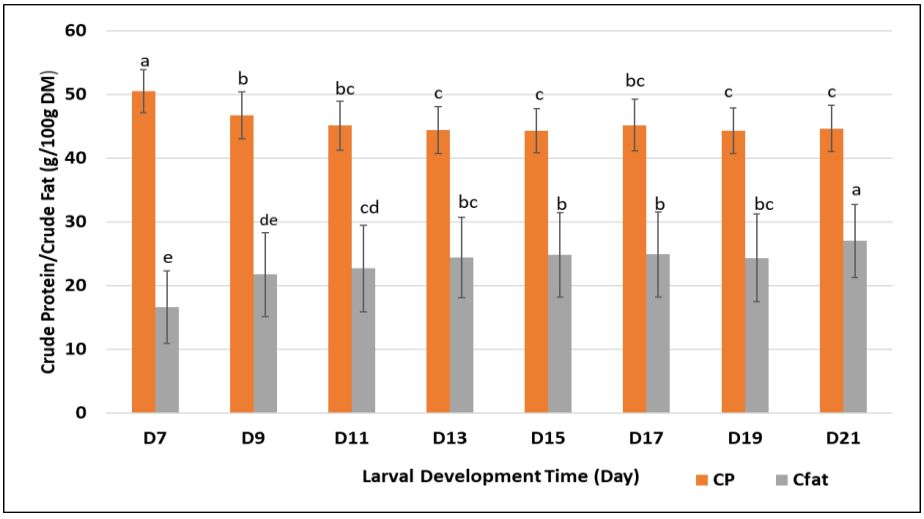


Figure 33: The overall mean of six feed substrates on crude protein (CP) and crude fat (Cfat) of BSFL at different sampling ages. D7-D21=Sampling of larvae between days 7 and 21.

[abcd]The mean values indicated by different superscripts within the same parameter (CP and Cfat, g/100g DM) are significantly different ($p < 0.05$). Error bars represent the standard errors of the mean of larval crude protein (n=24) and crude fat (n=24).

3.3.5 The Growth Performance of Black Soldier Fly Larvae

3.3.5.1 Total Yield of Black Soldier Fly Larvae

As indicated in Table 15, the fresh larval yield differed significantly between substrates (p = 0.0032); however, it was not different between high- and moderate-protein substrates. From a 0.25 g egg, the total number of larvae in the trays was varies which effected the yield of larvae in each substrate. In order to standardise the production performance of larvae, the results were reported based on 1000 larvae. The MECM produced the lowest yield (389.68 ± 50.22 g), whereas SBW produced the most (682.21 ± 49.58 g), although not significantly different from the other substrates. The SBW-fed larva had the highest weight of 145.18 ± 7.27 mg, followed by SG-fed larvae of 131.07 ± 7.04 mg, and the lowest was larva reared on WP, RB and MECM (p = 0.0135). Larva reared on RB of about 114.17 ± 1.37 mg, which was not significantly different from larva on MO, WP and MECM. There were no significant differences in the total number of larvae between the feed substrates (p > 0.05). However, in dry matter yield, the RB-fed larvae had the highest weight (133.56 ± 5.19), followed by MECM-fed larvae (111.08 ± 5.26 g) and the lowest (68.39 ± 3.45 g) were larvae reared on WP (p < 0.0001). There were no significant differences in the total number of larvae between the feed substrates (p > 0.05).

3.3.5.2 Feed Reduction and Bioconversion Efficiency

The performance of larvae is determined by the efficiency by which the larvae convert the dry matter in the substrate into biomass. In this study, the BSFL were shown to have the ability to reduce the amount of DM in six feed substrates with significantly different (p = 0.0003) in reduction percentages (Table 15). The DM of MO had the best-reduced substrate with the least residue of 24.97%, while MECM exhibited less feed reduction, where the remaining residue was 47.6%. Larvae consumed more on the high-nutrient substrates than other low-nutrient substrates

due to the palatability of the substrates. The bioconversion efficiency was statistically different (p = 0.0215) among all substrates. During the developmental period of 17 days, the DM of substrates reduced by 52.40 ± 2.80 – 75.03 ± 2.29%, of which 14.05 ± 0.96 – 22.20 ± 0.72% of the DM was converted into larval biomass. There were significant differences in bioconversion efficiency in dry matter (BCEDM) among larvae reared on the six feed substrates (p = 0.0123). The BCEDM of larvae reared on MO, SG, and SBW substrates was higher than that of larvae reared on moderate- and low-protein substrates, which were around 14%.

3.3.5.3 Protein Conversion Efficiency

The larvae fed on the substrates demonstrated different protein accumulation patterns according to the substrate they were reared on (Table 15). Protein conversion efficiency measures how well the larvae utilise the protein present in the substrate and convert it into their protein. The protein conversion efficiency of the larvae differed according to feed substrate (PrCEDM), which was significantly different (p < 0.0001) from each other. MECM had an excellent PrCEDM, at 138.79 ± 9.31%, followed by RB at 57.42 ± 2.80%. In contrast, the lowest efficiency of protein conversion was observed in high-protein substrates, SG- and MO-fed larvae at around 25.8%.

3.3.6 Cost of Substrates and Larvae

The cost of substrate used for rearing BSFL on SG, MO, SBW, WP, RB and MECM for 17 days was RM15.15, RM18.65, RM3.73, RM6.26, RM6.01 and RM0.81, respectively. The cost of substrate used to produce larvae on MECM was the lowest due to the lowest price of this substrate, which was only 0.20/kg, while the highest cost of the substrate was underutilised plant leaves, SG and MO, whereby leaves were sold for RM4/kg and RM2/kg, respectively. Due to that, the production of larvae on MECM showed the lowest cost of RM23.31/kg DM compared to the larval from SBW, WP, and RB substrates at RM39.32/kg, respectively RM103.77/kg, and RM108.65/kg in DM ($p < 0.0001$), respectively (Table 15). It is worthwhile to use MECM as a feed substrate for rearing BSFL; however, the protein produced from this substrate was the least among other substrates. These experiments are done at a small scale; however, the cost will decrease when it is conducted on a larger scale and in an environment that has been optimised and managed to provide greater output. It is essential to consider feed costs. A higher weight yield could be more cost-effective for the insect breeder when considering cost.

Table 15: Performance of black soldier fly larvae reared on the six feed substrates on day 17.

Substrate / Performance measurement	**SG**	**MO**	**SBW**	**WP**	**RB**	**MECM**	***p*-value**
No. of Larvae (Head)	5216 ± 369^{ab}	5789 ± 253^{a}	4578 ± 385^{ab}	5076 ± 907^{ab}	4658 ± 50^{ab}	3443 ± 585^{c}	0.0547
Individual Larval Wet Weight (mg)	100.04 ± 4.20^{b}	95.84 ± 4.45^{b}	91.37 ± 0.78^{b}	68.39 ± 3.45^{c}	133.56 ± 5.19^{a}	111.08 ± 5.26^{b}	<0.0001
Larval Wet Yield (g)	682.21 ± 49.58^{a}	675.46 ± 24.50^{a}	659.45 ± 25.30^{a}	598.67 ± 76.30^{a}	525.43 ± 6.60^{ab}	389.68 ± 50.22^{b}	0.0032
Larval DM Yield (g)	131.07 ± 7.04^{ab}	116.75 ± 0.94^{ab}	145.18 ± 7.27^{a}	110.57 ± 9.22^{b}	114.17 ± 1.37^{b}	114.73 ± 6.30^{b}	0.0135
Feed Reduction (%, DM)	65.68 ± 2.35^{ab}	75.03 ± 2.29^{a}	70.25 ± 1.39^{a}	64.16 ± 2.84^{ab}	54.54 ± 3.13^{bc}	52.40 ± 2.80^{c}	0.0003
BCEDM (%, DM)	17.62 ± 1.89a^{b}	17.72 ± 0.86a^{b}	22.20 ± 0.72^{a}	13.56 ± 1.51c	14.06 ± 0.96c	14.22 ± 1.88c	0.0123
PrCEDM (%, DM)	25.80 ± 2.69^{c}	25.85 ± 1.15^{c}	46.48 ± 0.46^{bc}	43.93 ± 5.26^{bc}	57.42 ± 2.80^{b}	138.79 ± 9.31^{a}	<0.0001
Cost of Substrate Used (RM)	15.15 ± 0.00^{b}	18.65 ± 0.35^{a}	3.73 ± 0.00^{d}	6.26 ± 0.07^{c}	6.01 ± 0.15^{c}	0.81 ± 0.01^{e}	<0.0001
Cost of Larvae (RM/kg, DM)	228.13 ± 19.41^{a}	262.73 ± 6.36^{a}	39.32 ± 2.23^{c}	103.77 ± 7.39^{b}	108.65 ± 4.72^{b}	23.31 ± 1.64^{c}	<0.0001

^{abcde} Means ± standard error of the mean (n=3) within a row followed with different superscripts are significant differences ($p < 0.05$). The BCEDM and PrCEDM were reported based on 1000 larvae.
BCEDM=Substrate-to-Biomass Conversion Efficiency in dry matter basis, PrCEDM=Protein Conversion Efficiency in dry matter basis.
SG=*Sesbania grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

3.3.7 Larval Nutrient Composition on Day 17

As previously shown, larvae reared on moderate protein substrates, WP and RB produced the greatest concentrations of CP, approximately 55.94 ± 0.82 and 54.33 ± 0.60 g/100g DM, respectively, compared to high protein substrates ($p < 0.0001$, Table 16). The same holds for the Cfat content of larvae, with MECM-fed larvae having the highest amount (46.22 ± 0.89 g/100g DM) and SG-fed larvae the lowest (7.27 ± 0.15 g/100g DM). Larvae on MECM contained the lowest crude fibre (CF) (6.58 ± 0.13 g/100g DM) of all larvae on other feed substrates ($p < 0.05$), as well as neutral detergent fibre, NDF (18.63 ± 0.64 g/100g DM, $p < 0.0001$) and acid detergent fibre, ADF (11.68 ± 1.30 g/100g DM, $p = 0.0002$) levels. In comparison, larvae on WP and RB contained higher NDF at 50.71 ± 3.60 g/100g DM and 42.67 ± 2.21 g/100g DM, respectively. The CF content of larvae showed a significant inversely related to the CF of the feed substrate (r = - 0.51868, $p = 0.0274$, Figure 34), which means that the higher CF in the feed substrate, the lower this nutrient in larvae. Larvae that fed high CF, may take long time to digest fibre from the substrate which in turn will slow the growth and have an impact on their body structure.

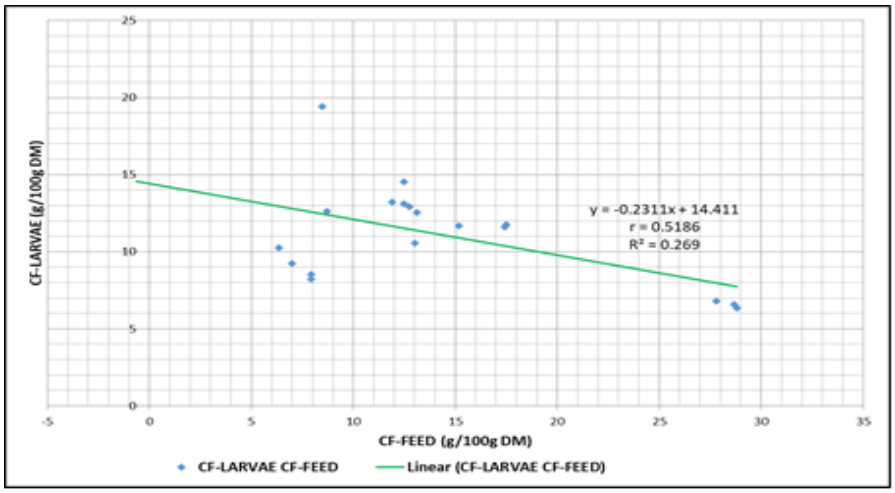


Figure 34: Negative linear relationship between crude fibre of BSFL produced from six feed substrates with varying crude fibre contents.

Table 16: Nutrient composition of black soldier fly larvae on day 17 reared on the six feed substrates.

Substrate / Nutrient composition (g/100g DM)	**SG**	**MO**	**SBW**	**WP**	**RB**	**MECM**	***p*-value**
CP	47.57 ± 0.26^{b}	48.02 ± 0.67^{b}	48.11 ± 0.36^{b}	55.94 ± 0.82^{a}	54.33 ± 0.60^{a}	31.63 ± 0.28^{c}	<0.0001
Cfat	7.27 ± 0.15^{d}	13.59 ± 0.46^{c}	12.95 ± 0.77^{c}	12.44 ± 0.84^{c}	31.54 ± 0.82^{b}	46.22 ± 0.89^{a}	<0.0001
CF	12.53 ± 1.15^{ab}	13.52 ± 3.18^{a}	11.67 ± 0.04^{ab}	13.08 ± 0.09^{ab}	9.23 ± 0.59^{ab}	6.58 ± 0.13^{b}	0.0301
NDF	38.00 ± 1.21^{bc}	37.54 ± 0.88^{bc}	31.06 ± 0.70^{c}	50.71 ± 3.60^{a}	42.67 ± 2.21^{ab}	18.63 ± 0.64^{d}	<0.0001
ADF	28.49 ± 1.48^{a}	24.55 ± 1.31^{ab}	14.55 ± 0.41^{de}	20.53 ± 0.37^{bc}	18.02 ± 0.60^{cd}	11.68 ± 1.30^{e}	0.0002

^{abcde}Means ± standard error of the mean (n=3) within a row followed by different superscripts indicated significant differences across feed substrates ($p < 0.05$).

CP=crude protein, Cfat=crude fat, CF=crude fibre, NDF=neutral detergent fibre, ADF=acid detergent fibre.

SG=*Sesbania grandiflora*, MO=*Moringa oleifera*, SBW=soybean waste, WP=wheat pollard, RB=rice bran, MECM=milk-extracted coconut meat.

3.4 Discussion

Limiting land resources and overfishing in the seas makes it no longer desirable to use soybean meal (SBM) and fishmeal (FM) as the primary source of protein in animal feed (Tacon and Metian, 2009; Van Huis *et al.*, 2013). The use of black soldier fly larval protein as a sustainable alternative to expensive protein sources from fish or plants is gaining attraction (Wasko *et al.*, 2016). BSFL is a bioconverter that processes a range of organic wastes into high protein and fat biomass (Siddiqui *et al.*, 2022). The quality of the meal impacts the growth of BSF, both in the larval and adult stages (Nguyen *et al.*, 2013). The macronutrient composition of feeding substrates, such as protein, fat, and carbohydrate contents (Barragán-Fonseca *et al.*, 2018; Meneguz *et al.*, 2018; Gold *et al.*, 2020) and micronutrients, such as minerals and vitamins, affects the nutritional quality of the BSF body (Barragan-Fonseca *et al.*, 2017; Spranghers *et al.*, 2017); however, it was not clearly understood (Chia *et al.*, 2020). Previous studies have discussed the importance of proteins and carbohydrates in feed substrates for larvae growth (St Hilaire *et al.*, 2007; Nguyen *et al.*, 2015; Tschirner and Simon, 2015; Cammack and Tomberlin, 2017; Beniers and Graham, 2019). On the other hand, the present study considers proteins and fats in the feed substrate. This is due to numerous studies have demonstrated that an insect diet's amount of protein and fat significantly affects its growth (Gobbi *et al.*, 2013; Karasov and Martnez del Rio, 2007).

During BSF development, the contents of larval dry matter (DM), crude protein (CP), and crude fat (Cfat) fluctuated over time. The CP content of larvae increased immediately after hatching, progressively decreasing, and remaining relatively stable. According to Liu *et al.* (2017), the CP increased rapidly and reached its highest level in mature larvae and prepupa. At the early stage of development, the larvae contained a high level of CP due to their requirement of protein in an excess amount for normal growth, development, and energy. However, it declined considerably as the age of larvae increased, irrespective of the rearing substrate used, until the larva pupated and progressed to the next stage of development (Liu

et al., 2017; Cockcroft, 2018). As the larvae developed, they accumulated enough protein to continue their development (Lalander *et al.*, 2019), whereby the energy for growth was also derived from proteins (Brookes and Fraenkel, 1958). The larvae accumulated nutrients present in the diets at an earlier stage of larval development, which also could be supplied by microbes such as bacteria and fungi in the feed substrates (Offor, 2010; Yu *et al.*, 2011). The drop in CP level might be related to the utilisation of protein in enzymatic activities to form the chitin layer (Kramer and Koga, 1986). It might be due to the diluting effect of the high-fat content during the late larvae stage (Cockcroft, 2018). Previous studies reported that the protein content of BSF varied according to feed types (Oonincx *et al.*, 2015b; Liu *et al.*, 2017; Müller *et al.,* 2017) and feeding rate (Diener *et al.*, 2009). Larvae reared on a high protein substrate produced a high level of larval protein, as shown by the larvae on leaf meals and soybean waste (SBW). Surprisingly, the CP level of larvae on moderate protein substrates such as wheat pollard (WP) and RB are comparable and even higher than larvae on high protein substrates (Danieli *et al*., 2019), which may be due to the high digestibility of protein of these substrates of about 95% (Mwesigwa *et al.*, 2013; Han *et al.*, 2015). The protein content of feed substrates is usually seen affecting the protein level of BSFL (Cammack and Tomberlin, 2017; Meneguz *et al*., 2018; Miranda *et al*., 2019), but the effects on the larvae were not always the same. Nguyen *et al.* (2015) reported that larvae reared on higher protein substrates like liver and beef had a higher protein content of 60.3% DM than the 38.5% DM in larvae on vegetable waste. In contrast, other studies reported that protein levels did not influence the nutrient composition in BSFL, as suggested by Daniel *et al.* (2018) and Shumo *et al.* (2019). This is because the high protein concentration increases the acidity of the feed substrates, which affects larval growth. Mineralisation of organic nitrogen into nitrite and nitrate and bioconversion of readily biodegradable organics into intermediate species of organic acids have further lowered the pH of feed substrates, slowing BSFL development and protein levels (Lim *et al.*, 2019). Other studies found that the CP content of BSFL increased as the CP content of the BSFL diet went down (Tschirner and Simon, 2015; Barragan-Fonseca *et al*., 2017). However, the BSF larvae have

been reported to perform better on feedstock with protein levels lower than 30% and are better bioconverters of plant proteins than animal proteins (Nguyen *et al.*, 2015).

In the present study, an equal proportion of protein and fat content in feed substrate are recommended for larval development. Several studies indicate that 20% of CP in feed substrate is sufficient for insect growth (Clifford and Woodring, 1990; Ramos-Elorduy *et al.*, 2002). However, Nyakeri *et al.* (2019) reported that a substrate containing 12.4% protein was optimal for larvae growth. The CP and Cfat ratio of 1:1 in RB substrate with a CP concentration of 12 g/100g and Cfat (11.86 g/100g DM) DM are likely adequate, as demonstrated by RB-fed larvae, whose weight exceeds that of larvae reared on other substrates with higher protein contents, such as leaf meals, with a CP and Cfat ratio that was considerably different. Therefore, utilising more than 20% protein substrates is impractical and may be considered wasteful, given the ideal protein content for larval development. The imbalance in protein and fat levels in the substrates (SG and MO) caused larvae on these substrates gained less weight than RB. In addition, these two macronutrients have been used and stored during larval development. In the MECM substrate, the low CP content of only 3.73 g/100g but high in Cfat (14.8 g/100g DM) could be the possible reason why larvae reared on this substrate had the lowest CP, small size and yield, but contained high fat which more than 50 g/100g DM. The nutritional imbalance had a deleterious effect on larval growth and development.

Overall, the BSFL reared on the six feed substrates had quality AAs and were higher than in other studies (Spranghers *et al.*, 2017; Shumo *et al.*, 2018), especially for the critical AAs in fish feed such as lysine, methionine, and threonine (St-Hillarie *et al.*, 2007; Sealey *et al.*, 2011). The highest level of AAs contents emerged in early larval development (4^{th} - 6^{th} day), followed by a steady decline until larval maturation and remained stable in the late stages of prepupae. Notably, the adult stage had a larger concentration of AAs than other life stages (Liu *et al.*, 2017). Several of the

AA profiles in this investigation were comparable to those in BSFL fed on three organic waste streams such as chicken dung, kitchen waste, and brewers' spent grain (Shumo *et al.*, 2019), except for leucine, which was shown to be significantly higher in all substrates. On the contrary, BSFL produced in this study had lower AA profiles than FM with a CP content of 58.5% (Barreto-Curiel *et al.*, 2016), except for cysteine, which was abundant in all substrates, but it was greater than soybean meal (Chen *et al.*, 2010; Thrane *et al.*, 2017), except for aspartic acid, glutamic acid, arginine, lysine, and phenylalanine.

MECM substrate contained the highest fat, which produced larvae with the highest Cfat amongst the other larvae. The high Cfat content of MECM-fed larvae has resulted in the highest dry matter amongst the other larvae, whereby the dry weight of these larvae was the second highest after RB-fed larvae. As reported by Beniers and Graham (2019), the heavier larvae significantly producing much fat than the lighter larvae. Larvae reared on leaf meals possessed the lowest Cfat in the larvae due to lower Cfat in the substrates (Li *et al.*, 2015; Spranghers *et al.*, 2017; Bava *et al.*, 2019; Danieli *et al.*, 2019). However, Shumo *et al.* (2019) showed no evidence of the effect of feeding substrates on the Cfat content of BSFL. The Cfat levels of MECM- and RB-fed larvae in the present study were considered to have an extremely high-fat content that exceeded the nutritional requirement of 15% for fat in seabass diets (Sakaras *et al.*, 1988; Catacutan and Coloso, 1995) whereby both larvae are useful for producing biodiesel, as suggested by Surendra *et al.* (2016). According to Liu *et al.* (2017), the fat level changed during the development of BSFL. The amount of Cfat in BSFL was low at the initial stage but rose significantly with age, and this pattern was inversely related to the CP content of BSFL, which was high at the beginning of larvae development. The BSFL accumulates fat in their bodies throughout the development period from the feed substrates as energy by a non-feeding adult (Sheppard *et al.*, 1994; Sheppard *et al.*, 2002; Cockcroft, 2018).

The fatty acid (FA) composition is a critical factor in applying BSFL in food and feeds production with fat content, especially the presence of the long-chain

polyunsaturated FA (PUFA) for feeding fish. In the present study, the larval fat was primarily composed of lauric acid (Li *et al.*, 2016; Barroso *et al.*, 2017; Spranghers *et al.*, 2017; Ewald *et al.*, 2020; Lu *et al*., 2022) and other saturated fatty acids (SFA), which the larvae synthesised. Lauric acid exhibits a broad spectrum of antimicrobial activity in combating potential environmental threats such as viruses, protozoa and harmful bacteria that found in organic waste stream (Ramos *et al*., 2009; Gasco *et al*., 2018, Matsue *et al*., 2019), and it is essential for the growth performance and gut health of animals, mainly fish feed contains such larvae (Schiavone *et al*., 2018). Previous studies discovered that the FA content of the substrate affected the larvae's FA profile (Ramos-Bueno *et al.*, 2016; Ewald *et al.*, 2020). For example, MECM substrate and MECM-fed larvae had the highest lauric acid content of the six feed substrates. The high SFA concentration in the larvae might be a concern for aquaculture, because fish needs omega-3 fatty acids for growth (Vidaković, 2015). Thus, it is important to focus on enhancing feed substrate in order to produce BSFL of good quality in PUFA. The FA contents of insects can also vary according to growth stage, temperature, and dietary regime (Kaczmarek *et al.*, 2020). Fat that accumulates throughout larvae development is stored in the form of SFA as energy because this form of FA is less prone to oxidation than unsaturated fatty acids (Ushakova *et al.*, 2016). The lauric acid and other SFA increase as the larvae grow (Liu *et al.*, 2017; Ewald *et al.*, 2020). Other FAs synthesised by the larvae are palmitic acid and heptadecanoic acid methyl ester since these FAs are undetectable in the substrates (Ewald *et al.*, 2020). The nutrient quality of the larvae can be increase by supplementing the feed substrates with fish offal (St-Hillaire *et al*. (2007a), and brown algae (*Ascophyllum nodosum*) (Liland *et al*., 2017). Linoleic and linolenic acids are common in MO and SG substrates and the larvae because the enzymes required to form these two FA are primarily found in plants and marine algae (Tvrzicka *et al.*, 2011) as well as in WP and RB (Ruan *et al*., 2015).

The weight of larvae reduced considerably when they obtained enough energy to initiate to pupa development. Manurung *et al.* (2016) reported a sharp decrease in larval weight gain when they metamorphosed into prepupae. Several factors might

contribute to the differences in the developmental period detected in the present study between feed substrates due to differences in palatability, texture, nutrient content, availability, the presence of anti-nutritional or toxin-producing factors and environmental temperature (Makkar and Becker, 1996; Adeyemo and Onilude, 2013; Harnden and Tomberlin, 2016; Holmes *et al.*, 2016; Raksasat *et al.*, 2020). The presence of anti-nutritional factors (ANFs) usually in plant materials such as tannins, saponins, mimosine, and phytate, although in negligible amounts, might reduce the quality of feed substrate, particularly the nutrient availability (Fojas *et al.*, 1982; Makkar and Becker, 1996), which is likely to occur in larvae reared on SG and MO where the larvae reared on these substrates showed lower CP, CFat, and weight compared to a similar CP in a substrate such as SBW and moderate substrate as well. These may have been caused by the high density of larvae, whereby they compete each other for feed and the limited availability of these substrates. The optimal feeding rate of 100 mg/larva/day (Diener *et al*., 2009) was not reached because there were more larvae than expected, which most likely indicates an error when weighing a 0.25 g egg due to the light weight of larval eggs. Feeding larvae directly with SG and MO leaves involved minimum processing. Still, direct ingestion of leaf meals by insects reduces food utilisation due to lack of enzymes to degrade cellulose and other polysaccharides (Friend, 1958), making it more appropriate to ferment the substrate before feeding the insect (Gao *et al.*, 2019). However, the presence of microbes in the larval gut and substrates may assist in hydrolysing the fibres, making the substrates available for larval growth (Kim *et al.*, 2014). The development time is shorter when the larvae have an adequate feed supply (Manurung *et al.*, 2016; Kinasih *et al.*, 2018; Lalander *et al.*, 2019). In the present study, the time was generally shorter than that observed in previous studies (Abduh *et al.*, 2017; Lalander *et al.*, 2019). The time might differ, although fed on similar substrates. Municipal organic solid waste-fed larvae take 16 – 37 days to reach the prepupae stage (Nyakeri *et al.*, 2017; Tinder *et al.*, 2017), dairy manure and soybean curd residue from 16 – 18 days and 19 – 23 days (Rehman *et al.*, 2017a), respectively. Larvae on semi-digested grass require a longer development time of 70 days which shows that this material is unsuitable for rearing BSFL due to its high

lignin content (Liu *et al.*, 2018). MECM-fed larvae showed a slower development rate due to low-quality nutrients and took a long time to pupate (Wong *et al.*, 2020), which was the same as the larvae reared on fruit and vegetables (Lalander *et al.*, 2019) and dairy manure (Myers *et al.*, 2008). Compensatory feeding on diets with a low concentration of nutrients forced larvae to feed for a more extended period (Gall and Behmer, 2014). Larvae consume more substrate to compensate for specific nutrients lacking in the substrates, particularly protein and carbohydrate, thus increasing the development time (Simpson *et al.*, 2006; Banks *et al.*, 2014).

In the present study, the moisture content of substrates offered to the larvae was standardised to 76 g/100g. During the feeding trial, the RB substrate was infected with a thick layer of fungus, particularly during the first week of larval development. However, there was a reduction in fungus infection when the bioconversion process was active (Tschirner and Simon, 2015). It was noted that the presence of this fungus did not impair the growth performance of RB-fed larvae, as the larvae's growth rate was the highest among other substrates. The colour of the substrates could change as early as 24 h after exposure to BSFL during their active phase where the substrate became darker due to bioconversion activities. The substrates were warm to touch, and there was a smell of urine coming out from the feed substrate due to the release of ammonia during compost formation (Cickova *et al.*, 2015; Lalander *et al.*, 2015). The BSFL were metabolically active during their first week of feeding, which decreased in the following weeks. The dipteran larval mass's metabolic activity generates heat with temperatures as high as 6°C (Brits, 2017), like microbial breakdown, which raises the temperature inside the growth media (Jay, 1998). The moisture content in biowaste of 70 – 80% is suitable and ideal for BSFL growth (Guo-Hui *et al.*, 2014; Liu *et al.*, 2018; Lalander *et al.*, 2020), and separation of BSFL from the residue can be performed easily (Cheng *et al.*, 2017; Dortmans *et al.*, 2017). Low moisture content in feed substrate caused the larvae develop more slowly due to inefficient digestion of waste (Fatchurochim *et al.*, 1989; Cheng *et al.*, 2017), which could not grow on diets containing less than 40% moisture (Cammack and Tomberlin, 2017). Substrates with a high moisture content of more than 80%

create a problem during the harvesting of larvae due to the wet substrates sticking to the larvae body (Diener *et al.*, 2011). On the other hand, it reduces the biomass conversion ratio and survival rate of the larvae (Barragan-Fonseca *et al.*, 2017; Lalander *et al.*, 2020).

The day 17 was chosen as the ideal day for larval harvesting due to the highest CP and weight of larvae, which would produce a higher quantity of protein than other days. The protein levels were increased in most individual substrates, including SG, WP, and RB. Early harvesting is problematic and impractical due to the high moisture content and small weight of the BSFL, which did not have the yield advantage (Yu *et al.*, 2021). Consequently, harvesting the final instar larvae on day 17 was appropriate in light of the nutritional value and performance of the larvae at that time. Using the same larval age for harvesting larvae on the six substrates allowed a more precise comparison of nutrient composition and larval performance during substrate conversion to larval biomass. In addition, greater detail was due to larval growth, and bioconversion occurred actively during the larval stages rather than the prepupal stages. Fowles and Nansen (2019) observed that the optimal timing to harvest BSFL was during the late immature stage to maximise biomass production. However, there is no systematic approach to determining the optimal day to harvest BSFL.

Performance is determined by the efficiency with which the larvae convert the substrate's dry matter into biomass, which depends on the nutrient composition of the waste stream (Brits, 2017; Barragán-Fonseca *et al*., 2018). Waste reduction and bioconversion are practical and straightforward approaches for determining the BSF larvae's feeding efficiency (Banks *et al.*, 2014; Cammack and Tomberlin, 2017; Lalander *et al.*, 2019; Gold *et al.*, 2020). The reduction of RB and MECM waste was the lowest compared to the other substrates; probably, the high-fat content in both substrates did not draw the interest of the larvae to eat them as much as indicated by the other substrates. The waste reduction of more than 50% in the present study was considered high compared to previous studies with other substrates, including

rice, maize straw, and wheat bran (Manurung *et al.*, 2016; Bulak *et al.*, 2018; Gao *et al.*, 2019). In addition, differences between treatments were detected due to the nature and quality of the substrates used (Nyakeri *et al.*, 2019). The feed reduction of substrates reflected the weight of larvae yield harvested on day 17, where a significant decrease in waste produced higher larvae weight (Scala *et al.*, 2020). Bioconversion helps solve the problem of organic waste processing and further benefit the environment. BSFL requires organic wastes rich in digestible nutritional components to function well in bioconversion activity (Kinasih *et al.*, 2020; Leong and Kutty, 2020). Oonincx *et al.* (2015a), on the other hand, stated that dietary protein was the most important factor in feed conversion efficiency. The substrate with higher protein improves bioconversion activity and greatly contributes to biomass yield than those with lower protein content (Lalander *et al.*, 2019). Like feed reduction, the feed bioconversion efficiency is significantly influenced by the number of larvae, feed availability, environmental conditions (such as temperature, humidity, and shaded environment), and dietary composition (Holmes *et al.*, 2012; Parra *et al.*, 2015). The number of larvae in each substrate differed from the expected 3,750, most likely due to various egg batches and parent stock. A 0.25 g of the egg was mixed from several patches of eggs. Mixing eggs from various parent stocks led to eggs functioning differently in terms of hatchability rate and quality, resulting in a variable number of larvae per tray. Larvae grown on MECM contained the less larvae was probably due to high Cfat content. According to Nguyen *et al*. (2015), feed substrate that contained less 90 g/kg was found to have detrimental effects on the survival of BSFL and adults.

A similar trend was observed in protein conversion efficiency, whereby MECM-fed larvae showed the highest efficiency compared to other feed substrates. MECM substrate contained five times more fat than protein than the other substrates. The low PrCEDM of larvae on MO and SG substrates was due to the adequate amount of protein available in the diet that met the growing requirement needs of larvae. The BSFL has been shown to convert poor-quality substrates in nutritional composition into larval biomass with superior nutritive value (Danieli *et al.*, 2019). The midgut

morphology and physiology of larvae on low-nutrient substrate differ from those on high-nutrient feed substrate due to their need to exploit feed nutrients' ingestion (Cappellozza *et al.*, 2019; Bonelli *et al.*, 2020). There were significant differences in gene expression for enzymes involved in metabolic activity in low- and high-protein content of the substrates (Bonelli *et al.*, 2020). The enzymes such as trypsin and trypsin-like, chymotrypsin, chymotrypsin-like, and other serine proteases that are involved in the proteolytic activity are significantly higher in the posterior midgut regions of larvae reared on a lower protein diet such as vegetable mixed diet compared to those on high-protein substrates (Bonelli *et al.*, 2020). On the other hand, the α-amylase activity was absent, which aids in digesting carbohydrates in the middle and posterior of the midgut of larvae when they are reared on low nutrient substrates (Bonelli *et al.*, 2019; Bonelli *et al.*, 2020). Genes involved in protein catabolism, translation, transport, and metabolic activities are upregulated, allowing the midgut to optimise protein catabolism, improve nutrient intake, and maintain high protein synthesis in low protein and energy contents in the diet (Bonelli *et al.*, 2020). These circumstances demonstrate that BSFL can survive on a low-quality diet, such as MECM, and produce larval biomass with a higher nutrient composition than the substrate (Tschirner and Simon, 2015).

The study found that the RB substrate was the best, as seen by the larvae that grew on it. The daily growth of larval weight, the highest weight and largest size are among the advantages of RB-fed larvae. Meanwhile, RB-fed larvae contained high dry matter, protein, and a short development time. However, the AA quality of these larvae is not significantly different from that of other substrates, but it has the highest percentage of PUFA. Although the excellent performance of larvae on RB was observed, this substrate was not chosen to continue the production of BSFL in this study. This is because RB has been used as an ingredient in animal feed, as well as the high cost of larval production and the presence of growth fungi on the substrate, making it uneasy about using this material, which may harmful to the fish in the future. Thus, the MECM substrate was chosen as the feed substrate for biomass production in this study, although the nutritional content of larvae fed

MECM was not as good as larvae fed other substrates. The high dry matter of MECM-fed larvae changes the perception of these larvae, as their dry matter yield was not significantly different from other substrates except for SBW-fed larvae. This choice benefits because this BSFL can convert low-value resources into high-quality biomass. The efficiency of larvae in accumulating protein in MECM was excellent, resulting in the CP of larvae on MECM multiple times that of the substrate protein. This condition makes the BSFL conversion preferable rather than providing it to animals directly with significantly lower nutritional qualities, particularly protein. Additionally, MECM is a biodegradable organic waste with low nutrient content, contributing to the waste's low cost. The utilisation of this waste helps to mitigate pollution to a degree (Siddiqui *et al.*, 2020). The shortcomings of MECM larvae that are low in CP content but high in Cfat can be solved and increased by supplementing with affordable high-nutrient substrates to develop a feed resource suitable for enhancing black soldier fly production (Raksasat *et al.*, 2020). SG is a choice of feed substrate for rearing BSFL because this unexploited plant is nutrient-rich, fast-growing, and easily found in the village area. Thus, to maximise the production of black soldier fly larvae, trials were conducted to evaluate the effect of combining MECM with high protein leaves of SG on larvae yield and nutrient composition. The results from these trials are presented and discussed in the following chapter.

3.5 Conclusions

All six feed substrates supported the development of BSFL growth, which took less than 20 days to enter the prepupal stage, except for MECM-fed larvae. BSFL differed in nutrient composition, depending on the feed substrates they were reared on. In this investigation, the larvae that were produced had a high concentration of either protein or fat, depending on the chemical make-up of the feed that was provided. Exploring neglected or underutilised plant species opens up new avenues in BSFL rearing, allowing for the development of an alternative protein

source to fishmeal in aquafeed. Even though BSFL can be reared on a variety of organic wastes, it's important to know and standardise the nutritional value of the substrates so that the nutrient content of the BSFL can be manipulated and maximised to best suit the dietary needs of the animals, such as fish. Also, using organic waste, such as agro-industrial by-products (AIBPs), to produce larvae helps the environment eliminate polluting materials that may eventually end up in landfills. BSFL reared on meals derived from underutilised plant species, and AIBPs provided indirect benefits to food production by filling the gaps for increased food security in the future. The BSFL showed an excellent capability in reducing feed substrates and converting them into larval biomass in the shortest growth period. The larvae exhibited different protein accumulation patterns based on the protein content of the feed substrate, such as MECM, which resulted in the most significant protein conversion efficiency. Day 17 was selected as the best day to harvest larvae for all feed substrates because the amount of crude protein produced was high due to high dry matter, and the weight of larvae was optimal on this day. Faster growing and shorter harvestable larvae days reared on the feed substrates allow the BSFL to be produced regularly as a sustainable protein source replacement, whereby at least twelve cycles of BSFL production can be performed in a year. The RB substrate was identified as the best substrate because larvae performed at their highest potential on it when compared to other substrates. However, MECM and SG have been chosen to continue the production of BSFL despite the limitations of the larvae produced. These two substrates are combined through a series of combinations in Chapter 4 to produce larvae that are better in terms of performance and food composition compared to a single feed substrate, to be used as a substitute for FM in the diet of Asian seabass.

CHAPTER 4

PRODUCTION OF BLACK SOLDIER FLY LARVAE ON STANDARDISED FEED SUBSTRATES

4.1 Introduction

Previous research has concentrated exclusively on using low-cost organic waste with low nutrient contents for growing black soldier fly larvae (BSFL) to reduce the voluminous organic solid waste via quick composting by larvae (Lim *et al.*, 2019). The BSFL that feeds on a single feed substrate containing low and imbalanced nutrient content will increase feed consumption levels to compensate for the low nutritional quality (Wright *et al.* 2003). Due to the nutritional composition of BSFL is influenced by the substrate used, dietary changes can increase the nutrient constituent of larvae (Anderson, 2000) and produce quality larvae (Liland *et al.*, 2017; Barragan-Fonseca *et al.*, 2018; Wong *et al.*, 2019). Researchers are currently producing BSFL by fortifying dietary components and increasing the substrate's absorption capacity in combination with organic waste (Rehman *et al.*, 2017; Julita *et al.*, 2018; Lim *et al.*, 2019).

The nutrient composition of larvae, particularly the protein content, can be improved by increasing the proportion of high-protein waste in the feed substrate (Lim *et al.*, 2019). High-quality feed substrates accelerate larvae development and improve feed conversion ratios and the ability to survive in some insect species (de Haas *et al.*, 2006). The resulting increase in biomass production efficiency and waste reduction capacity will enhance the BSF technology making it more attractive for waste conversion activities (Nyakeri *et al.*, 2019). Mixing nutritionally separated single streams into the mixed substrate may increase the performance of BSF (Rehman *et al.*, 2017a; 2017b). Larvae consume more nutrients from the mixed feed with improved nutrient composition, developing into more significant individuals

due to the diet's increased digestibility (Brits, 2017). Combining two ingredients in a mixed feed substrate may provide an alternative method of managing surplus by-products and compensating for nutrient deficiencies in any substrates to produce a better-tasting or more palatable product (Bonelli *et al*., 2020; Chia *et al.*, 2020; Li *et al.*, 2022). For instance, the incorporation of vegetable waste into horse manure enhances the performance, development, and nutritional content of the black soldier fly (Julita *et al.*, 2018) and the combination between soybean curd residue and kitchen waste was found to significantly increase larval yield, bioconversion rate, and bioaccumulation of lipid (Li *et al.*, 2022). However, more research is required to optimise diet formulation to ensure the production of BSFL with consistent quantity and quality (Broeckx *et al.*, 2021). Mohd-Noor *et al.* (2017) presented a potential use of discarded coconut endosperm that had undergone a self-fermentation process to improve its nutrient content as a feeding substrate for growing BSFL. In the present study, two feedstuffs, milk-extracted coconut meat (MECM) and an underutilised plant species known as *Sesbania grandiflora* (SG), were mixed in varying proportions to manipulate and improve the nutritional value of the substrate to determine the effect of mixed substrate on the nutrient composition of the BSFL. The previous chapter reported the growth performance of larvae on a single feed substrate (MECM and SG) (Chapter 3). These feedstuffs have been selected for the combinations considering that the nutrient content of SG is higher than the MECM nutrient content, which includes crude protein, amino acid, and fatty acid profiles. The nutritional content of SG-fed larvae was higher than that of MECM-fed larvae. Although the nutrient content of MECM larvae is relatively low, the efficiency of larvae to convert substrate into larval biomass and protein is high. In addition, the weight gain of MECM larvae was better than that of larvae grown on high protein substrates, and the cost of protein for these larvae was the lowest of any other type of larvae in this study. Thus, this chapter addresses the Phase 3 objective to examine the effects of varying the proportion of feed substrates in larvae diets on the nutrient profile of larval biomass and larval capability in converting the feed substrates into biomass. The best mixed feed

substrate will be determined based on the larval conversion capability and their optimal harvesting time for answering the Research Question 3 and 4;

3. Is using a combination of feed substrates superior to using a single substrate for larval development and nutrient composition? What is the impact on the larval ability to convert feed substrate into biomass?

4. Following on from question 3, what is the optimum mix of different substrates on larval development and nutritional value?

4.2 Materials and Methods

4.2.1 Preparation of Feed Substrates

The milk-extracted coconut meat (MECM) and *Sesbania grandiflora* (SG) were initially prepared according to the protocol described in Chapter 3. Water was added to MECM to achieve a moisture content of approximately 76 g/100g, as suggested by Fathurochim *et al.* (1989). In order to determine an ideal mixed feed substrate combination, the ground SG leaves were mixed with MECM at various proportions, as shown in Table 17. Thus, the single substrate diets, L1 and L7, were replicated as control diets containing only MECM and SG, respectively. The mixtures of MECM and SG were prepared by mixing the substrates in a grinder (The Baker, Taiwan) to obtain homogeneity of the combined substrates before placing them in the feeding trays, as shown in Figures 35 and 36.

Table 17: Proportion of *Sesbania grandiflora* and milk-extracted coconut meat mixed feed substrate.

	Diet						
Designation	L1	L2	L3	L4	L5	L6	L7
Portion of MECM:SG	5:0	4:1	3:2	1:1	2:3	1:4	0:5

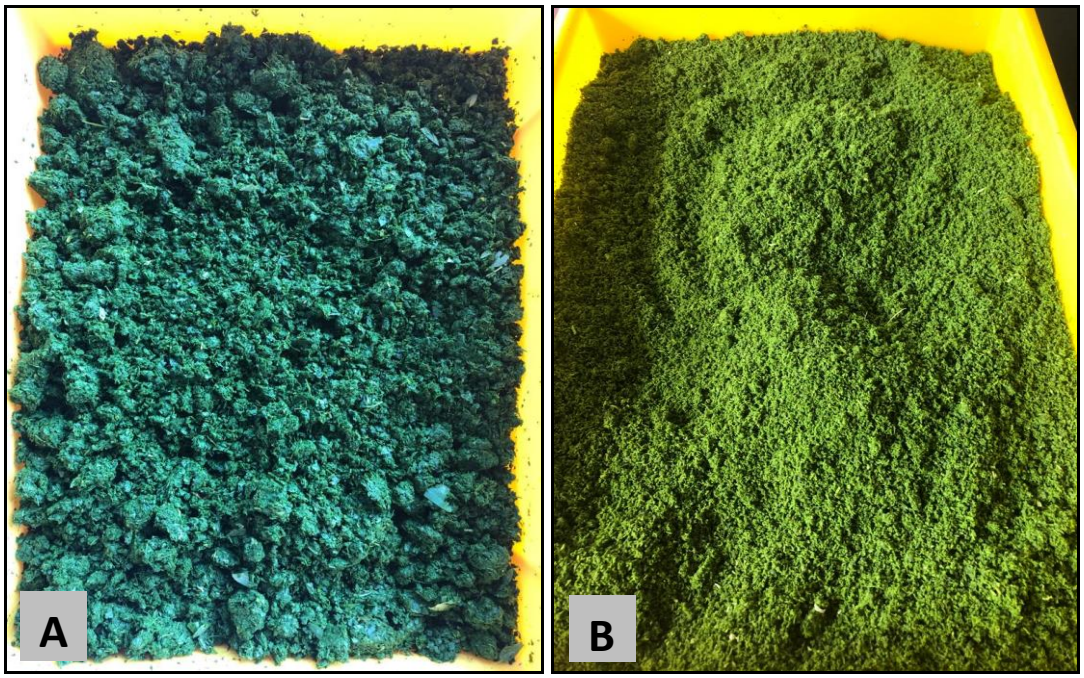


Figure 35: Trays containing single *Sesbania grandiflora* (SG) substrate as control diet in L7 (A) and mixed substrate of MECM and SG at a proportion of 1:4 in L6 diet (B).

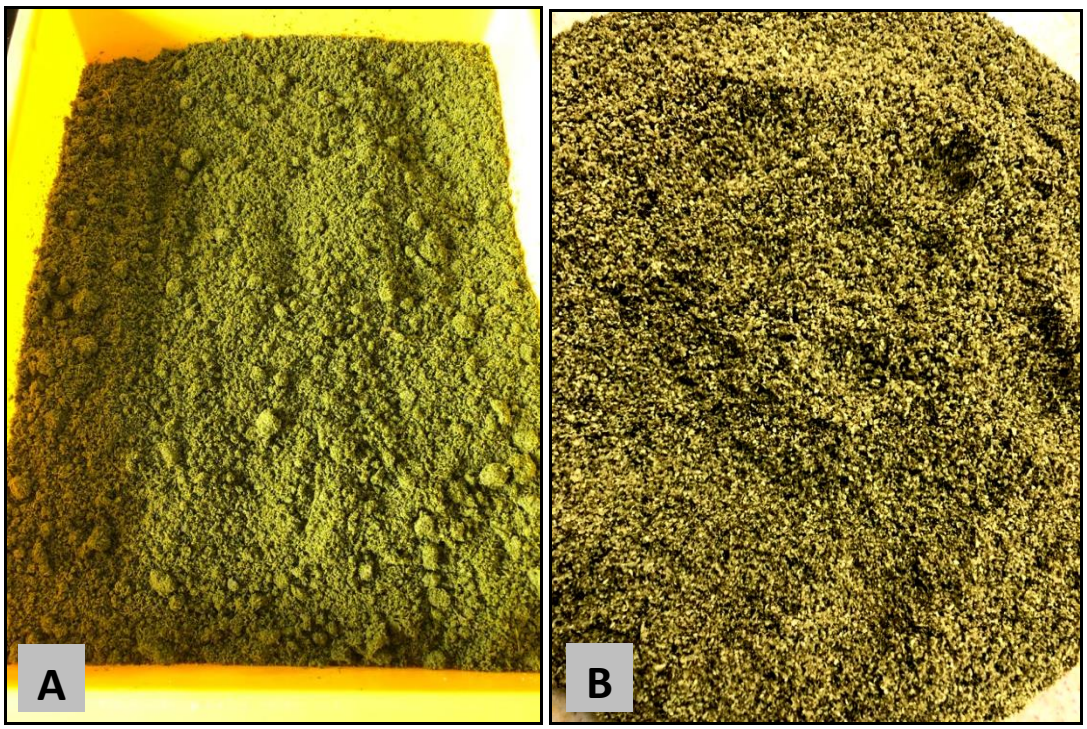


Figure 36: Trays containing MECM: SG mixed feed substrate at proportions of 2:3 (L5)(A) and 1:1 (L4)(B).

4.2.2 Rearing of Black Soldier Fly Larvae on the Mixed Feed Substrates

Twenty-one feeding trial trays containing five mixed substrates (L2 to L6), and two single substrates as controls (L1 and L7) in triplicate, were placed randomly on a portable steel rack tray stacker. Then, 0.25 g of fresh BSF eggs were put on a small piece of paper or leaf in each tray. The growth trial was carried out for 25 days in a room at 27 ± 1.3°C and 74 ± 5% relative humidity (HOBO U23 Pro v2 Temperature/Relative Humidity Data Logger, USA). Substrates were added to each incubation tray every three days at an optimum feeding rate of 100 mg/larva/day (Diener *et al.*, 2009; Abduh *et al.*, 2017). The feed substrates were added to each incubation tray on days 5, 8, 11, 14, 17, 21, and 23 based on the total number of larvae left in the tray after sampling. The tap water in the spray bottle was sprinkled on the feed substrate whenever they looked dry to ensure that the substrates were sufficiently moist and conducive for larval growth.

4.2.2.1 Sampling of Black Soldier Fly Larvae

About 470 larvae from each of the 21 incubation trays were randomly hand-picked for sampling every two days, from days 17 to 25. The sampling began on day 17 as it was determined to be the best harvesting age for BSFL reared on a single feed substrate, as discussed in Chapter 3. The sampling day was extended until day 25 due to the longer development time required by the MECM-fed larvae, which was 28 days compared to 17 days for the SG substrate. The sampling period of BSFL was also extended to day 25 due to the lack of information on how the MECM and SG mixed substrates affect larvae output and quality. The weight of 50 cleaned larvae was obtained by averaging the weight of each larva, whereby 20 out of them were measured individually for size (length and width). Each tray was sampled and recorded in triplicate. The larvae were dried at 60°C (Binder, Germany) to determine the nutrient composition of larvae on each meal substrate.

4.2.3 Proximate Analysis

The mixed feed substrates of L2, L3, L4, L5 and L6, and the single substrate in the controls (L1 and L7) were dried at 60˚C in an oven (Binder, Germany) for two days until they reached a constant weight and kept at 4°C in a refrigerator for future chemical analysis. The dried larvae harvested from L1, L2, L3, L4, L5, L6, and L7 on days 17 – 25 were subjected to proximate analyses, including dry matter (DM), ash, crude protein (CP), and crude fat (Cfat) content, crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF) and gross energy to evaluate the effects of various mixed feed substrates on the nutrient composition of larvae. The nutrient analyses were carried out by the protocols described in Chapter 2.

4.2.4 Substrate Consumption and Reduction Efficiency

A further set of feeding trials was carried out to determine the feed substrate reduction, feed-to-biomass conversion efficiency (BCEDM), and protein conversion efficiency (PrCEDM) on a DM basis of BSFL. In this trial, the mixtures of MECM and SG (MECM:SG), namely L1 (1:0), L4 (1:1), L5 (2:3), and L6 (1:4) and L7 (0:1) were used to investigate the effectiveness of BSFL in converting feed substrates to larval biomass. These substrates contained 50%, 60%, and 80% of SG in L4, L5, and L6, respectively, while L1 (100% MECM) and L7 (100% SG) served as controls. The production of larvae with the highest level of CP was the primary consideration in selecting the most appropriate mixed feed substrate for larvae rearing and the day for harvesting larvae. On the other hand, the composition of Cfat in larvae was also a factor in the selection of optimal mixed feed substrate. Substrates that yield larvae with a high-fat content were considered less desirable, as larvae with high-fat content will present difficulties during BSFL processing as alternative feed ingredients. The mixed feed substrates of L4, L5, and L6 were selected in this trial because they yielded higher larvae CP at an earlier time and acceptable Cfat contents. Additionally, L2 and L3 were eliminated since these substrates produced

BSFL with high-fat content, increasing rancidity and detrimental to storage (Borremans *et al.*, 2020). Based on previous studies to determine the optimal harvesting day, data were collected on days 17 and 19 of the trial.

The two feeding trials were performed concurrently, one trial ceased on day 17, while the other set stopped on day 19. Each trial consisted of 15 trays with three replicates for each feed substrate (L1, L4, L5, L6, L7). A sample of BSF eggs (0.25 g) was added to each tray (50 cm x 36 cm x 7.5 cm) containing 150 g of respective feed substrates, allowed to hatch into larvae. Substrates were added to each incubation tray every three days on days 5, 8, 11, and 14. The last substrates added to trays harvested on day 19 were also until day 14, for seeing that there is still enough food to sustain the larvae for two more days. The total mixed substrates offered to BSFL throughout the two days ranged from 813 to 880 g on a DM basis. While for SG and MECM single substrates, the total amount provided was around 1050 g and 860 g on a DM basis, respectively. Water was sprinkled inside the tray whenever the feed substrate looked dry to make the substrates sufficiently moist and conducive for larvae growth. After all the larvae were harvested on days 17 and 19, the amount of feed substrates offered, the biomass produced, and the feed residue was recorded to estimate the efficiency of BSFL in consuming and reducing feed substrates during the larvae bioconversion process. The data collected in this experiment was used in the calculation of feed substrate reduction, feed-to-biomass conversion efficiency (BCEDM), and protein conversion efficiency (PrCEDM) on a DM basis, as described by Banks *et al.* (2014) and Lalander *et al.* (2019), similar to the formulae 12 to 14 as described in Chapter 3 (3.2.6). BCEDM and PrCEDM was calculated by standardising the larvae number to 1000 larvae. The fresh larvae weight was used to determine larvae yield, while 50 larvae per tray for triplicate were weighed to determine the average weight of a larva. The larvae and feed substrate residues were dried in an oven at 60°C (Binder, Germany) until a constant weight was achieved. The larvae harvested on each day (days 17 and 19) were also analysed for nutrient content, including DM, CP, Cfat, CF, amino acid (AA), and fatty acid (FA) profiles.

4.2.5 Statistical Analysis

Data on the feeding trials of mixed feed substrates such as nutrient composition and growth performance and laboratory analyses (such as proximate analyses, amino acids and fatty acids) were checked for normal distribution using Kolmogorov-Smirnov. The data with normal distribution were subjected to the analysis of variance (2-way ANOVA) using the general linear model (GLM) procedure of the Statistical Analysis System Inc. version 9.4 (2013). Post-hoc multiple comparisons and Tukey's honest significant difference (HSD) test were used when significant treatment effects were observed to compare differences among the means within and between substrates throughout the larvae development period. The analysis determined the statistical differences in larval performance between the group means of each treatment on the respective sampling days on days 17, 19, 21, 23, and 25. In addition, data of nutrient composition and growth performance of larvae in converting feed substrates into larval biomass on days 17 and 19 were compared by GLM procedure followed by a Tukey's test to determine significant differences between the means of the various parameters, namely larval yield and number, waste reduction efficiency, feed-to-biomass conversion efficiency (BCEDM), protein conversion efficiency (PrCEDM), as well as nutrient composition such as DM, CP, Cfat, AAs and FAs profiles. The data in the tables are expressed as mean ± standard error of the mean (SEM); the differences were considered statistically significant when $p < 0.05$. Microsoft Excel was used for creating graphical representations of the data.

4.3 Results

4.3.1 Nutrient Composition of the Mixed Feed Substrate

There were significant differences in the nutrient composition of single feed substrates of MECM and SG as controls and the different combinations of these substrates ($p < 0.0001$, Table 18). As expected, the inclusion of higher levels of SG in the mixed substrates increased the levels of CP and ash. At the same time, the Cfat and CF contents, as well as NDF and ADF in mixed substrates, declined with the increase of SG amount in the mixed substrate. The gross energy (GE) of the substrates also decreased with a reduced proportion of MECM in the substrates. The ash content in the mixed feed substrates increased considerably with increasing inclusion levels of SG. The ash in L2 was 1.98 ± 0.06 g/100g DM compared to 4.84 ± 0.01 g/100g DM in L6. The CP of mixed feed substrates ranged from 7.72 to 21.51 g/100g DM when the proportion of SG increased from 20% (L2) to 80% (L6) in the mixed feed substrates. The CP in single feed substrate of SG (L1) and MECM (L7) was 27.94 ± 0.12 g/100g DM and 4.22 ± 0.01 g/100g DM, respectively. The Cfat content in L6 and L2 ranged between 11.39 g/100g and 23.76 g/100g DM, respectively. The addition of 20% MECM in L6 doubled the amount of Cfat in the mixed feed substrates, from 4.43 ± 0.02 g/100g DM in L7 to 11.39 ± 0.06 g/100g DM in L6. With decreasing amounts of MECM in the mixed feed substrates of L2 to L6, the crude fibre (CF) content reduced from 21.20 ± 0.40 g/100g DM to 12.42 ± 0.24 g/100g DM, respectively. This condition also caused a reduction of NDF and ADF in the mixed feed substrates from 56.16 to 44.00 g/100g DM and 42.67 to 20.62 g/100g DM, respectively.

Table 18: The nutritional composition of the single and mixed feed substrates of MECM and SG used for feeding BSFL.

Designa - tion	Portion of MECM:SG	Ash	CP	Cfat	CF	NDF	ADF	Energy
L1	5:0	1.05 ± 0.02^{d}	4.22 ± 0.01^{g}	27.57 ± 0.41^{a}	31.05 ± 0.44^{a}	59.04 ± 1.36^{a}	57.74 ± 3.24^{a}	19.62 ± 0.04^{b}
L2	4:1	1.98 ± 0.06^{c}	7.72 ± 0.06^{f}	23.76 ± 0.17^{b}	21.20 ± 0.40^{b}	56.17 ± 0.60^{a}	42.67 ± 0.48^{b}	20.91 ± 0.07^{ac}
L3	3:2	2.19 ± 0.33^{c}	11.98 ± 0.14^{e}	20.11 ± 0.09^{c}	18.02 ± 0.02^{c}	51.25 ± 0.65^{ab}	37.18 ± 0.73^{bc}	20.57 ± 0.17^{a}
L4	1:1	2.77 ± 0.06^{c}	14.11 ± 0.04^{d}	17.81 ± 0.27^{d}	16.20 ± 0.19^{d}	53.29 ± 0.11^{a}	36.71 ± 0.52^{bc}	19.81 ± 0.07^{b}
L5	2:3	4.02 ± 0.06^{b}	16.24 ± 0.11^{c}	15.84 ± 0.05^{e}	14.05 ± 0.09^{e}	51.85 ± 3.33^{ab}	29.89 ± 0.15^{c}	19.57 ± 0.28^{b}
L6	1:4	4.84 ± 0.01^{a}	21.51 ± 0.12^{b}	11.39 ± 0.06^{f}	12.42 ± 0.24^{f}	44.00 ± 0.26^{b}	20.62 ± 0.18^{d}	18.64 ± 0.04^{c}
L7	0:5	5.64 ± 0.16^{a}	27.94 ± 0.12^{a}	4.43 ± 0.02^{g}	8.63 ± 0.12^{g}	34.68 ± 1.14^{c}	14.09 ± 0.09^{d}	17.76 ± 0.06^{d}
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

^{abcd}Means ± standard error of the mean in g/100g DM, for ash, CP=crude protein, Cfat=crude fat, CF=crude fibre, NDF=neutral detergent fibre, and ADF=acid detergent fibre. GE is expressed in MJ/kg DM. The mean ± standard error of the mean within the same column with different superscripts are significantly different at $p < 0.0001$.

4.3.2 Influence of Feed Substrate on Growth Performance of the Black Soldier Fly Larvae

4.3.2.1 Mean Wet and Dry Weight of Black Soldier Fly Larvae

The results showed that during the 9-day sampling period (from days 17 to 25), the larvae on diets L1 to L7 had gained as much as 20.62 mg (L1), 19.88 mg (L2), 27.0 mg (L3), 33.80 mg (L4), 33.96 mg (L5), 35.83 mg (L6), and 27.6 mg (L7) wet weight, respectively (Table 19). The average wet weight for larvae reared on the L1 and L2 diets (100% MECM and greater amounts of MECM) increased steadily between days 17 and 25 ($p > 0.05$). With SG at 20% in the feed substrate, the L2 substrate produced larvae with a higher weight than the L1-fed larvae (Figure 37). The larval weight gain on diets L3 and L4, peaked at day 23 and then declined. In contrast, larvae reared on diets with higher levels of SG (L5, L6 and L7), whereby the wet weight of larvae peaked around day 19 or 21 and declined after that. Larva reached the maximum weight on different development days, such as L2 reached 111.66 ± 6.04 mg on day 25, L4 at 156.02 ± 1.91 mg on day 23 and day 19 for L5-fed larvae at around 159.16 ± 7.25 mg. The addition of MECM to SG substrate at 20% (L6), 40% (L5) and 50% (L4) markedly enhanced the weight of larvae compared to those reared on L7 (SG only). The weight was observed to increase with the increase of protein composition, peaking when the protein content reached 21.51 ± 0.127% in the L6 mixed feed substrate. Although not significant, all larval weights decreased on day 25 across the treatments except for larvae reared on L1 and L2. The protein constituent was the lowest in L1 substrate, which is made up of only MECM substrate but high in Cfat. The presence of 20% of SG in the L2 mixed substrate generally increased the weight of larvae compared to L1.

Dry matter accumulation followed a similar pattern to the wet weight. The dried weight of BSF larva reared on the single and mixed substrate combinations significantly differed throughout the development time ($p \leq 0.0040$, Table 19). Significance differences were observed in the larval dried weight ($p < 0.05$) within

the feed substrates except for larvae on L1 (p = 0.5571) and L2 (p = 0.1492). The dried weight of larva on the L6 substrate showed the highest amongst the mixed feed substrates tested, followed by L5 and L4, and the lowest was the larva on L2 (Figure 38). The dried weight of larva on L6 and L4 gradually increased until it attained the highest weight on day 23 of 45.46 ± 2.77 mg and 41.53 ± 0.25 mg, respectively, before dropping to 37.51 ± 1.36 mg and 38.81 ± 2.32 mg, respectively on day 25. Larvae on L5 increased to 40.57 ± 2.21 mg on day 19 but plateaued until day 23, dropping drastically to 30.14 ± 1.47 mg on day 25. The dried larvae on L1 and L2 increased progressively over time, similar to the larval wet weight, but were not significantly different (p = 0.5571 and p = 0.1492, respectively).

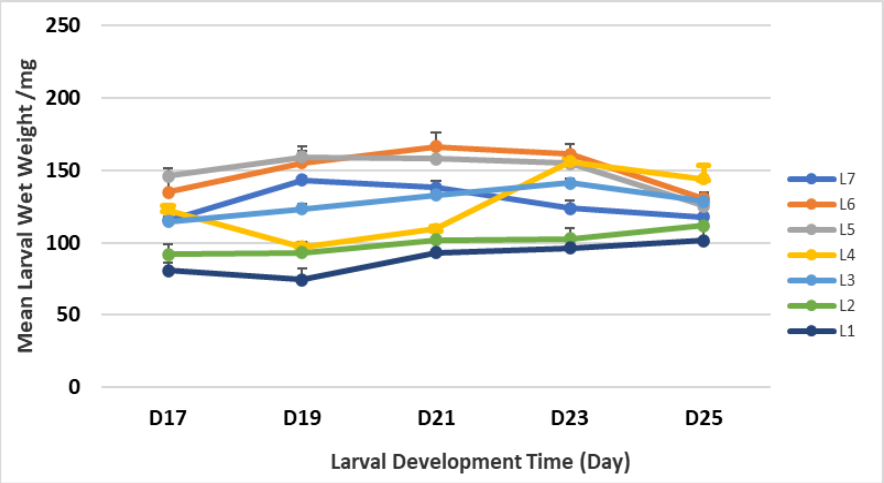


Figure 37: Mean wet weight (mg) of individual BSF larva reared on mixed feed substrate over development time. Error bars represent the standard errors of the mean larval Cfat of triplicate trays. Mixtures of MECM and SG (MECM:SG): L1 (5:0), L2 (4:1), L3 (3:2), L4 (1:1), L5 (2:3), L6 (1:4) and L7 (0:1). D17-D25=Sampling of larvae between days 17 and 25.

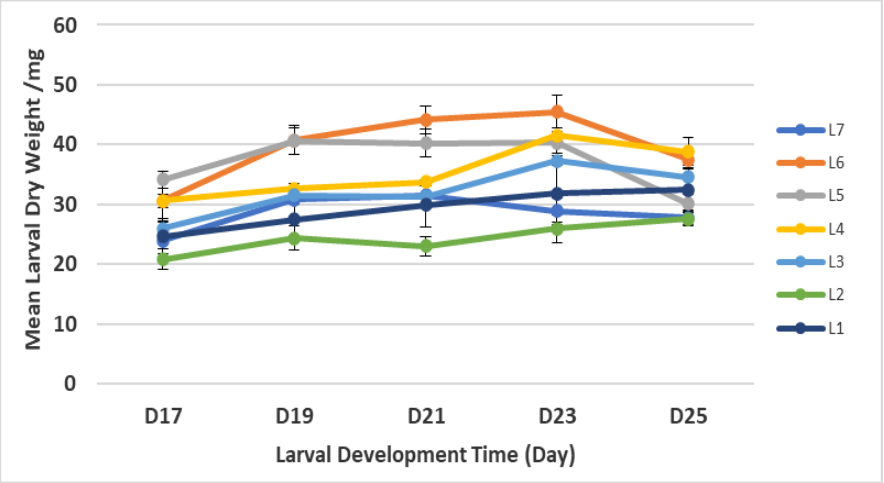


Figure 38: Mean dry weight (mg) of individual BSF larva reared on mixed feed substrate over development time. Error bars represent the standard errors of the mean larval Cfat of triplicate trays. Mixtures of MECM and SG (MECM:SG): L1 (5:0), L2 (4:1), L3 (3:2), L4 (1:1), L5 (2:3), L6 (1:4) and L7 (0:1). D17-D25=Sampling of larvae between days 17 and 25.

4.3.2.2 Mean Size of the Black Soldier Fly Larva

During the larval development period from day 19 to day 25, the mean larval length varied considerably within ($p < 0.05$) and between feed substrates ($p \leq 0.0017$, Table 19). At the end of the trial (day 25), the shortest larvae (16.45 mm) were those reared on MECM-rich substrates. Larvae reared on SG alone or SG-rich substrates gained longer lengths (18.11mm). In general, larvae on all diets reached their maximum length by day 21 or 23. On day 23, the mean length of L6-fed larvae surpassed that of L5-fed larvae, while the shortest larvae were recorded in the L1 and L2 diets. Despite their largest weight, larvae on L6 attained the longest length of 21.23 ± 0.02 mm with 20% MECM on day 23. The width data of larvae was inconsistent and uniform in all larvae because of the larva unpredictable movement during measurement. Larva reared on L6 had the largest mean width of around 5.09 ± 0.11 mm, while larvae fed 100% MECM (L1) had the smallest mean width, although not significant from L6. Adding SG to MECM at a rate of 40% (L3) resulted in larger BSFL width than L1 and L2. Furthermore, L3 - L6 larvae were larger than L7, but the differences were insignificant. The larval width was not significantly different between feed substrates on day 25 ($p = 0.7052$).

Table 19: Growth performance of BSFL on different feed mixtures of SG and MECM at different larval ages.

Substrate	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	*p*-value
MECM:SG Proportion	**5:0**	**4:1**	**3:2**	**1:1**	**2:3**	**1:4**	**0:5**	
Day	Larval Wet Weight (mg)							
17	80.62 ±4.69^{d}	91.78 ±7.22^{cd}	114.44 ±3.58^{bc,y}	122.22 ±3.58^{ab,y}	146 ±5.05^{a,wx}	134.89 ±9.46^{ab}	115.49 ±2.50^{bc,y}	<0.0001
19	86.36 ±7.03^{c}	93.25 ±6.79^{c}	123.51 ±3.46^{b,xy}	126.31 ±2.99^{b,xy}	159.16 ±7.25^{a,w}	155.38 ±7.61^{a}	143.09 ±2.91^{ab,w}	<0.0001
21	93.00 ±8.70^{c}	101.84 ±5.49^{c}	133.00 ±3.31^{b,wx}	145.71 ±1.04^{ab,wx}	157.84 ±7.82^{ab,w}	166.44 ±9.37^{a}	138.13 ±4.80^{ab,wx}	<0.0001
23	96.13 ±8.15^{c}	102.73 ±7.41^{c}	141.44 ±2.88^{ab,w}	156.02 ±1.91^{a,w}	154.73 ±6.76^{a,wx}	161.00 ±6.81^{a}	123.73 ±5.49^{bc,wxy}	<0.0001
25	101.24 ±8.99^{b}	111.66 ±6.04^{b}	128.59 ±5.22^{ab,wxy}	144.13 ±9.44^{a,wxy}	125.2 ±4.50^{ab,x}	130.61 ±3.93^{ab}	117.89±5.56^{ab,xy}	0.0093
p-value	0.4007	0.2803	0.0054	0.0027	0.0201	0.0278	0.0043	
	Larval Dry Weight (mg)							
17	24.65 ±2.97^{ab}	20.77 ±1.70^{b}	26.00 ±1.08^{ab,y}	30.62 ±1.11^{ab,y}	34.10 ±1.44^{a,wx}	30.65 ±3.53^{ab,x}	23.91 ±1.38^{b,x}	0.0061
19	27.45 ±3.20^{b}	24.35 ±2.00^{b}	31.52 ±1.28^{ab,x}	32.66 ±0.78^{ab,y}	40.57 ±2.21^{a,w}	40.69 ±2.43^{a,wx}	30.83 ±0.79^{b,wx}	0.0003
21	29.93 ±3.68^{cd}	23.01 ±1.63^{d}	31.29 ±0.31^{bcd,x}	33.74 ±0.34^{bc,xy}	40.23 ±2.32^{ab,w}	44.10 ±2.38^{a,w}	31.51 ±1.66^{bcd,w}	<0.0001
23	31.81 ±4.71^{bcd}	25.93 ±2.25^{d}	37.27 ±0.83^{abcd,w}	41.53 ±0.25^{ab,w}	40.36 ±1.76^{abc,w}	45.46 ±2.77^{a,w}	28.86 ±2.19^{cd,wx}	0.0006
25	32.47 ±3.37^{ab}	27.55 ±1.14^{b}	34.55 ±1.59^{ab,wx}	38.81 ±2.32^{a,wx}	30.14 ±1.47^{ab,x}	37.51 ±1.36^{a,wx}	27.73 ±1.03^{b,wx}	0.0040
p-value	0.5571	0.1492	0.0004	0.0004	0.0081	0.0155	0.0330	

continue….

Substrate	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	*p*-value
SG:MECM Proportion	**5:0**	**4:1**	**3:2**	**1:1**	**2:3**	**1:4**	**0:5**	
				Larval Length (mm)				
17	15.85 ±0.34^{c,wx}	15.70 ±0.63^{c}	17.78 ±0.37^{ab,wx}	17.78 ±0.24^{ab,x}	19.44 ±0.26^{a,x}	18.41 ±0.30^{ab,x}	16.83 ±0.20^{bc,y}	<0.0001
19	14.48 ±0.28^{c,x}	15.16 ±0.16^{c}	16.45 ±0.19^{b,x}	16.22 ±0.13^{b,y}	17.55 ±0.19^{a,z}	17.83 ±0.33^{a,x}	16.97 ±0.14^{ab,y}	<0.0001
21	16.99 ±0.60^{d,w}	17.20 ±0.44^{d}	18.57 ±0.05^{dc,w}	19.15 ±0.23^{abc,w}	20.74 ±0.29^{a,w}	20.51 ±0.38^{ab,w}	19.02 ±0.11^{bc,w}	<0.0001
23	16.89 ±0.49^{cd,w}	16.73 ±0.41^{d}	18.43 ±0.35^{bc,w}	18.80 ±0.24^{b,wx}	19.05 ±0.26^{b,xy}	21.23 ±0.02^{a,w}	19.47 ±0.27^{b,w}	<0.0001
25	16.90 ±0.19^{bc,w}	16.45 ±0.31^{c}	17.55 ±0.24^{abc,wx}	18.58 ±0.23^{a,wx}	18.01 ±0.23^{ab,yz}	18.81 ±0.53^{a,x}	18.11 ±0.21^{ab,x}	0.0017
p-value	0.0107	0.0501	0.0023	<0.0001	<0.0001	0.0001	<0.0001	
				Larval Width (mm)				
17	4.29 ± 0.16^{bc}	4.15 ± 0.01^{c}	4.71 ± 0.08^{ab,w}	4.76 ± 0.08^{a}	4.95 ± 0.01^{a,w}	4.70 ± 0.01^{ab,wx}	4.53 ± 0.03^{abc,wx}	0.0003
19	4.23 ± 0.23^{b}	4.55 ± 0.06^{ab}	4.84 ± 0.05^{a,w}	4.83 ± 0.03^{a}	4.89 ± 0.03^{a,w}	4.91 ± 0.06^{a,w}	4.81 ± 0.05^{a,w}	0.0017
21	4.07 ± 0.13^{c}	4.22 ± 0.12^{c}	4.69 ± 0.05^{ab,wx}	4.68 ± 0.07^{ab}	4.97 ± 0.01^{a,w}	4.96 ± 0.06^{a,w}	4.43 ± 0.08^{bc,x}	<0.0001
23	4.40 ± 0.21^{c}	4.59 ± 0.05^{abc}	4.80 ± 0.12^{abc,w}	4.87 ± 0.03^{abc}	4.95 ± 0.01^{ab,w}	5.09 ± 0.11^{a,w}	4.56 ± 0.08^{bc,wx}	0.0056
25	4.28 ± 0.03	4.35 ± 0.15	4.32 ± 0.01^{x}	4.50 ± 0.16	4.30 ± 0.14^{x}	4.47 ± 0.01^{x}	4.30 ± 0.04^{x}	0.7052
p-value	0.7603	0.0281	0.0177	0.0856	0.0001	0.0062	0.0015	

^{abcd}Means ± standard error of the mean within row showed comparison within treatment by day followed by different superscripts are significantly different ($p < 0.05$).

^{xyz}Means ± standard error of the mean within the column showed a comparison between treatments by day followed by different superscripts are significantly different ($p < 0.05$).

4.3.3 The Influence of Feed Substrates on Nutrient Composition of the Black Soldier Fly Larvae

4.3.3.1 Dry Matter Content of Black Soldier Fly Larvae

The nutrient composition of BSFL reared on different diets is presented in Table 20. The DM content increased as the age of larvae increased ($p < 0.05$), except for the larvae reared solely on MECM and SG as single substrates, L1 ($p = 0.7918$) and L7 ($p = 0.0546$). The addition of 20% MECM in the SG substrate (L6) increased the DM content of larvae from 22.59 ± 1.23 to 28.71 ± 0.24 g/100g compared to L7 throughout the development time. During the 9-day observation, the larvae reared on L1 contained an average DM of 31.75 g/100, followed by 26.44 g/100 in L6, while the lowest value of 22.37 g/100g DM was observed in larvae fed with 100% SG (L7). The DM larvae reared on L2, L3 and L4 did not show any trend compared to the other larvae, which increased with the development time.

Table 20: Nutrient composition of BSFL reared on different mixed feed substrates at various larval ages.

Substrate	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	***p-value***
MECM:SG Proportion	**5:0**	**4:1**	**3:2**	**1:1**	**2:3**	**1:4**	**0:5**	
Day	Dry Matter (g/100g of wet weight)							
17	30.36 ± 1.81^{a}	22.62 ± 0.10^{b,x}	22.70 ± 0.24^{b,x}	25.05 ± 0.45^{b,wx}	23.35 ± 0.25^{b,x}	22.59 ± 1.23^{b,x}	20.68 ± 0.86^{b}	<0.0001
19	31.62 ± 1.04^{a}	26.09 ± 0.52^{b,w}	25.50 ± 0.38^{b,w}	25.86 ± 0.05^{b,wx}	25.47 ± 0.26^{b,wx}	26.18 ± 0.74^{b,wx}	21.56 ±0.59^{c}	<0.0001
21	32.01 ± 0.89^{a}	22.55 ± 0.42^{d,x}	23.54 ± 0.39^{cd,x}	23.16 ± 0.28^{cd,x}	25.47 ± 0.54^{bc,wx}	26.51 ± 0.34^{b,w}	22.79 ± 0.54^{d}	<0.0001
23	32.76 ± 1.96^{a}	23.25 ± 0.77^{c,w}	25.19 ± 0.43^{bc,wx}	26.35 ± 0.30^{bc,w}	26.62 ± 0.17^{bc,w}	26.09 ± 0.24^{bc,w}	23.25 ± 0.77^{b}	0.0001
25	32.00 ± 0.49^{a}	24.77 ± 1.23^{c,wx}	26.86 ± 0.23^{bc,w}	27.02 ± 1.26^{bc,w}	24.08 ± 0.87^{c,wx}	28.71 ± 0.24^{ab,w}	23.54 ± 0.26^{c}	<0.0001
p-value	0.7918	0.0109	<0.0001	0.0089	0.0153	0.0018	0.0546	
	Crude Protein (g/100g DM)							
17	28.39 ± 0.43^{d,y}	42.95 ± 0.98^{c,x}	48.11 ± 0.31^{b,wx}	48.89 ± 0.66^{b,x}	49.91 ± 0.27^{b}	51.00 ±1.00^{ab,w}	53.59 ± 0.37^{a,x}	<0.0001
19	35.26 ± 2.37^{c,wx}	47.27 ± 0.44^{b,wx}	52.03 ± 0.10^{a,w}	53.96 ± 0.34^{a,w}	53.40 ± 0.39^{a}	54.30 ± 1.19^{a,w}	56.27 ± 0.51^{a,w}	<0.0001
21	34.40 ± 0.48^{b,wx}	48.26 ± 0.67^{a,w}	47.90 ± 1.52^{a,wx}	48.36 ± 0.11^{a,x}	51.09 ± 1.42^{a}	51.11 ± 0.96^{a,w}	50.51 ± 0.22^{a,y}	<0.0001
23	38.42 ± 0.89^{b,w}	49.88 ± 0.62^{a,w}	44.21 ± 1.38^{abc,xy}	47.19 ± 1.31^{ab,x}	50.15 ± 2.37^{a}	40.56 ± 0.62^{b,x}	47.83 ± 0.83^{a,z}	<0.0001
25	31.88 ± 0.69^{c,xy}	33.49 ± 1.08^{c,y}	43.01 ± 0.57^{b,y}	46.14 ± 1.03^{ab,x}	48.99 ± 0.32^{a}	42.82 ± 0.61^{b,x}	44.41 ± 0.15^{b,z}	<0.0001
p-value	0.0006	<0.0001	0.0005	0.0008	0.3586	<0.0001	<0.0001	

continue…

Substrate	L1	L2	L3	L4	L5	L6	L7	*p*-value
MECM:SG	**5:0**	**4:1**	**3:2**	**1:1**	**2:3**	**1:4**	**0:5**	
				Crude Fat (g/100g DM)				
17	54.91 ± 1.65^{a,wx}	33.52 ± 0.87^{b,w}	23.31 ± 0.99^{c}	16.79 ± 0.31^{de,y}	18.90 ± 0.56^{dc}	15.52 ± 1.38^{de,wx}	12.31 ± 0.50^{e,w}	<0.0001
19	53.28 ± 0.90^{a,wx}	33.81 ± 0.72^{b,w}	23.86 ± 0.73^{c}	17.03 ± 0.78^{d,y}	18.84 ± 0.48^{d}	18.53 ± 1.45^{d,w}	8.91 ± 0.87^{e,xy}	<0.0001
21	55.55 ± 1.38^{a,w}	27.76 ± 0.95^{b,x}	21.24 ± 1.54^{c}	19.92 ± 0.61^{c,x}	19.13 ± 1.52^{c}	12.92 ± 0.22^{d,x}	10.71 ± 1.05^{d,wx}	<0.0001
23	49.42 ± 1.04^{a,xy}	25.25 ± 0.18^{b,x}	21.99 ± 0.53^{c}	23.12 ± 0.11^{bc,w}	15.29 ± 0.39^{d}	16.77 ± 0.78^{d,wx}	7.61 ± 0.53^{e,xy}	<0.0001
25	46.06 ± 0.91^{a,y}	24.89 ± 0.82^{b,x}	23.47 ± 0.31^{b}	24.21 ± 0.69^{b,w}	15.89 ± 0.24^{c}	17.14 ± 0.58^{c,wx}	7.17 ± 0.22^{d,y}	<0.0001
p-value	0.0011	<0.0001	0.2908	<0.0001	0.0397	0.025	0.002	
				Ash (g/100g DM)				
17	3.22 ± 0.06^{d,x}	6.68 ± 0.68d^{c,x}	8.87 ± 0.40^{bc}	8.47 ± 0.49^{bc,y}	10.34 ± 0.38^{bc,y}	11.87 ± 1.43^{ab,x}	15.51 ± 1.71^{a}	<0.0001
19	3.40 ± 0.19^{e,x}	7.76 ± 0.03^{d,wx}	9.73 ± 0.07^{c}	10.20 ± 0.06^{c,x}	10.85 ± 0.19^{c,xy}	13.72 ± 0.33b^{,wx}	16.47 ± 0.58^{a}	<0.0001
21	3.64 ± 0.12^{f,wx}	8.64 ± 0.07^{e,w}	9.69 ± 0.91d^{e}	10.90 ± 0.08^{cd,wx}	11.96 ± 0.60^{c,xy}	14.43 ± 0.26^{b,wx}	18.29 ± 0.34^{a}	<0.0001
23	3.10 ± 0.25^{f,x}	7.96 ± 0.02^{e,wx}	10.26 ± 0.04^{d}	10.53 ± 0.19^{cd,wx}	12.59 ± 0.20^{c,wx}	16.27 ± 1.12^{b,w}	18.68 ± 0.34^{a}	<0.0001
25	4.37 ± 0.14^{f,w}	9.11 ± 0.67^{e,w}	10.89 ± 0.10^{d}	11.50 ± 0.28^{d,w}	14.21 ± 0.17^{c,w}	16.80 ± 0.13^{b,w}	19.28 ± 0.04^{a}	<0.0001
p-value	0.0078	0.0163	0.1591	0.0001	0.0006	0.0268	0.0826	

^{abcdef} Means ± standard error of the mean within row showed the comparison between treatments by day followed by different superscripts are significantly different ($p < 0.05$).

^{vwxyz} Means ± standard error of the mean within the column showed comparison within treatment by day followed by different superscripts are significantly different ($p < 0.05$).

4.3.3.2 Crude Protein Content of Black Soldier Fly Larvae

The CP content of BSFL showed significant differences throughout the development time ($p < 0.05$) except for the larvae on L5, MECM:SG of proportion 2:3 ($p = 0.3586$, Table 20). The results showed increased CP content of larvae reared on L3 to L7 from days 17 to 19. The CP content peaked on day 19 and reduced gradually until day 25 (Figure 39). The CP content of larvae on mixed feed substrates L3 – L6 did not differ significantly from those in L7 (100% SG) on day 19, although the CP in the substrates differed considerably from 12.0 to 27.9 g/100g DM while the Cfat ranged between 4.4 to 20.1 g/100g DM. Interestingly, the accumulation trend of larval protein on these substrates was similar. Larvae reared on L1 and L2 showed a similar trend of CP content which increased considerably and reached a maximum peak on day 23 of around 38 g/100g and 50 g/100g DM (Figure 38), respectively. While, in the other substrates, of L3 to L7, there was a significant reduction in CP on day 23. The CP of BSFL revealed highly significant differences between feed substrates at all larval ages ($p < 0.0001$). The larvae reared on L7 that contained only SG produced the highest content of CP of about 56 g/100g DM on day 19, while the larvae on L2 to L6 substrates ranged from 47.2 to 54.3 g/100g DM. At all larvae ages, the CP content increased when the proportion of SG in the mixed feed substrates increased, albeit it dropped throughout the development day.

The stability of macronutrients between CP and Cfat in a balanced ratio (1:1) in L5 substrate produced BSFL with quite consistent protein content throughout development time ($p > 0.05$). The BSFL on MECM:SG of proportions 5:0 (L1) and 4:1 (L2) contained low CP in the mixtures, 4.2 g/100g DM and 7.7 g/100g DM, respectively but higher in fat content of 23.8 g/100g DM and 27.5 g/100g DM, respectively as shown in Table 14 accumulated less protein took a longer time to reach the peak. The mixed feed substrate containing 20% and 50% MECM in L6 and L4 showed an increase of larvae CP content by approximately 40 − 53% compared to larvae reared on L1 at day 19. Compared to L7 (100% SG), the CP contents of

larvae reduced in descending order in L6, L4, L5, L3 and L2 on day 19. Larvae reared on 100% MECM in L1 had the lowest CP value compared to 100% SG or all the mixed feed substrate combinations.

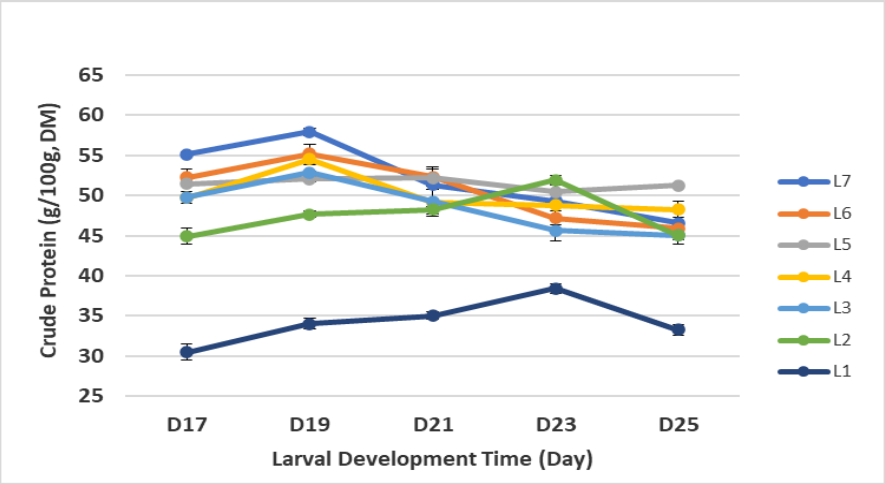


Figure 39: Crude protein (CP, g/100g DM) content of BSFL reared on mixed feed substrate over development time. Error bars represent the standard errors of the mean larval CP of triplicate trays. Mixtures of MECM and SG (MECM:SG): L1 (5:0), L2 (4:1), L3 (3:2), L4 (1:1), L5 (2:3), L6 (1:4) and L7 (0:1). D17-D25=Sampling of larvae between days 17 and 25.

4.3.3.3 Crude Fat Content of Black Soldier Fly Larvae

The crude fat content (Cfat) in the mixed feed substrates reduced as the proportion of SG to MECM increased in the feed substrate, as shown in Figure 40. On day 17, the Cfat in larvae was from 15.52 ± 1.3 g/100g DM to 33.52 ± 0.87 g/100g DM, when the amount of MECM in the mixed substrates increased from 20%, to 80% (Table 20). The Cfat in BSFL showed significant differences throughout the development time except for larvae reared on 60% MECM, L3 (p = 0.2908). The Cfat content in the larvae varied significantly between substrates (p <0.0001) throughout the larval development from day 17 to day 25. The results showed that the level of Cfat in the larvae resembled the trend of Cfat in the substrate, whereby the mixed

feed substrates that contained a higher proportion of MECM produced higher Cfat levels in the BSFL.

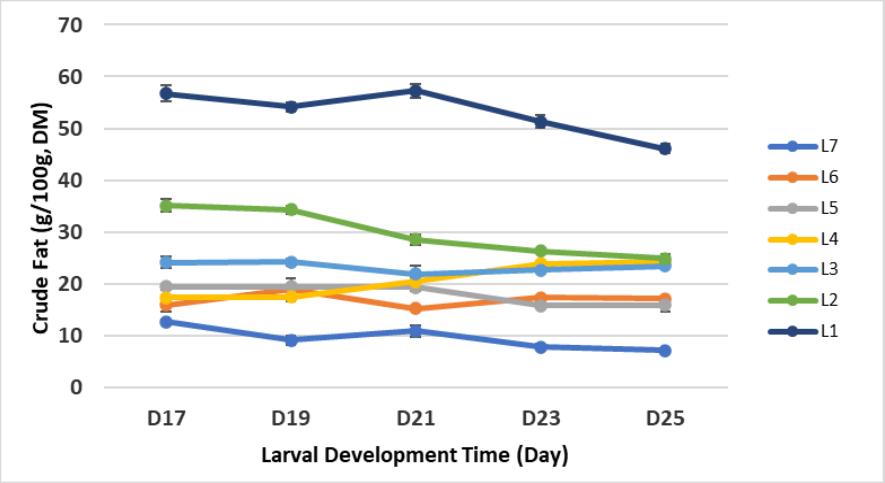


Figure 40: Crude fat (Cfat, g/100g DM) content of BSFL reared on mixed feed substrate over development time. Error bars represent the standard errors of the mean larval Cfat of triplicate trays. Mixtures of MECM and SG (MECM:SG): L1 (5:0), L2 (4:1), L3 (3:2), L4 (1:1), L5 (2:3), L6 (1:4) and L7 (0:1). D17-D25=Sampling of larvae between days 17 and 25.

4.3.3.4 Ash Content of Black Soldier Fly Larvae

The ash content of BSFL in the mixed feed substrates increased gradually during larval development. Significant differences were observed in the ash content of BSFL reared on the different feed substrate combinations throughout their 25-day development period ($p < 0.05$, Table 20) except for the larvae reared on L3, MECM:SG at 3:2 ($p = 0.1591$) and L7 ($p = 0.0826$). There were significant differences ($p < 0.0001$) in the ash content of larvae between feed substrate combinations from days 17 to 25 of development. The ash content of larvae reared on 100% SG (L7) had the highest amount of ash, and the least was observed in larvae on 100% MECM (L1). The ash accumulation in the larvae reared on the MECM, and SG mixtures declined when the proportion of SG in feed mixtures decreased. The 100%

SG leaves (L7) contained 5.64 g/100g ash compared to only 1.05 g/100g in 100% MECM. A reduction of 20% SG in the mixed substrate combination of L6 resulted in a decrease in the percentage of ash in BSFL compared to L7. In L7 substrate, the larval ash concentration ranged between 15.51 ± 1.71 g/100g DM and 19.28 ± 0.04 g/100g DM, while on L6, it ranged between 11.87 ± 1.43 and 16.80 ± 0.13 g/100g DM, indicating a drop in ash concentration of around 12.9% to 23.5% during the nine days development. The addition of 20% SG to MECM (L2) increased the level of ash in the larvae that ranged between 6.68 ± 0.68 to 9.11 ± 0.67 g/100g DM by more than twice the level in L1 of 3.22 ± 0.06 – 4.37 ± 0.14 g/100g DM at all larval ages.

4.3.4 The Effects of Mixed Feed Substrates on the Substrate Reduction and Bioconversion Efficiency of Black Soldier Fly Larvae

The effects of the mixed feed substrates containing different proportions of MECM and SG leaves on biomass production, substrate reduction, and bioconversion performance are shown in Table 21. Except for protein conversion efficiency, all the other parameters showed improvements when MECM was supplemented with SG. The performance of BSFL reared on the different feed substrates led to different results ($p \leq 0.0449$) in terms of larval dry matter yield weight and total number of larvae. For both days (days 17 and 19), the weight of larvae on mixed feed substrate, including the control, was significantly different. Larvae reared on L1, L4, and L5 weighed more on day 19, but larvae reared on greater SG, L6 and L7 weighed less on that day. L4 substrate contained the most larvae (approximately 4731 ± 127 heads per tray), followed by L6-fed larvae (4362 ± 168 heads per tray). On both days, the number of larvae in the substrates of L1, L5, and L7 did not differ significantly. The L1 substrate generated the fewest larvae, with 3369 ± 297 heads per tray on day 17 and 3264 ± 239 heads per tray on day 19. The BSFL DM yield varied significantly ($p \leq 0.0196$) between the controls of L1 and L7 single substrates on days 17 and 19. The differences in the larvae DM yield on all substrates were not

significant difference between day 17 and day 19 except in L4-fed larvae (p = 0.0009). In terms of larval yield (DM), L4-fed larvae had the highest dry weight yield of 123.96 ± 3.98 g DM per tray, followed by L6 of 112.68 ± 12.08 g DM per tray, while the lowest was observed in larvae reared on 100% MECM (L1) of about 82.54 ± 5.15 g DM per tray on day 19.

The presence of SG proportion of 50 – 80% in the MECM:SG combination considerably increased ($p \leq 0.0039$) the reduction of dry matter substrate by the larvae for both days. Large amounts of feed are ingested by larvae, leading to a larger decline in the amount of feed available. The amount of substrate ingested by the larvae rose when the percentage of MECM was decreased while SG was increased, possibly due to the substrate's greater palatability. The percentage of substrate reduction increased with the increase of SG in a mixture. However, only L7 showed significant differences in the feed reduction between days 17 and 19 (p = 0.0020). On day 17, the biggest reduction was 69.5% for L6, while the lowest was 44.52 ± 1.66% in L1. On day 19, the highest feed reduction was observed in L7 at 76.33 ± 1.01% DM compared to the lowest figure observed in L1 at 50.08 ± 5.22% DM. The dry matter reduction of mixed feed substrates was greater than single feed substrate of L1, ranging from 62.07 ± 1.32% DM in L4 to 69.45 ± 0.66% DM in L6 on day 17 ($p < 0.0001$) and 67.07 ± 2.88% DM in L5 to 70.93 ± 4.66% DM L6 on day 19 (p = 0.0039).

The bioconversion efficiency on dry matter basis (BCEDM) of larvae increased from day 17 to 19, except for L7. The BCEDM of larvae fed on L4, L5, and L6 increased in ascending order in accordance with the reduction of MECM proportions in the feed substrates; however, they did not differ significantly except when compared to SG alone. The BCEDM of the L4 substrate of 23.02 ± 1.41% increased by about 73.2% from the larvae reared on L7, which recorded a BCEDM of 13.30 ± 0.22% on day 19. The BCEDM in L4 increased from 16.88 ± 0.35% to 23.02 ± 1.41% DM (p = 0.0132) in two-day period with an increment of 36.4%. This trend was also observed in the

other mixed feed substrates, but the increment was insignificant except for larvae on L7, which reduced from 20.56 ± 2.22% to 13.30 ± 0.22% DM (p = 0.0310).

The efficiency of protein substrate conversion to larvae protein based on a dry matter basis (PrCEDM) varied significantly (p < 0.0001) for both days, ranged from 38.2% (L7) to 142.6% DM (L1), and from 26.1% (L7) to 151.6% (L1) DM, respectively. The reduction of SG proportion in mixed substrate caused an increase of PrCEDM by the larvae. Among the mixed feed substrates, the PrCEDM increased from 54.4% to 84.1% DM (p = 0.0127) in L4. This led to an increase in PrCEDM by about 54.6% whilst others did not differ significantly (p > 0.05) during two-day growth period.

Table 21: The comparative performance of BSFL between days 17 and 19 on the mixed feed substrates.

Substrate	**L1**	**L4**	**L5**	**L6**	**L7**	***p*-value**
MECM:SG Proportion	**5:0**	**1:1**	**2:3**	**1:4**	**0:5**	
			Larvae Number (head)			
17	3369 ± 297^{b}	$3605 \pm 313^{ab,y}$	3762 ± 135^{ab}	$3770 \pm 67^{ab,y}$	4452 ± 127^{a}	0.0449
19	3264 ± 239^{b}	$4731 \pm 127^{a,x}$	3713 ± 506^{ab}	$4362 \pm 168^{ab,x}$	4002 ± 239^{ab}	0.0372
p-value	0.7970	0.0290	0.9309	0.0306	0.1707	
Day			Larval Wet Weight (mg)			
17	76.78 ± 8.40^{b}	117.13 ± 9.18^{ab}	121.27 ± 3.29^{a}	120.31 ± 3.59^{a}	135.37 ± 14.30^{a}	0.0083
19	87.25 ± 6.55^{b}	127.69 ± 2.30^{a}	127.62 ± 10.16^{a}	118.62 ± 9.95^{ab}	130.69 ± 2.31^{a}	0.0084
p-value	0.3810	0.3272	0.5838	0.8809	0.7629	
			Larval Dry Yield (g)			
17	70.85 ± 1.23^{b}	$88.77 \pm 0.49^{b,y}$	91.53 ± 3.98^{b}	100.53 ± 0.63^{ab}	129.17 ± 15.00^{a}	0.002
19	82.54 ± 5.15^{b}	$123.96 \pm 3.98^{a,x}$	99.54 ± 7.41^{ab}	112.68 ± 12.08^{a}	106.54 ± 1.79^{a}	0.0196
p-value	0.0921	0.0009	0.3946	0.3720	0.2084	

continue…

Substrate	L1	L4	L5	L6	L7	*p*-value
MECM:SG Proportion	**5:0**	**1:1**	**2:3**	**1:4**	**0:5**	
Feed Reduction (%)						
17	44.52 ± 1.66^{c}	62.07 ± 1.32^{b}	64.19 ± 0.96^{ab}	69.45 ± 0.66^{a}	$61.52 \pm 1.80^{b,y}$	<.0001
19	50.08 ± 5.22^{b}	63.73 ± 1.92^{ab}	67.07 ± 2.88^{a}	70.93 ± 4.66^{a}	$76.33 \pm 1.01^{a,x}$	0.0039
p-value	0.3675	0.5161	0.3968	0.7696	0.0020	
Substrate-to-Biomass Conversion Efficiency, BCEDM (%)						
17	18.60 ± 0.74	16.88 ± 0.35^{y}	17.53 ± 0.65	16.45 ± 0.16	20.56 ± 2.22^{x}	0.1379
19	19.40 ± 1.08^{a}	$23.02 \pm 1.41^{a,x}$	18.33 ± 1.60^{ab}	17.96 ± 0.92^{ab}	$13.30 \pm 0.22^{b,y}$	0.0022
p-value	0.5762	0.0132	0.6664	0.1826	0.0310	
Protein Conversion Efficiency, PrCEDM (%)						
17	142.56 ± 7.66^{a}	$54.36 \pm 1.69^{b,y}$	50.71 ± 2.16^{b}	$41.49 \pm 0.63^{b,y}$	38.24 ± 4.57^{b}	<0.0001
19	151.58 ± 3.12^{a}	$84.08 \pm 6.71^{b,x}$	58.14 ± 5.70^{c}	$52.42 \pm 3.00^{c,x}$	26.05 ± 3.26^{d}	<0.0001
p-value	0.337	0.0127	0.2897	0.0234	0.0955	

^{abc} Means $\pm$ standard error of the mean within the row showed a comparison between treatments by day followed by different superscripts are significantly different ($p < 0.05$).

^{xyz} Means $\pm$ standard error of the mean within the column showed a comparison within treatment by day followed by different superscripts are significantly different ($p < 0.05$).

BCEDM and PrCEDM were reported based on 1000 larvae.

4.3.5 Nutritional Value of Black Soldier Fly Larvae on Days 17 and 19 of Development

4.3.5.1 Nutrient Composition

Data on the nutrient composition of BSFL reared on the five selected feeds consisting of single and mixed substrates of L1, L4, L5, L6 and L7 on days 17 and 19 are presented in Table 22. The nutrient composition of the larvae was substantially different ($p < 0.05$) for both days. On day 17, the larvae DM reared on the five different feed substrates ranged from 17.4 ± 2.39 g/100g in L7 to 28.5 ± 0.56 g/100g in L1, which differed significantly at $p = 0.0011$ (Table 22). On day 19, significant differences in the DM content were similarly observed at $p < 0.0001$, which ranged from 20.08 ± 1.39 g/100g in L7 to 29.97 ± 0.36 g/100g in L1. Except for the larvae fed on L4 ($p = 0.0350$), the DM content in larvae showed no significant differences between days 17 and 19. The L1-fed larvae had the most DM content on both days, while the L7-fed larvae had the least. The DM larvae seemed to increase at high proportions of MECM in the feed substrate.

The CP contents of larvae varied significantly ($p < 0.0001$) between feed substrates on both days of sampling (Table 22). The larvae fed on L7 had the greatest CP of 52.51 g/100g DM on day 17, which differed considerably from those feds with other feed substrates. The L6 substrate generated larvae with the highest CP content of 54.55 g/100g DM on day 19, albeit not significantly different from L4, L5, and L7 substrates. In the two-day development period, the CP of larvae increased significantly in all mixed feed substrates of L4, L5 and L6 compared to L1 and L7. For example, the larvae reared on L4 (MECM:SG; 1:1) showed an increase of CP content from 44.22 ± 0.45 to 50.06 ± 0.95 g/100g DM ($p = 0.0050$), 45.44 ± 0.39 to 49.75 ± 0.78 g/100g DM ($p = 0.0077$) in L5 and 47.14 ± 0.2 to 54.55 ± 0.96 g/100g DM ($p =$ 0016) in L6.

The Cfat content in BSFL revealed significant ($p < 0.0001$) variations on both days between the single and mixed feed substrates (Table 22). The BSFL grown on MECM had the highest Cfat content of more than 40 g/100g DM on day 17, whereas the larvae reared on SG had the lowest content of approximately 7.54 ± 1.08 g/100g DM. The Cfat content in larvae increased as the proportion of MECM in the substrates increased. The addition of 50% MECM in the mixed feed substrate in L4 impacted an increase of more than 100% of larvae Cfat compared to the L7-fed larvae, which had 100% SG. The Cfat content of larvae fed on the mixed substrates of L4, L5 and L6 decreased on day 19 and in L7; however, the differences were not significant. However, L1-fed larvae increased the concentration of Cfat from 42.21 ± 3.36 g/100g DM on day 17 to 53.04 ± 0.61 g/100g DM on day 19 ($p = 0.8109$).

The combination of MECM and SG in the feed substrate increased the concentration of ash in the BSFL, albeit lower in L4 compared to L6 for both days ($p < 0.0001$) and were significantly different from larvae reared on 100% MECM (L1). The ash concentration was reduced from day 17 to 19 except for larvae reared on L1 ($p = 0.1936$) and L7 ($p = 0.0199$). On day 17 and day 19, L6-fed larvae had significantly higher ($p < 0.0001$) ash levels of around 15.05 ± 0.36 g/100g, and 13.9 ± 0.53 g/100g, respectively, compared to 12.17 ± 0.37 g/100g and 11.75 ± 0.16 g/100g, respectively in L4 which was significantly different from L1-fed larvae. However, the highest ash concentration among the feed substrates was recorded at 17.63 ±0.03 g/100g on day 19 in L7 at 100%SG which was significantly higher ($p = 0.0199$) than the figure of 14.04 ± 0.96 g/100g observed in the same substrate on day 17.

There was a significant difference in the crude fibre (CF) of the larvae on the feed substrates on day 17 ($p = 0.0029$) and day 19 ($p = 0.0203$). On both days, the larvae reared on 100% SG (L7) contained the highest amount of CF, while the lowest level was recorded in L1-fed larvae which was 100% MECM. There was no significant difference in larval CF between mixed-feed substrates and L7-fed larvae. The CF of L7-fed larvae increased significantly ($p = 0.0019$) from 12.03 ± 0.29 to 15.03 ± 0.29

g/100g DM, while the rise from 7.81 ± 0.38 to 9.31 ± 0.45 g/100g DM in L1-fed larvae was not significant (p = 0.0667) in two-day development periods. The CF of larvae reared on mixed feed substrates in two-days of development time was not significantly different (p > 0.05).

Table 22: The nutrient composition of BSFL on days 17 and 19 on the mixed feed substrates.

Substrate	**L1**	**L4**	**L5**	**L6**	**L7**	***p*-value**
MECM:SG Proportion	**5:0**	**1:1**	**2:3**	**1:4**	**0:5**	
Day	Dry Matter (g/100g of wet weight)					
17	28.46 ± 0.56^{a}	23.46 ± 0.86^{ab,x}	23.46 ± 0.86^{ab}	22.69 ± 0.33^{bc}	17.38 ± 2.39^{c}	0.0011
19	29.97 ± 0.36^{a}	21.96 ± 0.38^{b,y}	22.48 ± 0.80^{b}	22.13 ± 0.28^{b}	20.08 ± 1.39^{b}	<0.0001
p-value	0.0850	0.0350	0.4526	0.2624	0.3852	
	Crude Protein (g/100g DM)					
17	26.39 ± 0.71^{c}	44.22 ± 0.45^{b,y}	45.44 ± 0.39^{b,y}	47.14 ± 0.21^{y,b}	52.51 ± 1.52^{a}	<0.0001
19	27.05 ± 1.19^{b}	50.06 ± 0.95^{a,x}	49.75 ± 0.78^{a,x}	54.55 ± 0.96^{x,a}	50.97 ± 1.42^{a}	<0.0001
p-value	0.6590	0.0050	0.0077	0.0016	0.4983	
	Crude Fat (g/100g DM)					
17	42.21 ± 3.36^{a}	19.34 ± 2.49^{b}	14.77 ± 1.10^{bc}	11.23 ± 0.60^{bc}	7.54 ± 1.08^{c}	<0.0001
19	53.04 ± 0.61^{a}	12.58 ± 0.30^{b}	12.07 ± 1.41^{b}	9.55 ± 0.89^{bc}	7.18 ± 0.91^{c}	<0.0001
p-value	0.8109	0.0542	0.206	0.1939	0.8109	
	Ash (g/100g)					
17	3.17± 0.10^{c}	12.17 ± 0.37^{b}	13.13 ± 0.32^{ab,x}	15.05 ± 0.36^{a}	14.04 ± 0.96^{ab,y}	<0.0001
19	3.34 ± 0.02^{d}	11.75 ± 0.16^{c}	12.01 ± 0.25^{c,y}	13.9 ± 0.53^{b}	17.63 ±0.03^{a,x}	<0.0001
p-value	0.1936	0.3540	0.0495	0.1469	0.0199	

Substrate	**L1**	**L4**	**L5**	**L6**	**L7**	***p*-value**
MECM:SG Proportion	**5:0**	**1:1**	**2:3**	**1:4**	**0:5**	
Day	Crude Fibre (g/100g DM)					
17	7.81 ± 0.38^{b}	11.12 ± 0.16^{a}	11.39 ± 0.59^{a}	10.18 ± 0.99^{ab}	12.03 ± 0.29^{a,y}	0.0029
19	9.31 ± 0.45^{b}	10.77 ±0.16^{ab}	10.97 ± 0.57^{ab}	11.89 ± 2.03^{ab}	15.03 ± 0.29^{a,x}	0.0203
p-value	0.0667	0.1849	0.6334	0.4891	0.0019	

^{abc}Means ± standard error of the mean within row showed comparison between treatments by day followed by different supercripts are significantly different ($p < 0.05$).

^{xy}Means ± standard error of the mean within column showed comparison within treatment by day followed by different supercripts are significantly different ($p < 0.05$).

4.3.5.2 Amino Acid Profiles of Black Soldier Fly Larvae

Tables 23 and 24 illustrate the amino acid (AA) profiles of BSFL reared on mixed feed substrate on days 17 and 19. The concentrations of AA in larvae reared on the six feed substrates on day 17 were significantly ($p < 0.05$) different, except for arginine. The non-essential amino acids (non-EAAs), such as alanine and glutamic acid, were the most prevalent in the mixed feed substrates. The alanine levels in BSFL reared on L4 (MECM:SG, 1:1), L5 (MECM:SG, 2:3), and L6 (MECM:SG, 1:4) were 5.01 ± 0.26 g/100g DM, 4.66 ± 0.05 g/100g DM, and 4.30 ± 0.09 g/100g DM, respectively, which were significantly ($p < 0.0001$) higher than those in L7 consisting of 100% SG (3.11 g/100g DM) and L1 of 100% MECM (1.47 ± 0.13 g/100g DM). Although the glutamic acid content was highest in SG-fed larvae (L7) at 5.66 g/100g, it was not significantly different from L4-fed larvae at 4.96 ± 0.27 g/100g DM and L6-fed larvae at 4.83 ± 0.26 g/100g DM. The essential amino acids (EAAs) of leucine were more prevalent than the other EAAs, particularly in L5 at 3.19 ± 0.03 and L6 at 3.27 ± 0.02 g/100g DM, compared to L7-fed larvae at around 2.89 ± 0.28 g/100g DM. However, the differences in the leucine content in the mixed feed substrates were insignificant. The differences in lysine levels in the mixed feed substrates ranged from 2.66 ± 0.36 to 2.97 ± 0.08 g/100g DM were not significant. The lysine level of 3.03 ± 0.33 g/100g was higher in the larvae fed on L7 substrate, while it was lowest in L1-fed larvae, of 1.49 ± 0.13 g/100g DM.

On day 19, alanine and glutamic acid were also prevalent in the larvae, as shown in Table 24. For example, glutamic acid levels in mixed feed substrates (L4-L6) were between 5.04 ± 0.16 and 5.69 ± 0.33 g/100g DM, compared to the L7- and L1-fed larvae concentrations which were 5.33 ± 0.35 g/100g DM and 3.39 ± 0.05 g/100g DM, respectively. Although these figures were not significantly different, they were greater than the contents recorded in the larvae collected on day 17. The EAAs of isoleucine, lysine, phenylalanine and valine in the larvae reared on the mixed feed substrates were not significantly different from the L7-fed larvae. Larvae reared on L6 substrate had the greatest concentration of leucine at 3.50 ± 0.01 g/100g DM,

which was significantly (p < 0.0001) higher than the 1.96 ± 0.01 g/100g DM recorded in larvae given L1.

Most AA levels were higher on day 19 than day 17 (p > 0.05), particularly in larvae reared on L4 and L5, as shown in Table 25. The levels of AAs in BSFL reared on L7 substrates steadily reduced over the two development days. Only a few AAs showed significant differences between days 17 and 19, particularly the larvae reared on L6 (Table 25). For example, glycine, serine, cysteine, histidine, leucine, methionine and threonine exhibited higher levels on day 19 compared to day 17. The total AA concentrations increased roughly 8.5%, 5.6% and 13.4% in L4-, L5- and L6-fed larvae, respectively, from day 17 to day 19. Proline was undetectable in the larvae reared on L5 on day 17 but was detected in a trace amount on day 19. The AA profiles of larvae fed with L4 were comparable between the two-day development periods except for tryptophan, 0.80 ± 0.15 (day 17) and 1.37 ± 0.08 (day 19).

Table 23: Amino acid profiles of black soldier fly larvae reared on mixed feed substrate at day 17.

Substrate / Amino Acid	L1	L4	L5	L6	L7	*p*-value
Alanine	1.47 ± 0.13^{d}	5.01 ± 0.26^{a}	4.66 ± 0.05^{ab}	4.30 ± 0.09^{b}	3.11 ± 0.03^{c}	<0.0001
Aspartic Acid	1.67 ± 0.08^{b}	2.92 ± 0.94^{ab}	3.86 ± 0.07^{a}	4.06 ± 0.08^{a}	3.83 ± 0.11^{a}	0.0133
Glutamic Acid	2.97 ± 0.09^{c}	4.96 ± 0.27^{ab}	4.60 ± 0.17^{b}	4.83 ± 0.26^{ab}	5.66 ± 0.05^{a}	<0.0001
Glycine	1.42 ± 0.01^{b}	2.48 ± 0.09^{ab}	2.42 ± 0.01^{ab}	2.51 ± 0.01^{ab}	2.81 ± 0.12^{a}	<0.0001
Proline	2.26 ± 0.03^{a}	0.74 ± 0.08^{b}	ND	2.21 ± .03^{a}	2.48 ± 0.21^{a}	0.0001
Serine	0.90 ± 0.05^{b}	1.46 ± 0.06^{a}	1.44 ± 0.01^{a}	1.54 ± 0.05^{a}	1.41 ± 0.04^{a}	<0.0001
Tyrosine	1.53 ± 0.24^{c}	3.92 ± 0.27^{ab}	4.42 ± 0.07^{a}	2.91 ± 0.37^{bc}	3.63 ± 0.47^{ab}	0.0006
Arginine	1.73 ± 0.37	2.24 ± 0.13	2.26 ± 0.04	2.30 ± 0.06	2.33 ± 0.19	0.2430
Cysteine	1.12 ± 0.06^{b}	0.10 ± 0.01^{c}	0.12 ± 0.00^{c}	0.22 ± 0.01^{c}	2.15 ± 0.10^{a}	<0.0001
Histidine*	0.97 ± 0.02^{b}	1.50 ± 0.05^{a}	1.28 ± 0.06^{ab}	1.50 ± 0.02^{a}	1.53 ± 0.06^{a}	0.0102
Isoleucine*	1.21 ± 0.09^{b}	1.98 ± 0.04^{a}	1.97 ± 0.02^{ab}	2.01 ± 0.01^{a}	2.09 ± 0.35^{a}	0.0198
Leucine*	1.61 ± 0.16^{b}	2.80 ± 0.51^{ab}	3.19 ± 0.03^{a}	3.27 ± 0.02^{a}	2.89 ± 0.28^{a}	0.0094
Lysine*	1.49 ± 0.13^{b}	2.66 ± 0.36^{a}	2.74 ± 0.12^{a}	2.97 ± 0.08^{a}	3.03 ± 0.33^{a}	0.0056
Methionine*	1.18 ± 0.10^{a}	0.67 ± 0.01^{b}	0.62 ± 0.01^{b}	0.73 ±0.04^{b}	0.63 ± 0.09^{b}	0.0003
Phenyl-Alanine*	0.74 ± 0.12^{b}	2.46 ± 0.70^{a}	1.78 ± 0.05^{ab}	1.91 ± 0.02^{ab}	1.71 ± 0.20^{ab}	0.0501
Threonine*	0.62 ± 0.02^{b}	1.15 ± 0.09^{a}	1.15 ± 0.08^{a}	1.17 ± 0.04^{a}	1.11 ± 0.02^{a}	<0.0001
Tryptophan*	0.22 ± 0.01^{b}	0.80 ± 0.15^{b}	0.56 ± 0.00^{b}	0.66 ± 0.05^{b}	1.61 ± 0.03^{a}	<0.0001
Valine*	1.73 ± 0.03^{b}	2.73 ± 0.09^{a}	2.66 ± 0.02^{a}	2.75 ± 0.10^{a}	2.81 ± 0.22^{a}	0.0003
Total AA	24.81 ± 0.16	40.55 ± 1.93	39.72 ± 0.11	41.83 ± 0.40	44.84 ± 1.31	
Total non-EAA	15.05 ± 0.16	23.81 ± 1.77	23.78 ± 0.20	24.88 ± 0.30	27.41 ± 0.03	
Total EAA	9.77 ± 0.22	16.74 ± 0.28	15.94 ± 0.23	16.96 ± 0.10	17.42 ± 1.29	
Total CP	26.39 ± 0.71	44.22 ± 0.45	45.44 ± 0.39	47.14 ± 0.21	52.51 ± 1.52	

^{abcd}Means ± standard error of the mean is in g/100 g on the DM basis (n=3) in the same row followed with different superscripts are significantly different at $p < 0.05$. *=essential amino acids, ND=not detected.

Mixtures of MECM:SG; L1=5:0, L4=1:1, L5=2:3, L6=1:4, L7=0:5.

Table 24: Amino acid profiles of black soldier fly larvae reared on mixed feed substrate at day 19.

Substrate/ Amino Acid	L1	L4	L5	L6	L7	*p*-value
Alanine	1.75 ± 0.01^{b}	4.71 ± 0.16^{a}	5.86 ± 0.07^{a}	3.73 ± 0.01^{ab}	5.04 ± 0.94^{a}	0.0027
Aspartic Acid	2.22 ± 0.01^{b}	3.99 ± 0.19^{a}	3.58 ± 0.03^{a}	4.00 ± 0.09^{a}	3.76 ± 0.10^{a}	<0.0001
Glutamic Acid	3.39 ± 0.05^{b}	5.69 ± 0.33^{a}	5.04 ± 0.16^{a}	5.17 ± 0.08^{a}	5.33 ± 0.35^{a}	0.0024
Glycine	1.63 ± 0.01^{c}	2.75 ± 0.17^{b}	2.47 ± 0.03^{b}	3.66 ± 0.06^{a}	2.60 ± 0.13^{b}	<0.0001
Proline	2.15 ± 0.09^{b}	0.40 ± 0.13^{c}	0.67 ± 0.02^{c}	2.72 ± 0.03^{a}	2.06 ± 0.11^{b}	<0.0001
Serine	1.04 ± 0.00^{c}	1.65 ± 0.10^{b}	1.38 ± 0.01^{bc}	2.41 ± 0.05^{a}	1.41 ± 0.11^{bc}	<0.0001
Tyrosine	1.59 ± 0.07^{c}	2.71 ± 0.44^{bc}	3.46 ± 0.10^{ab}	2.41 ± 0.03^{bc}	4.10 ± 0.34^{a}	0.0016
Arginine	1.40 ± 0.01^{b}	3.46 ± 0.58^{a}	2.18 ± 0.02^{ab}	2.23 ± 0.04^{ab}	2.19 ± 0.06^{ab}	<0.0113
Cysteine	1.06 ± 0.05^{b}	0.33 ± 0.12^{c}	0.59 ± 0.00^{c}	2.69 ± 0.13^{a}	2.38 ± 0.03^{a}	<0.0001
Histidine*	0.99 ± 0.01^{c}	1.32 ± 0.13^{b}	1.52 ± 0.02^{ab}	1.66 ± 0.02^{a}	1.58 ± 0.04^{ab}	0.0009
Isoleucine*	1.25 ± 0.01^{b}	2.27 ± 0.16^{a}	1.95 ± 0.03^{a}	1.84 ± 0.01^{a}	1.84 ± 0.16^{a}	0.0030
Leucine*	1.96 ± 0.01^{b}	3.12 ± 0.06^{a}	3.16 ± 0.02^{a}	3.50 ± 0.01^{ab}	3.07 ± 0.20^{a}	<0.0001
Lysine*	1.86 ± 0.01^{c}	3.00 ± 0.11^{a}	2.86 ± 0.13^{ab}	2.45 ± 0.00^{b}	2.78 ± 0.10^{ab}	0.0002
Methionine*	0.44 ± 0.00^{c}	1.37 ± 0.00^{b}	1.25 ± 0.00^{b}	2.38 ± 0.16^{a}	0.61 ± 0.08^{c}	<0.0001
Phenyl-Alanine*	0.89 ± 0.00^{b}	1.58 ± 0.13^{a}	1.65 ± 0.03^{a}	1.78 ± 0.00^{a}	1.63 ± 0.13^{a}	0.0011
Threonine*	0.70 ± 0.00^{c}	1.16 ± 0.08^{b}	1.02 ± 0.01^{b}	2.16 ± 0.04^{a}	1.08 ± 0.07^{b}	<0.0001
Tryptophan*	0.23 ± 0.01^{b}	1.37 ± 0.08^{a}	0.55 ± 0.00^{b}	0.44 ± 0.00^{b}	1.41 ± 0.15^{a}	<0.0001
Valine*	2.07 ± 0.00^{b}	3.13 ± 0.16^{a}	2.77 ± 0.03^{a}	2.20 ± 0.00^{b}	2.77 ± 0.13^{a}	0.0003
Total AA	26.41 ± 0.22	44.00 ± 0.71	41.96 ± 0.15	47.42 ± 0.66	45.65 ± 1.03	
Total non-EAA	16.16 ± 0.09	25.68 ± 0.61	25.23 ± 0.04	29.03 ± 0.47	28.87 ± 0.67	
Total EAA	10.25 ± 0.14	18.32 ± 0.20	16.74 ± 0.12	18.40 ± 0.20	16.78 ± 0.65	
Total CP	27.05 ± 1.19	50.06 ± 0.95	49.75 ± 0.78	54.55 ± 0.96	50.97 ± 1.42	

^{abc}Means ± standard error of the mean is in g/100 g on the DM basis (n=3) in the same row, followed by different superscripts that are significantly different at $p < 0.05$. *=essential amino acids, ND=not detected.

Mixtures of MECM:SG; L1=5:0, L4=1:1, L5=2:3, L6=1:4, L7=0:5.

Table 25: The amino acid profiles comparison of black soldier fly larvae within feed substrates between days 17 and 19.

Substrate	**L1**		**L4**		**L5**		**L6**		**L7**	
Day / Amino Acid	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**
Alanine	1.47 ± 0.13	1.75 ± 0.01	5.01 ± 0.26	4.71 ± 0.16	4.66 ± 0.05[b]	5.86 ± 0.07[a]	4.30 ± 0.09[a]	3.73 ± 0.01[b]	3.11 ± 0.03	5.04 ± 0.94
Aspartic Acid	1.67 ± 0.08[b]	2.22 ± 0.01[a]	2.92 ± 0.94	3.99 ± 0.19	3.86 ± 0.07[a]	3.58 ± 0.03[b]	4.06 ± 0.08	4.00 ± 0.09	3.83 ± 0.11	3.76 ± 0.10
Glutamic Acid	2.97 ± 0.09[b]	3.39 ± 0.05[a]	4.96 ± 0.27	5.69 ± 0.33	4.60 ± 0.17	5.04 ± 0.16	4.83 ± 0.26	5.17 ± 0.08	5.66 ± 0.05	5.33 ± 0.35
Glycine	1.42 ± 0.01[b]	1.63 ± 0.01[a]	2.48 ± 0.09	2.75 ± 0.17	2.42 ± 0.01	2.47 ± 0.03	2.51 ± 0.01[b]	3.66 ± 0.06[a]	2.81 ± 0.12	2.60 ± 0.13
Proline	2.26 ± 0.03	2.15 ± 0.09	0.74 ± 0.08	0.40 ± 0.13	ND	0.67 ± 0.02	2.21 ± 0.03	2.72 ± 0.03	2.48 ± 0.21	2.06 ± 0.11
Serine	0.90 ± 0.05	1.04 ± 0.00	1.46 ± 0.06	1.65 ± 0.10	1.44 ± 0.01[a]	1.38 ± 0.01[b]	1.54 ± 0.05[b]	2.41 ± 0.05[a]	1.41 ± 0.04	1.41 ± 0.11
Tyrosine	1.53 ± 0.24	1.59 ± 0.07	3.92 ± 0.27	2.71 ± 0.44	4.42 ± 0.07[a]	3.46 ± 0.10[b]	2.91 ± 0.37	2.41 ± 0.03	3.63 ± 0.47	4.10 ± 0.34
Arginine	1.73 ± 0.37	1.40 ± 0.01	2.24 ± 0.13[a]	3.46 ± 0.58[b]	2.26 ± 0.04	2.18 ± 0.02	2.30 ± 0.06	2.23 ± 0.04	2.33 ± 0.19	2.19 ± 0.06
Cysteine	1.12 ± 0.06	1.06 ± 0.05	0.1 ± 0.01	0.33 ± 0.12	0.12 ± 0.00[b]	0.59 ± 0.00[a]	0.22 ± 0.01[b]	2.69 ± 0.13[a]	2.15 ± 0.10	2.38 ± 0.03

Substrate	L1		L4		L5		L6		L7	
Day / Amino Acid	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**
Histidine	0.97 ± 0.02	0.99 ± 0.01	1.50 ± 0.05	1.32 ± 0.13	1.28 ± 0.06[b]	1.52 ± 0.02[a]	1.50 ± 0.02[b]	1.66 ± 0.02[a]	1.53 ± 0.06	1.58 ± 0.04
Isoleucine	1.21 ± 0.09	1.25 ± 0.01	1.98 ± 0.04	2.27 ± 0.16	1.97 ± 0.02	1.95 ± 0.03	2.01 ± 0.01[a]	1.84 ± 0.01[b]	2.09 ± 0.35	1.84 ± 0.16
Leucine	1.61 ± 0.16	1.96 ± 0.01	2.80 ± 0.51	3.12 ± 0.06	3.19 ± 0.03	3.16 ± 0.02	3.26 ± 0.02[b]	3.50 ± 0.01[a]	2.89 ± 0.28	3.07 ± 0.20
Lysine	1.49 ± 0.13	1.86 ± 0.01	2.65 ± 0.36	3.00 ± 0.11	2.74 ± 0.12	2.86 ± 0.13	2.97 ± 0.08[a]	2.45 ± 0.00[b]	3.03 ± 0.33	2.78 ± 0.10
Methionine	1.18 ± 0.10[a]	0.44 ± 0.00[b]	0.67 ± 0.01[b]	1.37 ± 0.00[a]	0.62 ± 0.01[b]	1.25 ± 0.00[a]	0.73 ± 0.04[b]	2.38 ± 0.16[a]	0.63 ± 0.09	0.61 ± 0.08
Phenyl-alanine	0.74 ± 0.12	0.89 ± 0.00	2.46 ± 0.70	1.58 ± 0.13	1.78 ± 0.05	1.66 ± 0.03	1.91 ± 0.02[a]	1.77 ± 0.00[b]	1.71 ± 0.20	1.63 ± 0.13
Threonine	0.62 ± 0.02[b]	0.70 ± 0.00[a]	1.15 ± 0.09	1.16 ± 0.08	1.15 ± 0.02[a]	1.02 ± 0.01[b]	1.17 ± 0.04[b]	2.16 ± 0.04[a]	1.11 ± 0.02	1.08 ± 0.07
Tryptophan	0.22 ± 0.01	0.23 ± 0.01	0.80 ± 0.15[b]	1.37 ± 0.08[a]	0.56 ± 0.01	0.55 ± 0.00	0.66 ± 0.05[a]	0.44 ± 0.00[b]	1.61 ± 0.03	1.41 ± 0.15
Valine	1.73 ± 0.03[b]	2.07 ± 0.00[a]	2.73 ± 0.09	3.13 ± 0.16	2.66 ± 0.02[b]	2.77 ± 0.03[a]	2.75 ± 0.10[a]	2.20 ± 0.00[b]	2.81 ± 0.22	2.77 ± 0.13

[ab]Means ± standard error of the mean is in g/100 g on the DM basis (n=3) between two columns of days 17 and 19 within same substrate with different superscripts are significantly different at $p < 0.05$. Mixtures of MECM:SG; L1=5:0, L4=1:1, L5=2:3, L6=1:4, L7=0:5.

4.3.5.3 The Fatty Acid Profiles of Black Soldier Fly Larvae

The BSFL fatty acid (FA) profiles on days 17 and 19 are shown in Tables 26 and 27, respectively. On day 17, the FA profiles between the single and mixed feed substrates were significantly different ($p < 0.05$) except for myristoleic acid (C14:1) and eicosapentaenoic acid, EPA (20:5n3). Only small levels of EPA were detected in L4- and L5-fed larvae. The BSFL demonstrated the greatest levels of lauric acid (C12:0) ranging from 28.85 ± 0.27% to 36.82 ± 0.12% of total FA in the mixed feed substrates, while at 41.30 ± 0.46% in L1-fed larvae (100% MECM) and 27.35 ± 0.64% in L7-fed larvae (100% SG). The level of lauric acid (C12:0), myristic acid (C14:0) and myristoleic acid (C14:1) in BSFL on day 17 increased as the proportion of MECM in the feed substrate increased (Table 26). In the mixed feed substrates, the larvae fed with L4 had significantly ($p < 0.0002$) higher in C12:0 content than L5 and L6. Stearic acid (C18:0) was also high in the larvae fed with mixed feed substrates, ranging from 22.63 ± 0.71% to 27.51 ± 2.71% of total FA, which was significantly ($p = 0.0010$) greater than in the larvae reared on the primary substrates of L1 and L7. Heptadecanoic acid (C17:0) was absent in L1 and L4 substrates but was present in L5, L6 and L7 as the proportion of SG in the substrate increased. The monounsaturated fatty acid (MUFA) of cis-10-heptadecanoic acid (C17:1) in the mixed substrates, L5 and L6, was higher than in L7-fed larvae but not detected in L1- and L4-fed larvae. Linoleic acid (C18:2n6) was not significantly different amongst mixed feed substrates and L1-fed larvae but different from L7-fed larvae ($p = 0.0033$). Linolenic acid (C18:3n3) was detected at less than 1% of total FA in the larvae fed with substrates containing less than 80% SG, while not detectable in L1, which had no SG.

The larvae FA composition on day 19 is shown in Table 27. The FA profiles in BSFL were significantly ($p < 0.05$) different amongst the substrates except for myristic acid, pentadecanoic acid, heptadecanoic acid, cis-10-heptadecanoic acid, cis-oleic acid, linoleic acid, and arachidic acid. Lauric acid was found in abundance in the larvae on day 19 across all feed substrates and its level increased as the inclusion

level of MECM in the substrates rose, as in L4, L5, and L6. Only a few FA levels changed significantly between days 17 and 19. For instance, the polyunsaturated fatty acid (PUFA) of linolenic acid (C18:3n3) in BSFL rose on day 19 ($p < 0.05$), particularly in mixed feed substrate of L6 and L7 (Table 27). Including 20% MECM in L6 increased this FA at 7.9% compared to L7. However, the level of C18:3n3 dropped in descending order in L6, L5 and L4 as the proportions of SG declined in the mixed feed substrates and were undetectable in L1-fed larvae. The FA profiles in BSFL revealed significant ($p < 0.05$) differences between the two-day development period except for lauric acid, cis-oleic acid, heptadecanoic acid, pentadecanoic acid, eicosanoic, eicosapentaenoic acid, behenic acid, erucic acid methyl ester and docosadienoic acid (Table 28). The level of linolenic acid (C18:3n3) significantly ($p < 0.05$) increased in the larvae on day 19. The levels of this FA in L4-, L5- and L6-fed larvae increased by 54%, 100% and 77%, respectively, in just two days. The increase in linoleic acid (C18:2n6) levels from day 17 to day 19 was not significant, although the larvae on L4 showed the highest level of about 6.5% compared to L5 and L6 on day 19. Other FAs showed decreasing trends with the development time. Several FAs that were absent on day 17, such as behenic acid (C22:0), erucic acid methyl ester (C22:1), and docosadienoic acid (22:2n6), reappeared at modest levels on day 19.

The total saturated fatty acids (SFA) were high on days 17 and 19, which ranged from 75.56 ± 0.37% – 87.76 ± 0.04% (Table 26) and from 74.80 ± 2.48% – 89.00 ± 0.09% of total FA (Table 27), respectively. The SFA levels in larvae increased in ascending order from L1 to L7. The MUFA ranged from 6.90 ± 0.07% to 17.28 ± 0.87% on day 17 of total FA (Table 26) and 6.14 ± 0.07% to 15.64 ± 1.67% of total FA on day 19 (Table 27). The PUFA were the least abundant FA in BSFL, although the levels rose throughout the time, ranging from 4.54% ± 0.05% − 8.76 ± 0.49% on day 17 and 4.86 ± 0.00% − 9.72 ± 0.49% on day 19. Except for the larvae reared on L1, the levels of SFA in larvae grown on L4, L5, L6, and L7 dropped as the proportions of SG increased during the two-day development. Although the trend in the levels of MUFA was ambiguous on day 17, it was clearer on day 19. This was aided by an

increase in the percentage of PUFA in BSFL, including linoleic acid and linolenic acid. However, eicosapentaenoic acid (EPA, 20:5n3) only appeared on day 17 in L4- and L5-fed larvae with small quantities of 0.23 ± 0.02% and 0.79 ± 0.14%, respectively, and was not detected in day 19.

Table 26: Fatty acid profiles of black soldier fly larvae reared on mixed feed substrate at day 17.

Substrate/ Fatty Acid (% of total FA)	**L1**	**L4**	**L5**	**L6**	**L7**	***p-value***
Lauric Acid (C12:0)	41.30 ± 0.46^{a}	36.82 ± 0.12^{b}	29.45 ± 1.53^{c}	28.85 ± 0.27^{c}	27.35 ± 0.64^{c}	0.0002
Myristic Acid (C14:0)	16.28 ± 0.02^{a}	13.55 ± 0.01^{ab}	12.70 ± 1.54^{ab}	9.57 ± 0.37^{bc}	5.58 ± 0.28^{c}	0.0009
Myristoleic Acid (C14:1)	1.04 ± 0.02	0.93 ± 0.00	0.7125 ± 0.19	ND	ND	0.2247
Palmitic Acid (C16:0)	13.33 ± 0.02^{b}	13.49 ± 0.04^{b}	15.20 ±0.23^{ab}	14.83 ± 0.06^{ab}	17.24 ± 0.96^{a}	0.0079
Palmitoleic Acid (C16:1)	3.53 ± 0.15^{c}	5.97 ± 0.07^{a}	6.00 ± 0.04^{a}	5.52 ± 0.08^{a}	4.51 ± 0.07^{b}	<.0001
Heptadecanoic Acid (C17:0)	ND	ND	0.48 ± 0.09^{b}	0.47 ± 0.04^{b}	0.90 ± 0.07^{a}	0.0364
cis-10-heptadecanoic Acid (C17:1)	ND	ND	0.64 ± 0.11^{c}	1.24 ± 0.04^{b}	2.23 ± 0.04^{a}	0.0013
Stearic Acid (C18:0)	12.64 ± 0.33^{b}	23.55 ± 0.11^{a}	27.51 ± 2.71^{a}	22.63 ± 0.71^{a}	10.54 ± 0.91^{b}	0.0010
cis-oleic Acid (C18:1n9cis)	5.92 ± 0.05^{b}	ND	ND	10.52 ± 0.99^{a}	8.94 ± 0.23^{ab}	0.0252
Linoleic Acid (C18:2n6)	4.54 ± 0.05^{b}	4.26 ± 0.00^{b}	4.85 ± 0.38^{b}	4.08 ± 0.04^{b}	6.63 ± 0.37^{a}	0.0033
Linolenic Acid (C18:3n3)	ND	0.85 ± 0.01^{b}	0.99 ± 0.03^{b}	2.23 ± 0.02^{a}	2.13 ± 0.12^{a}	0.0002
Arachidic Acid (C20:0)	1.43 ± 0.38^{b}	0.36 ± 0.01^{b}	0.69 ± 0.00^{b}	0.40 ± 0.02^{b}	13.96 ± 1.43^{a}	<0.0001
Eicosapentaenoic Acid (20:5n3)	ND	0.23 ± 0.02	0.79 ± 0.14	ND	ND	0.0582
ΣSFA	84.96 ± 0.24	87.76 ± 0.04	86.03 ± 0.50	76.42 ± 0.81	75.56 ± 0.37	
ΣMUFA	10.49 ± 0.18	6.90 ± 0.07	7.35 ± 0.04	17.28 ± 0.87	15.68 ± 0.11	
ΣPUFA	4.54 ± 0.05	5.34 ± 0.03	6.62 ± 0.54	6.31 ± 0.06	8.76 ± 0.49	

[abcd]Means ± standard error of the mean on a DM basis (n=3) in the same row followed with different superscripts are significantly different at $p < 0.05$. ΣSFA=Saturated Fatty Acid, ΣMUFA=Total Monounsaturated Fatty Acid, ΣPUFA=Total Polyunsaturated Fatty Acid.

Table 27: Fatty acid profiles of black soldier fly larvae reared on mixed feed substrate at day 19.

Substrate / Fatty Acid (% of total FA)	**L1**	**L4**	**L5**	**L6**	**L7**	***p*-value**
Lauric Acid (C12:0)	39.04 ± 0.60^{a}	36.41 ± 2.25^{a}	32.41 ± 1.56^{ab}	27.84 ± 1.10^{c}	27.29 ± 0.78^{c}	0.0065
Myristic Acid (C14:0)	17.28 ± 0.68	12.59 ± 0.87	12.23 ± 0.47	13.18 ± 0.30	14.75 ± 1.71	0.0578
Myristoleic Acid (C14:1)	0.98 ± 0.02^{ab}	0.78 ± 0.01^{cd}	1.15 ± 0.06^{a}	0.67 ± 0.03^{d}	0.87 ± 0.03^{bc}	0.0009
Pentadecanoic Acid (C15:0)	ND	ND	0.26 ± 0.05	0.36 ± 0.05	0.37 ± 0.04	0.2190
Palmitic Acid (C16:0)	13.48 ± 0.71^{b}	12.83 ±0.10^{b}	12.73 ± 0.51^{b}	17.78 ± 0.29^{a}	18.46 ± 0.53^{a}	0.0008
Palmitoleic Acid (C16:1)	3.74 ± 0.04^{b}	5.44 ± 0.34^{ab}	6.46 ± 0.05^{a}	4.59 ± 0.02^{ab}	4.06 ± 0.66^{b}	0.0108
Heptadecanoic Acid (C17:0)	ND	ND	0.41 ± 0.02	0.49 ± 0.02	0.30 ± 0.08	0.1324
cis-10-heptadecanoic Acid (C17:1)	ND	ND	0.77 ± 0.01	0.80 ± 0.05	0.35 ± 0.31	0.2909
Stearic Acid (C18:0)	18.80 ± 0.47^{ab}	23.26 ± 1.92^{a}	19.03 ± 2.14^{ab}	16.85 ± 0.98^{ab}	13.09 ± 0.45^{b}	0.0273
cis-oleic Acid (C18:1n9cis)	ND	ND	6.11 ± 0.19	6.22 ± 0.74	10.02 ± 1.42	0.0944
Linoleic Acid (C18:2n6)	4.70 ± 0.00	6.50 ± 0.62	5.18 ± 0.03	5.68 ± 0.17	5.61 ± 0.41	0.0864
Linolenic Acid (C18:3n3)	ND	1.31 ± 0.07^{b}	2.00 ± 0.09^{b}	3.94 ± 0.31^{a}	3.65 ± 0.36^{a}	0.0043
Arachidic Acid (C20:0)	ND	0.23 ± 0.00	0.41 ± 0.01	0.46 ± 0.04	0.33 ± 0.06	0.1336
Eicosenoic (C20:1n9)	1.28 ± 0.10^{b}	0.35 ± 0.11^{a}	0.37 ± 0.01^{a}	0.44 ± 0.03^{a}	0.30 ± 0.15^{a}	0.0035

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Substrate / Fatty Acid (% of total FA)	**L1**	**L4**	**L5**	**L6**	**L7**	***p*-value**
Behenic Acid (C22:0)	0.39 ± 0.25	0.14 ± 0.04	0.25 ± 0.03	0.33 ± 0.01	0.21 ± 0.09	0.6281
Erucic Acid methyl ester (C22:1)	0.15 ± 0.02	0.13 ± 0.02	0.08 ± 0.01	0.11 ± 0.01	0.04 ± 0.03	0.068
Docosadienoic Acid (22:2n6)	0.17 ± 0.00[a]	0.15 ± 0.03[ab]	0.16 ± 0.03[ab]	0.12 ± 0.01[ab]	0.06 ± 0.01[b]	0.0417
ΣSFA	89.00 ± 0.07	85.34 ± 1.18	77.72 ± 0.39	77.30 ± 1.39	74.80 ± 2.48	
ΣMUFA	6.14 ± 0.07	6.70 ± 0.46	14.94 ± 0.30	12.82 ± 0.78	15.64 ± 1.67	
ΣPUFA	4.86 ± 0.00	7.96 ± 0.72	7.33 ± 0.09	9.72 ± 0.49	9.31 ± 0.78	

[abcd]Means ± standard error of the mean on a DM basis (n=3) in the same row followed with different superscripts are significantly different at $p < 0.05$.

ΣSFA=Saturated Fatty Acid, ΣMUFA=Total Monounsaturated Fatty Acid, ΣPUFA=Total Polyunsaturated Fatty Acid.

Mixtures of MECM:SG; L1=5:0, L4=1:1, L5=2:3, L6=1:4, L7=0:5.

Table 28: The fatty acid profile comparison of black soldier fly larvae within feed substrates between days 17 and 19.

Substrate	**L1**		**L4**		**L5**		**L6**		**L7**	
Day / Fatty Acid (% of total FA)	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**
Lauric Acid (C12:0)	41.30 ± 0.46	39.04 ± 0.60	36.82 ± 0.12	36.41 ± 2.25	29.45 ± 1.53	32.41 ± 1.56	28.85 ± 0.27	27.84 ± 1.10	27.35 ± 0.64	27.29 ± 0.78
Myristic Acid (C14:0)	16.28 ± 0.02	17.28 ± 0.68	13.55 ± 0.01	12.59 ± 0.87	12.70 ± 1.54	12.23 ± 0.47	9.57 ± 0.37[b]	13.18 ± 0.30[a]	5.58 ± 0.28[b]	14.75 ± 1.71[a]
Myristoleic Acid (C14:1)	1.04 ± 0.02	0.98 ± 0.02	0.93 ± 0.00[a]	0.78 ± 0.01[b]	0.71 ± 0.19	1.15 ± 0.06	ND	0.67 ± 0.03	ND	0.87 ± 0.03
Pentadecanoic Acid (C15:0)	ND	ND	ND	ND	ND	0.26 ± 0.05	ND	0.36 ± 0.05	ND	0.37 ± 0.04
Palmitic Acid (C16:0)	13.33 ± 0.02	13.48 ± 0.71	13.49 ± 0.04[a]	12.83 ± 0.10[b]	15.20 ± 0.23[a]	12.73 ± 0.51[b]	14.83 ± 0.06[b]	17.78 ± 0.29[a]	17.24 ± 0.96	18.46 ± 0.53
Palmitoleic Acid (C16:1)	3.53 ± 0.15	3.74 ± 0.04	5.97 ± 0.07	5.44 ± 0.34	6.00 ± 0.04[a]	6.46 ± 0.05[b]	5.44 ± 0.08[a]	4.59 ± 0.02[b]	4.51 ± 0.07	4.06 ± 0.66
Heptadecanoic Acid (C17:0)	ND	ND	ND	ND	0.48 ± 0.09	0.41 ± 0.02	0.47 ± 0.04	0.49 ± 0.02	0.90 ± 0.07[a]	0.30 ± 0.08[b]
cis-10-heptadeca-noic Acid (C17:1)	ND	ND	ND	ND	0.64 ± 0.11	0.77 ± 0.01	1.24 ± 0.04[a]	0.80 ± 0.05[b]	2.23 ± 0.04[a]	0.35 ± 0.31[b]
Stearic Acid (C18:0)	12.64 ± 0.33[b]	18.80 ± 0.47[a]	23.55 ± 0.11	23.26 ± 1.92	27.51 ± 2.71	19.03 ± 2.14	22.63 ± 0.71[a]	16.85 ± 0.98[b]	10.54 ± 0.91	13.09 ± 0.45

continue…

Substrate	L1		L4		L5		L6		L7	
Day / Fatty Acid (% of total FA)	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**	**17**	**19**
cis-oleic Acid (C18:1n9cis)	5.92 ± 0.05	ND	ND	ND	ND	6.11 ± 0.19	10.52 ± 0.99	6.22 ± 0.74	8.94 ± 0.23	10.02 ± 1.42
Linoleic Acid (C18:2n6)	4.54 ± 0.05	4.70 ± 0.00	4.26 ± 0.00	6.50 ± 0.62	4.85 ± 0.38	5.18 ± 0.03	4.08 ± 0.04[b]	5.68 ± 0.17[a]	6.63 ± 0.37	5.61 ± 0.41
Linolenic Acid (C18:3n3)	ND	ND	0.85 ± 0.01[b]	1.31 ± 0.07[a]	0.99 ± 0.03[b]	2.00 ± 0.09[a]	2.23 ± 0.02[b]	3.94 ± 0.31[a]	2.13 ± 0.12	3.65 ± 0.36
Arachidic Acid (C20:0)	1.43 ± 0.38	ND	0.36 ± 0.01	0.23 ± 0.00	0.69 ± 0.00[a]	0.41 ± 0.01[b]	0.40 ± 0.02	0.46 ± 0.04	13.96 ± 1.43[a]	0.33 ± 0.06[b]
Eicosenoic (C20:In9)	ND	1.28 ± 0.10	ND	0.35 ± 0.11	ND	0.37 ± 0.01	ND	0.44 ± 0.03	ND	0.30 ± 0.15
Eicosapentaenoic Acid (C20:5n3)	ND	ND	0.23 ± 0.02	ND	0.79 ± 0.14	ND	ND	ND	ND	ND
Behenic Acid (C22:0)	ND	0.39 ± 0.25	ND	0.14 ± 0.04	ND	0.25 ± 0.03	ND	0.33 ± 0.01	ND	0.21 ± 0.09
Erucic Acid methyl-ester (C22:1)	ND	0.15 ± 0.02	ND	0.13 ± 0.02	ND	0.08 ± 0.01	ND	0.11 ± 0.01	ND	0.04 ± 0.03
Docosadienoic Acid (22:2n6)	ND	0.17 ± 0.00	ND	0.15 ± 0.03	ND	0.16 ± 0.03	ND	0.12 ± 0.01	ND	0.06 ± 0.01

[ab]Means ± standard error of the mean (n=3) is in percentage between two columns of days 17 and 19 within the same substrate with different superscripts are significantly different at $p < 0.05$. ND=not detected. Mixtures of MECM:SG; L1=5:0, L4=1:1, L5=2:3, L6=1:4, L7=0:5.

4.4 Discussion

Utilising low nutrient quality organic waste in combination with other substrates has been known to improve the nutrient composition of the resulting mixed feed substrates suitable for rearing BSFL (Lim *et al.*, 2019; Nyakeri *et al.*, 2019, Li *et al.*, 2022). Enhancing the nutrient constituents of the substrate is important to produce high-quality BSFL in terms of nutrient composition and growth performance since diet plays a major role in larvae production (Salomone *et al.*, 2017; Spranghers *et al.*, 2017; Lim *et al.*, 2019). In addition, the larval feed substrates greatly influence the nutrients accumulated in larvae, which they utilise during the non-feeding period of their life cycle (Tomberlin *et al.*, 2002; Manzano-Agugliaro *et al.*, 2012). In most situations, no single meal provides an insect with a balanced diet. Dietary selections and absorption rates must be adjusted to achieve and maintain a balanced diet (Simpson and Raubenheimer, 2012). Rehman *et al.* (2017) demonstrated synergistic effects of co-digestion of dairy manure and soybean curd, as well as between coconut endosperm and soybean curd as mixed feed substrates in enhancing the biological growth of BSFL (Lim *et al.*, 2019). In this study, the incorporation of MECM and SG improved the palatability and quality of the mixed feed substrates for the CP, Cfat, ash, and CF content compared to using either MECM or SG as a single feed substrate.

Supplementing MECM with SG, which is higher in protein, could increase the protein content of the larvae. In this study, the optimal level of substitution (MECM:SG) was determined. Overall, the nutrient composition and growth performance of larvae improved on the mixed feed substrates, irrespective of SG proportion, compared to the single substrate of MECM (L1). On the contrary, in some cases, the performance of larvae on the single feed of 100% SG (L7) was better than the mixed feed substrates, particularly for L5- and L6-fed larvae, although the differences were not significant. Adding 20% to 80% SG produced the highest weight at different times. The BSFL developed faster and gave better weight on the mixed feed substrate compared to the single feed substrate of 100% MECM

(L1). Larvae are known to consume different rates of mixed feed substrates with varying nutrient compositions to compensate for the deficient nutrients and eventually acquire their maximum weight later (Nyakeri *et al.*, 2019). The wet weight of larvae was observed to increase with the rise in the CP content of the substrate compared to the single substrate of MECM (L1). The absence of sufficient nutrient sources in L1 (100% MECM) and L2 (80%MECM:20%SG) impacted larval weight gain. The nutrient composition of L1 and L2 substrates possibly was insufficient to excite the palatability of BSFL, due to the substrate being high in fat content but low in CP. In this regard, BSFL grows more slowly while taking up more substrate to obtain sufficient nutrients and achieve maximum weight before pupation (Lim *et al.*, 2019). However, the dry weight of L3-fed larvae − L6-fed larvae surpassed L1-fed larvae due to the higher weight on these mixed feed substrates, whereby the nutritional contents, particularly CP of the substrates, rose with the amount of SG in feed substrate combinations. Nyakeri *et al.* (2019) reported that BSF larvae developed faster and gave better yields on a mixture of food waste and faecal sludge than faecal sludge alone. The mixed substrate improved the palatability of the substrates in terms of nutrient composition and taste, which led to an increase in the amount of feed consumed by the larvae and enhanced larval development (Bonelli *et al*., 2020; Chia *et al.*, 2020; Li *et al.*, 2022). The same condition happened with MECM and SG combinations. Lim *et al.* (2019) reported that larvae grown on the mixed feed substrate of soybean curd residue and coconut endosperm waste reached the highest total weight with a protein content of 12.4% and Cfat at 31.5% in the substrate. At 31%, the fat concentration of the mixed feed substrate is deemed high, producing larvae with high-fat content. This is due to a strong correlation between the fat content of the substrate and fat larvae (as determined in Chapter 3). In the present study, the larvae reared on the mixed feed substrate produced higher wet weight than those reared on the single substrates of MECM and SG. The wet weight gain of larvae on MECM:SG substrates was greater than that of the weight gained on soybean curd residue and coconut endosperm waste (Lim *et al.*, 2019) as well as on soybean curd residue and kitchen waste mixtures (Li *et al.*, 2022). The CP and Cfat contents in feed substrates have been

shown to influence the levels of these macronutrients in the larvae (Barragán-Fonseca *et al.*, 2018; Gold *et al.*, 2020). Nyakeri *et al.* (2019) reported that a protein-to-fat ratio of greater than 2.4 in the feed substrate was desirable because these nutrients were important for larval growth, especially the protein nutritional constituent. In the present study, the protein-to-fat ratios in L4, L5 and L6 at 0.8, 1.0 and 1.9, respectively, were sufficient for larval growth requirement. The growth performance of larvae on L2 (MECM:SG; 4:1) with a CP content of 7.7 g/100g and a high Cfat content of about 23.8 g/100g was not significantly different from the larvae on L1 (5:0). The extremely low CP: Cfat ratio is detrimental to larval growth in terms of weight and sizes. Determining the ideal CP:Cfat ratio was difficult due to the nutritional makeup of feed substrates. Nutrients other than CP and Cfat, such as carbohydrates, amino acids, ADF, NDF, and minerals, are likely to be involved in the development of BSFL (Beniers and Graham, 2019; Shumo *et al.*, 2019). Adding MECM into SG in the mixed feed substrates resulted in a substantial increase of larval DM yield, implying the benefits of utilising the low-nutrient quality organic waste substrates for improved BSFL performance. The protein threshold for rearing larvae on mixed feed substrate of MECM and SG of approximately 11.98 g/100g DM (3:2, L3) was able to support the physiological growth of BSFL compared to L1 and L2 substrates, which was similar to the level reported by Lim *et al.* (2019), while the figure of 21.51 g/100g in L6 was similar to the level reported by Kassahun (2012). The high larval CP of about 50 g/100g DM allowed for its utilisation as an alternative feed ingredient in animal husbandry and aquaculture (Cullere *et al.*, 2016; Magalhaes *et al.*, 2017).

The findings in the present study indicated that the protein levels in mixed feed substrates with suitable fat content should be prioritised to improve the performance of BSFL (Gobbi *et al.*, 2013; Nyakeri *et al.*, 2019). The addition of 50% MECM to SG (L4) increased CP content in larvae by about 67.5% and 84.5% on days 17 and 19, respectively, compared to the L1-fed larvae (Table 16). Gregory *et al.* (2019) reported that the mixture of *Moringa oleifera* leaves and palm kernel cake produced larvae with a CP content of 50%. However, it was not substantially greater

than the control palm kernel cake, which had 54% CP. Compared to L4 – L6, the nutritional imbalance between CP and Cfat in the substrates was noticeable in L7 (high protein but low Cfat) as well as in L1 (high Cfat but low protein). Larvae reared on L4 – L6 substrates produced larvae with CP content comparable to SG-fed larvae because the lack of protein and Cfat contents in the substrate were offset by the addition of SG and MECM, respectively. Nutritional imbalance leads to compensatory feeding in BSFL (Banks *et al.*, 2014). Larvae reared on L7 substrate with 27.9 g/100g DM of CP and 4.43 g/100g DM of Cfat were supposed to show better performance, but the low-fat content in the substrate hindered larval growth rate. The presence of 20%, 40%, and 50% MECM in L6, L5, and L4 substrates increased larval wet weight. Larvae on high-protein substrates may lose more energy during protein detoxification than those on low-protein substrates (Tschirner and Simon, 2015; Lim *et al.*, 2019). For example, methylated arginine released during the protein turnover is toxic to BSFL, where it must be broken down prior to removal from the body. The reduction in energy used in this catabolic metabolism affects the fresh weight and growth of the larvae.

Fishmeal (FM) has generally been used as the principal protein source in commercial aquatic feeds due to its high protein content and well-balanced EAA profile (Oliva-Teles *et al*., 2015). FM has high digestible essential amino acids (EAA) such as lysine, methionine, and leucine (Hall, 1992). The BSFL produced from mixed feed substrates was thought to have a better amino acid (EAA) profile than controls. BSFL reared on mixed substrate had higher levels of amino acids such as alanine, aspartic acid, glutamic acid, and tyrosine than control, L1-fed larvae. Histidine, isoleucine, lysine, tyrosine, methionine, and phenylalanine were the EAAs better than soybean meal (Chen *et al*., 2010). Similarly, some AA is higher in mixed substrate larvae than in L7-fed larvae. The levels of AA are observed to fluctuate independently of the concentration of SG or MECM in the feed substrate. Since the BSFL produced in the present study is used for the FM replacement in seabass diet, lysine and arginine were some of the AA that took in consideration whereby these AA are important in maintaining electrolyte balance in fish and for energy

metabolism (lysine) (Murillo-Gurrea *et al*., 2001). These AA were observed to be high in L4-fed larvae at day 19. Manipulating AA content in larvae with feed is quite challenging because AA in larvae is not influenced by the nutrient quality of the feed substrate (Spranghers *et al.,* 2017; Barragan-Fonseca *et al.,* 2018). Nevertheless, the amount of AA in the feed should be high to ensure it is also high in the larvae since they can't make EAA on their own and get it from their diet. It is suggested to increase the EAA content of BSFL by supplementing feed substrate with waste products derived from grains, nuts, or seeds. On day 19, larvae growing on mixed substrates were found to have a higher AA content than on day 17, particularly for the larvae that had been fed L1 and L4, despite the differences between the two days were not statistically significant.

The saturated fatty acids (SFA), notably lauric acid, are abundant in BSFL fat reared on MECM and SG mixed substrates, whereby the SFA increased with a higher percentage of MECM in the mixtures (Ewald *et al*., 2020). The high concentration of SFA in BSFL may be difficult to use in aquafeed, which may cause the fish's nutritional quality. Therefore, producing BSFL with high-nutrient values in PUFA needs to be considered by utilising the feed substrate with materials rich in PUFA, such as mussels and fish (Ewald *et al*., 2020). The SFA levels in BSFL observed in this study were higher than in previous studies by Ewald *et al.* (2020), Rawski *et al.* (2020) and de Souza *et al.* (2021), but the PUFA levels were lower than those reported by Barroso *et al.* (2014). Nevertheless, BSFL fed on cow manure and fish offal contained considerable amounts of ALA, EPA, and DHA (St-Hilaire *et al*., 2007a). Thus, it is possible to manipulate the substrate to produce BSFL with a better fatty acid composition. In addition, previous reports (Meneguz *et al*., 2018; Spranghers *et al*., 2017; Ewald *et al*., 2020; Franco *et al.*, 2022) indicated that the rearing substrate affects BSFL fatty acid content. Although the exact mechanisms are unknown, to produce a high-quality BSFL, a further understanding of how feed substrate affects the fatty acid makeup of larvae is needed. Linoleic acid, a major component of larval PUFA, was found in high concentrations in L4-fed larvae on day 19, similar to that found in soybean seed-fed larvae (Banaszkiewicz, 2011). A high

PUFA content in BSFL is an advantage since PUFA has been shown to be beneficial to human health (Calder and Grimble, 2002; Sahena *et al.*, 2009), implying that they may also assist in animal health (Gao *et al.*, 2019). At lower proportions of SG in the mixed feed substrates, certain FAs become diluted since SG contains a higher concentration of FAs than MECM. Some concentrations of FA were accumulated as the larvae grew (Liu *et al*., 2017). A longer development period may increase some FA while decreasing others (Ewald *et al*., 2020). Several FAs that were not detected on day 17 were present on day 19. This is due to the nature of FA profiles in insects, which differ considerably throughout their developmental stages, where some FAs might appear while others disappear (Kazek *et al.*, 2019; Kaczmarek *et al.*, 2020). It was demonstrated that the tremendous diversity of FAs found at different phases of insect development: C23:0 and C25:0 was found exclusively in the larvae, C28:0 in the pupal cuticle, and C12:1 and C18:3 in adults. Changes in the FA content of insects are also correlated with changes in body composition, lifestyle, nutrition, environment (Kaczmarek *et al.*, 2020) and the age (Liu *et al*., 2017), with adults containing more FA than the larval stages of *Calliphora vomitoria*. Hence, it is important for the BSFL to have a harvesting day in order to avoid losing some of the important FAs that are necessary for the development of fish. Several insects exhibit a preference for certain FAs in their diet. However, the preference may vary during larval development; for example, *Drosophila melanogaster* larvae prefer unsaturated FAs, while the adults do not (Fougeron *et al.*, 2011).

High levels of substrate reduction of more than 60% were achieved in all the different proportions of feed substrates studied. Selecting the organic waste, particularly agro-industrial by-products such as MECM for rearing BSFL, was a good choice due to the high reduction weight of substrate for producing larval biomass in a shorter period (19 days) than the conventional composting. The substrate reduction of MECM and SG mixed feed substrates was even better than the combinations of other substrates such as soybean curd residue and kitchen waste, soybean curd residue and dairy manure mixtures and dairy and chicken manures, which were 32.71 – 58.36% (Li *et al.*, 2022), 26 – 72% (Rehman *et al.*, 2017a) and of

43.17 – 55.04% (Rehman *et al.*, 2017b), respectively. The bioconversion efficiency on dry matter basis (BCEDM) of L4 was also better than in previous studies (Rehman *et al.*, 2017a; Rehman *et al.*, 2017b; Li *et al.*, 2022). In addition, it was found that the co-digestion of dairy and chicken manures significantly improved the performance of BSFL, including larvae production, waste reduction and feed conversion ratio (Rehman *et al.*, 2017b). The differences in feed composition, such as nutrient, pH, moisture (Banks *et al.*, 2014) and environmental condition (Holmes *et al.*, 2012) influenced the capability of larvae to consume and reduce the organic matter content in the feed substrates.

In the present study, the mixed feed substrate of L4 (MECM:SG; 1:1) was chosen as the optimal feed substrate with the CP and Cfat levels of 14.11 g/100g DM and 17.81 g/100g DM (protein to fat ratio of 0.8), respectively. The CP content of L4-fed larvae of 50 g/100g DM was reasonably high and thus acceptable as an alternative replacement for fishmeal in the Asian seabass diet. Furthermore, compared to L5- and L6-fed BSFL, the Cfat content in larvae produced from L4 mixed feed substrate met the Cfat requirement of seabass (Sakaras *et al.*, 1988; Catacutan and Coloso, 1995). By feeding the BSFL with an optimal mixture of organics, maximum lipid and protein accumulation in the larvae could be obtained (Lim *et al.*, 2019). Another reason for selecting this feed substrate combination was the tremendous performance of BSFL in converting substrate to larvae biomass. The wet larval weight, larval DM yield and BCEDM were greater in L4 than in L5 and L6 and were even better than those fed on the primary substrates of MECM or SG, particularly on day 19. There were no significant differences in the larval performance in the mixed feed substrates of L5 and L6, and SG as single feed in L7, whereby the performance of larvae on these feed substrates was significantly better than the larvae fed with 100% MECM (L1).

The selection of L4 as the optimal substrate for BSFL rearing was more appropriate because it utilised more MECM than L5 or L6, which were abundant and inexpensive in Malaysia. The cost of raising larvae on L4 is much less than it would

be with any other mixed substrate since MECM is less expensive than SG. Using a large amount of MECM in rearing BSFL can help reduce environmental pollution and the economic burdens of organic waste disposal (Kim *et al.*, 2021). Besides, the selection of day 19 as the best harvesting time in this study was also because the larval protein on feed substrates (L4, L5, and L6) peaked on this day. However, the protein content of larvae on these substrates was not significantly different, although the CP and Cfat contents in the feed substrates varied significantly. The harvesting time of larvae on the mixed feed substrates, particularly L4, differed by two days from the single feed substrates (MECM and SG) on day 17, as decided in Chapter 3. The BSFL fed on mixed feed substrates attained only the highest protein level and other parameters on day 19. In addition, most of the amino acid levels on day 19 are higher than on day 17. The peak CP content in BSFL on day 19 could indicate that the larvae are maturing and about to enter the prepupae stage (Liu *et al.*, 2017). Although the development time of larvae reared on mixed feed substrate was not examined in the present study, the protein peak on day 19 might be associated with the development of the final larval instar occurring on that day. According to Fowles and Nansen (2019), the best time to harvest BSFL was during the late immature stage to maximise biomass production. Thus, based on the abovementioned parameters, the ideal day to harvest larvae on mixed feed substrate was day 19. Therefore, BSFL can provide a high number of annual growth cycles and a better capability to produce large quantities of protein to ensure the sustainability of this alternative material (Koutsos *et al*., 2019).

4.5 Conclusion

The findings in this study demonstrated that mixing of the low-nutrient substrate, milk-extracted coconut meat (MECM) with underutilised plant species rich in protein, *Sesbania grandiflora* (SG), improved the nutritional values and its suitability as feed substrate for BSFL production. The nutrient composition of feed substrate affects larvae body composition. The results showed that the levels of CP and Cfat in the larvae resembled the nutrient composition of the feed substrates whereby Cfat content increased with the rise of MECM in the mixed feed substrates. Higher biomass production was obtained at 20% (L2) supplementation rates and followed by 50% (L4) of SG in mixed feed substrates. An increase in protein content in the feed substrates caused an increase in the total larval weight gained and bioconversion performance. The best palatability of BSFL was achieved when the protein content in the feed reached the optimum level. The amount of protein and fat required is equal as both macronutrients are essential for larvae and adult stages. The growth performance of BSFL, substrate reduction, and bioconversion rate were either higher than those for MECM-fed larvae, better or comparable to the SG-fed larvae. The study found that using MECM, and SG leaves at the 1:1 ratio in L4 (50% MECM and 50%SG) was a good choice whereby the nutrient composition of larvae was comparable to larvae reared on the other substrates, L5, L6 and L7. The optimal day to harvest larvae on the mixed feed substrates was on a day 19. The selection was supported by the high bioconversion efficiency values obtained whilst applying the MECM:SG ratio of 1:1. The CP and Cfat contents of L4 were sufficient to produce larvae with high CP content comparable to larvae reared on SG leaves alone (double concentration of CP but lower in Cfat). The mixed feed substrate of MECM and SG can potentially be utilised for sustainable production of BSFL and improving bioremediation of MECM both at a small-scale and large-scale level. BSFL from L4 substrates were mass-produced to replace fishmeal in the diet of Asian seabass. A feeding trial employing a series of BSFL proportions in the diet of Asian seabass was conducted and described in Chapter 5.

CHAPTER 5

APPARENT DIGESTIBILITY COEFFICIENT OF BLACK SOLDIER FLY LARVAE

5.1 Introduction

Insect meal has the potential to be used as an alternative source of protein in aquaculture (Lock *et al*., 2016; Borgogno *et al*., 2017; Li *et al*., 2017; Magalhaes *et al*., 2017; Belghit *et al*., 2019; Fawole *et al*., 2020; Fisher *et al*., 2020). However, very little is known about the nutrient digestibility of insect-base protein, particularly the black soldier fly (BSF, *Hermetia illucens*). The nutritional value of a diet is determined by the digestibility of its constituents (Lupatsch *et al*., 1997). The fish's ability to digest feed is directly correlated to the quantity of available nutrients that can be absorbed in the small intestine. This is important to ensure that the BSFL-based diet formulation meets the fish nutrient requirements to achieve the expected rapid growth rate. Therefore, more research is needed to determine the digestibility and ideal inclusion levels of each ingredient to mitigate or reduce the negative effects of high inclusion levels of certain ingredients on fish growth performance (St-Hilaire *et al*., 2007; Belforti *et al*., 2015). This chapter addresses the phase 4 objective to determine the apparent nutrient digestibility of nutrients in diets and black soldier fly larvae as well.

5.2 Materials and Methods

5.2.2 Digestibility Study

5.2.2.1 Preparation of Diets for Digestibility Study

Two isocaloric and isonitrogenous diets, namely the reference diet (RD) and test diet (TD) were prepared for the digestibility study. Both contained 50 g/100g crude protein and 14 g/100g crude fat, the nutrient requirements of Asian seabass. The protein source in the reference diet was high-quality fishmeal (Triple Nine, 999 Fish Protein, Denmark) with a protein content of 70.8 g/100g DM and fat of 5.1 g/100g DM. The test diet contained fishmeal (50.4 g/100g) and dried BSFL meal (30 g/100g) (Table 29) (Cho *et al*., 1982). Both diets (RD and TD) contained corn starch, mineral premix, and chromium oxide (Cr_2O_3). Diets were prepared by hand, mixing all the ingredients except oil and vitamin premix. A 0.5% Cr_2O_3 was added as a digestible marker to estimate the apparent digestibility coefficient of BSFL. A volume of water, equal to about 25% of the total feed, was added to the ingredients and hand-mixed into a moist dough. The dough was then extruded into 3 mm pellets using a single-screw extruder (SIMA Experimental Extruder Model EXJ 320, Teck Seng Machinery, Kuala Lumpur). The pellets were oven-dried overnight at 60°C (Binder GmbH, Germany), and the remaining diet ingredients (vitamin premix was mixed with fish oil) were added gradually to coat the pellet and mixed for 10 min in a mixer (AICOH Mixer, Thailand). The pellets were kept at -20°C before being used to determine digestibility. The chemical compositions of the pellets were analysed for CP, and Cfat using the method described in Chapter 2 (2.2.3.3 and 2.2.3.4).

Table 29: Formulation and proximate composition of the reference (0% BSFL) and the test diet (30% BSFL) for the determination of the apparent digestibility coefficient.

Ingredient	**Reference Diet (g)**	**Test Diet (g)**
BSFL	0.0	30.0
Peruvian Fishmeal	70.8	50.4
Corn starch	14.5	10.4
Fish Oil	10.3	5.9
Mineral premix[1]	2.0	1.4
Vitamin premix[2]	2.0	1.4
Chromium	0.5	0.5
Total (g)	100	100
Chemical Analysis		
Ash (g/100g)	15.17 ± 0.51	14.91 ± 0.93
Crude Protein (g/100g)	51.30 ± 1,77	50.08 ± 1.71
Ether Extract (g/100g)	13.50 ± 0.15	14.00 ± 0.55
Gross Energy (MJ/kg)	19.48 ± 0.54	19.89 ± 0.57

Mineral premix contains (content per 1 kg) iron: 25,000 mg, copper: 785 mg, zinc: 1,894 mg, iodine: 150 mg, manganese: 800 mg, cobalt: 100 mg, sodium: 20 mg, DCP: 723,251 mg, sodium chloride: 60,000 mg, anti-caking: 1000 mg, potassium chloride: 50,000 mg, magnesium sulphate: 137,000 mg.

Vitamin premix contains (content per 10 kg/MT feed) vitamin A: 2,000,000 IU, vitamin D3: 400,000 IU, vitamin E: 20,000 mg, vitamin B1: 6,000 mg, vitamin B2: 6,000 mg, vitamin B6: 4,800 mg, vitamin B12: 10 mg, vitamin K3: 2,000 mg, vitamin C: 120,000 mg, Biotin: 200 mg.

5.2.2.2 Fish Rearing and Commencing of Digestibility Trial

The saltwater Asian seabass was purchased from Gelang Patah, Johor, following an acclimatisation period of about 10 days in freshwater by the supplier. Upon arrival at the MARDI Serdang Station, the fish were re-acclimatised again for about three weeks and fed with commercial feed. Once the fish condition was stabilised, a total of 90 juvenile fish (45.04 ± 1.50 g) were randomly transferred to six aerated

aquariums (100 L) each with 15 fish. In order to determine the apparent digestibility of diet, an *in vivo* digestibility trial was conducted. The digestibility study lasted for three weeks until the faeces sample collected from each aquarium was sufficient for chemical analysis. The fish were not fed a day before the start of the trial to ensure that their intestine was empty. Feed was given twice a day at 8:00 am and 15:00 pm at approximately 2% of wet body weight/day (Katya *et al*., 2017). The amount of feed consumed was recorded daily, and uneaten feed was scooped out with a hand net one hour after feeding. The uneaten feed was rinsed with distilled water and dried in the oven at 60^{o}C for three days. The daily feed consumption was calculated by subtracting the weight of the uneaten feed from the initial weight of the feed given. Approximately 3 hours after feeding, faecal samples were collected by siphoning using a 200 µm net (Mohamad-Zulkifli *et al*., 2019). The faeces and uneaten feed were rinsed with distilled water before scraping into a small aluminium bowl. The samples were then dried at 60°C for 48 h. The dried faeces were pooled into a container for each tank, weighed, and stored at -20^{o}C. The chemical analyses of the dried faeces were conducted particularly for CP and Cfat to calculate the apparent digestibility coefficient (ADC) of RD and TD, as well as the test ingredient of BSFL.

5.2.2.3 Chromium Oxide Measurement

The chromium oxide (Cr_2O_3, Prince Minerals, Inc. New York, USA, 1308-31-2; 100% Purity) content in the RD, TD and faeces samples collected from the fish fed with both diets was determined according to the method described by Hill and Anderson (1958) with modifications. A universal bottle was filled with two grams of feed sample or 1 gram of faeces containing approximately 10 mg of Cr_2O_3. A volume of 15 ml of digestion buffer (10 mg of sodium molybdate dihydrate dissolved in 500 ml mixture containing 150:150:200 v/v/v of distilled water, concentrated sulphuric acid, 98% (Fisher Scientific, USA) and 70% perchloric acid v/v (Merck, USA) was added to each bottle. The mixture was heated on a hot plate filled with sand to

achieve a temperature of around 300°C. Samples containing the buffer were heated in a fume hood until the colour turned reddish when they were immediately removed from the hot plate and chilled in cold water. Samples were then transferred into volumetric flasks and diluted with 90 ml of distilled water. After cooling, the mixture was brought to 100 ml and allowed to stand overnight to precipitate the inorganic components. Concurrently, 50 mg of pure Cr_2O_3 was prepared in duplicate using the same procedure as described above. A series of dilutions from the oxidised Cr_2O_3 was prepared, from 0 – 440 µg equivalent per millilitre, diluting with distilled water to plot the standard curve. Each dilution of about 200 µl was then transferred to a micro-plate to determine the optical density (OD) at 430 mµ in an Epoch spectrophotometer (BioTek, USA). The concentration of chromium in the samples was calculated using the equation, y = 1.3152x + 0.0193 (R^2 = 0.9963) (Appendix 3).

5.2.2.4 Analysis of Apparent Digestibility Coefficient

Faecal recovery (FR) of the Cr_2O_3 marker was calculated to show that the techniques used did not affect the Cr_2O_3 determination using Formula 17 (Guzman-Cedillo *et al*., 2017). The apparent digestibility coefficients (ADC) of CP and Cfat in reference and test diets (Formula 18) were calculated using the equations below (Cho and Kaushik, 1990; Mohamad-Zulkifli *et al*., 2019). The ADC of the test ingredient (BSFL) was calculated based on the digestibility of the RD and TD using Formula 19 (Bureau and Hua, 2006; Mohamad-Zulkifli *et al*., 2019).

Formula 17

$$FR\ (\%) = \frac{MFE}{D} \times 100$$

where FR is the faecal recovery of marker, MFE is the faecal excretion of marker (g/100g) and D is the dose of external marker (g/100g).

Formula 18

$$\text{a. } ADC\ of\ nutrient = 100 \times \left\{1 - \left(\frac{\%\ faeces\ nutrient}{\%\ dietary\ nutrient} \times \frac{\%\ dietary\ Cr_2O_3}{\%\ faeces\ Cr_2O_3}\right)\right\}$$

Formula 19

$$a.\ ADC_I = ADC_{TD} + (ADC_{TD} - ADC_{RD}) \times \left(\frac{0.7\ D_{RD}}{0.3 \times D_I}\right)$$

where: ADC_I = ADC of the test ingredients; ADC_{TD} = ADC of the test diet; ADC_{RD} = ADC of the reference diet; D_{RD} = % nutrient of the reference diet; D_I= % nutrient of the test ingredients.

5.2.3 Statistical Analysis

The normality of data from the ADC_{CP} and ADC_{Cfat} of reference and test diets was tested using the Shapiro-Wilk test. The normalised data was then analysed by one-way ANOVA followed by Tukey's multiple range test at a 5% probability level. The statistical analysis was performed using SAS version 9.4 software (2013, SAS Institute, Inc., Cary, NC, USA). Data is expressed as the mean and standard error of the means (SEMS), with $p < 0.05$ indicating statistical significance.

5.4 Results

5.4.1 Digestibility of Diets and Black Soldier Fly larvae

In the digestibility study, the recovery of chromium oxide from both reference and test diets was 76.9% and 85.7%, respectively (p = 0.4625), as shown in Table 30. The ADC of crude protein (ADC_{CP}) in the reference diet (RD) was significantly higher ($p < 0.0001$) than in the test diet (TD). The highest ADC_{CP} of 88.03 ± 0.31% was found in RD which contained 70% FM, compared to 78.28 ± 3.45% TD, which had a mixture of 30% BSFL and 50.4% FM. The ADC of crude fat ($ADC_{Cfat)}$ was higher than that of protein in both RD (94.23 ± 0.50%) and TD (91.02 ± 1.55%), but not significantly different (p = 0.1270). In contrast, the ADC of protein and fat of the tested ingredient (BSFL) was 71.02 ± 0.25% and 88.68 ± 2.53%, respectively.

Table 30: Apparent digestibility coefficient (ADC) of crude protein and crude fat of the reference diet, test diet and BSFL.

Parameter	**Reference Diet, RD**	**Test Diet, TD**	***p* - value**
Recovery marker of a chromium	76.93 ± 0.03	85.67 ± 0.04	0.4625
Apparent Digestibility Coefficient (ADC, %) of Nutrients			
ADC_{CP}	88.03 ± 0.31[a]	78.28 ± 3.45[b]	< 0.0001
ADC_{Cfat}	94.23 ± 0.50	91.02 ± 1.59	0.1270
	Ingredient (BSFL)		
ADC_{CP}	-	71.02 ± 0.25	
ADC_{Cfat}	-	88.68 ± 2.53	

[ab]Means ± standard error of the mean in g/100g DM are significantly different at $p < 0.05$.
ADC_{CP}=Apparent digestibility coefficient of crude protein, ADC_{Cfat}=Apparent digestibility coefficient of crude fat.

5.5 Discussion

Typically, the feeds used in the commercial aquaculture industry are comprised of a variety of complementary ingredients to supply an adequate quantity of digestible protein, digestible energy, and essential nutrients. The high energy value, quantity and quality of protein, and rich content of lipids, minerals and vitamins make fishmeal (FM) the standard and favoured dietary component for rearing fish and a benchmark ingredient in aquaculture diets (Udo *et al*., 2012). In order to formulate a cost-effective fish diet from alternative ingredients, an initial determination of the digestibility of the nutrients, especially protein and lipid, should be conducted to establish their nutritional values (Glencross *et al*., 2007; Liu *et al*., 2009). The apparent digestibility coefficient (ADC) of nutrients in the reference diet containing FM was higher than in the test diets. When fed to Asian seabass, the protein digestibility (ADC_{CP}) of diets containing BSFL was much lower than those based on FM, ranging from 88 – 98%. The superiority of FM over BSFL was probably due to its high palatability and a good balance of essential amino acids and fatty acids, as suggested by Hardy (2010) and Hussain *et al*. (2011). The ADC_{CP} in the BSFL-based test diets (78.3%) was significantly lower than in other previous studies, such as in European bass, *Dicentrarchus labrax* (91 – 93%) (Magalhães *et al*., 2017) and hybrid grouper, *Epinephelus fuscoguttatus* x *Epinephelus lanceolatus* (90 – 92%) (Mohamad-Zulkifli *et al*., 2019) but higher than plant meal such as flaxseed and rice bran (Gaylord and Barrows, 2008). It has been suggested that the variability in the ADC value of BSFL could be due to several factors, including the species of fish, drying method or processing of feed ingredients (Weththasinghe *et al*., 2021), the chemical composition of ingredients used in the diet (Rahman *et al*., 2016), inclusion level (Gasco *et al*., 2019a) and the feed substrates used to produce BSFL (Belghit *et al*., 2019). The growth reduction in Asian seabass given BSFLM could be due to lower protein digestibility of BSFL. The presence of anti-nutritional factors (ANFs) may also cause the weight of Asian seabass reduced when the percentage of BSFL inclusion increase. Plants contain all sorts of ANFs, such as protease inhibitors, which prevent protein hydrolysis in the cell lysate. It is possible that larvae

accumulated components such as tannins during their growth phase (Bhatta *et al*., 2012; Mat *et al*., 2022) since they were reared on plant materials (*Sesbania grandiflora* and milk-extracted coconut meat).

A high digestibility coefficient (ADC) of nutrients in BSFL-fed European seabass (Magalhaes *et al*., 2017) and mealworm beetle (Gasco *et al*., 2016), suggests that seabass can efficiently digest insect meals. It is also possible that the adaptation of seabass to a new environment, from marine to freshwater environment, may impact the fish digestive tract with the introduction of new microflora in the gastrointestinal tract when digesting BSFL-based feed, thereby affecting the absorption of protein and fat required for growth. The process of drying BSFL at high temperatures during the meal production stages can have a significant impact on the nutritional quality of the final product (Weththasinghe *et al*., 2021). Besides, the stage or form of BSFL used, either as full-fat larvae, defatted or hydrolysed larvae, can significantly affect nutrient absorption rate (English *et al*., 2021). According to Opstvedt *et al*. (2003), in non-ruminants, the overall digestibility of BSFL is improved when the larvae was dried at 90°C. It is possible that the drying temperature of 60°C used in this study was low to open the polypeptide chain and make the protein more accessible to digestive enzymes, which can increase the absorption rate of protein or fat by fish. Other species have a different capacity to digest the feed components, whereby the structure and function of their digestive systems are adapted to their feeding habits (Li *et al*., 2014).

The other key nutrient of concern in fish diets is the fat component, including the quantity, quality, and apparent digestibility of the fats. Kroeckel *et al*. (2012) reported that the inclusion of BSFL in the diets of turbot (*Psetta maxima*) resulted in a decreased ADC of fat (ADC_{Cfat}). Nevertheless, the values of ADC_{CP} and ADC_{Cfat} of BSFL meal in the present study were higher than those reported by Kroeckel *et al*. (2012) at approximately 63.1% and 78%, respectively, showing that BSFL in turbot had a low to moderate digestibility. However, the ADC_{CP} of BSFL was substantially lower than those reported by Mohamad-Zulkifli *et al*. (2019), who reported a range

of 81 - 88%, depending on the method of heating used. The value of protein digestibility in this study was also lower than those obtained from other insects, such as mealworm (*Tenebrio molitor*) when fed to Pacific white shrimp (*Penaeus vannamei*) at 76.1%, and cricket meal when fed to North African catfish (*Clarias gariepinus*) at 81.21% (Taufek *et al*., 2016). The protein quality in the feed ingredients has the greatest influence on fish performance and protein digestibility as it is the first indicator of its availability for fish (Yu *et al*. 2013). The low digestibility of protein in Asian seabass indicates an insufficient quantity of this nutrient in the diet, negatively affecting seabass growth, particularly in a diet high in BSFL, such as the BSFL60 diet.

It is possible that the method used to measure digestibility in this study (the recovery of chromium oxide (Cr_2O_3) resulted in a low value for protein digestibility. Chromium is an inert–indigestible marker that should not be absorbed in the gastrointestinal tract and must not react with the microbial population residing in the fish gastrointestinal tract (Ng and Wilson, 1997). To be an effective indicator of digestibility, the marker used must be completely recovered in faeces, not absorbed (de Souza *et al.,* 2015), or toxic to the fish (Ng and Wilson, 1997). However, a few studies have suggested that Cr_2O_3 is not inert and may have carcinogenic properties that affect diet digestibility with implications for nutrition and feeding practices (Shiau and Liang, 1995; Sedman *et al*., 2006). This indicates that it may be absorbed and retained in the fish body (Shiau and Shy, 1998). Faeces may be pulverised while samples are taken, resulting in chromium lost in the water, which alters the chromium concentration that is detected. Some considerations must be considered due to the low recovery of the marker, which finally impacts the digestibility coefficient. In addition, the determination of chromium in the sample diets and faeces showed variability in ADC results. The absence of suitable tools for the digestion of organic matter and solubilisation of chromium at 300^{o}C may influence the completion of the digestion process and consequently, the detection of low levels of the markers in the samples. This result will affect both the estimation of ADC of diets and BSFL. Higher ADC values of CP and Cfat in a previous study

obtained by Mohamad-Zulkifli *et al*. (2019), who determined the chromium marker using atomic absorption spectrometry (AAS). Using an AAS, Kavanagh *et al*. (2001) showed a recovery rate of 96% of Cr_2O_3 when added to pig diets. On the other hand, Jagger *et al*. (1992) obtained recovery rates of 74% and 80% for diets supplemented with 1 g and 5 g Cr_2O_3/kg, respectively, when determined using a UV spectrophotometer similar to the method used in the present study. These findings showed that AAS tended to measure a higher percentage of ADC compared to other instruments. The precise instrument and selected practical method are crucial for accurately estimating the chromium concentration in samples, which can affect the feed ingredient's digestibility. The variations in the total digestibility results were lower if the marker was determined using an AAS, as suggested by Kozloski *et al*. (1998).

5.6 Conclusions

The current findings show that BSFL reared on a combination of *Sesbania grandiflora* and milk-extracted coconut meat has moderate crude protein and crude fat digestibility in Asian seabass, implying that BSFL reared on a combination of Sesbania grandiflora and milk-extracted coconut meat is a potential alternative ingredient for aquafeed formulation, particularly to replace FM in the diet of Asian seabass (*Lates calcarifer*). This will result in better FM utilisation in the diet and less demand for this scarce and expensive component.

CHAPTER 6

FEEDING TRIALS OF BLACK SOLDIER FLY LARVAE MEAL IN ASIAN SEABASS (*Lates calcarifer*) DIET

6.1 Introduction

The survival and development of the aquaculture industry are threatened by the decline in the availability of fishmeal (FM). One of the ways to assist the industry is to eliminate or reduce the dependence on FM as protein ingredient in aquafeed. Tacon and Metian (2015) reported that FM inclusion rates in fish diets have gradually declined over the previous two decades. Typically, FM has been employed as the primary source of protein in diets formulated for carnivorous fish with high protein requirements, such as European seabass (Infante *et al.*, 1997), salmon (Ytrestøyl *et al*., 2015) and trout (Yaseen *et al*., 2016). Asian seabass requires a high protein diet of between 45 and 50%, depending on the size of the fish (Glencross, 2016) and up to 65% for feeding post-hatch larvae (Ambasankar, *et al*., 2009). Currently, the commercial fish feeds in the market contain a mixture of several plant-based and animal by-product ingredients, including soybean meal, canola meal, maize protein concentrate, and chicken by-products, as well as insects' meals (Fisher *et al*., 2020; Shukla *et al*., 2019). Past experiments have shown that a complete or selective replacement of FM with alternative proteins, such as insect meal, does not adversely affect fish development (Sealey *et al.*, 2011; Dumas *et al.*, 2018; Xiao *et al.*, 2018; Belghit *et al.*, 2019). Considerable progress has been demonstrated in using BSFLM to replace FM as an essential protein source in aquafeed. The Black soldier fly (BSF), which has a short rearing period and significant biomass growth, appears to be the most promising insect species (Wang and Shelomi, 2017). Most Black soldier fly larvae (BSFL) feeding trials focused on the salmonid species, with very little information on the Asian seabass, *Lates calcarifer* (Katya *et al.*, 2017). The huge reduction in the use of FM in fish feed gives great

hope that insects can be used widely as a replacement for the traditional protein sources of FM and soybean meal (SBM). However, the fish performance may vary depending on the percentage of insect meal replacement as well as the species of fish used. The quality of the harvested fish might differ in terms of physical, chemical, and sensory characteristics compared to the fish fed on FM- and SBM-based fish feeds, which also affect the acceptance of the fish product by the consumers (Bruni *et al*., 2019). Therefore, it is hypothesised that the Asian seabass (*Lates calcarifer*) fed a BSFLM replacement diet formulated based on similar nutritional and energy contents will perform comparably to the seabass fed an FM-based diet as its protein source. This chapter addresses the phase 4 objective to assess the growth performance of fish, chemical composition and the sensory evaluation of the fish fillet. The ideal BSFLM inclusion levels that would not compromise the Asian seabass growth were also determined. Additionally, this chapter addresses the Research Question No. 5;

Can black soldier fly larval meal (BSFLM) replace fishmeal in the Asian seabass diet? What is the ideal percentage of BSFLM replacement in the diet that does not affect the growth and development of Asian seabass?

6.2 Materials and Methods

6.2.1 Source of Black Soldier Fly Larvae

The production of BSFL as a protein replacement for FM in the Asian seabass diet was studied using a feed substrate that consisted of *Sesbania grandiflora* (SG) and agro-industrial by-product, milk-extracted coconut meat (MECM) in a ratio of 1:1 (50% SG and 50% MECM). After rearing the BSFL until day 19, they were washed and dried in an oven heated to 60^{o}C for three days. The powdered dried larvae were utilised in the preparation of fish feed.

6.2.2 Replacement of Black Soldier Fly Larvae in Seabass Diet

6.2.2.1 Feed Formulation and Preparation of Experimental Diets

Four experimental diets were formulated to be isonitrogenous (48 g/100g CP) and isolipidic (14 g/100g Cfat) to meet the optimum nutrient requirement of experimental fish as recommended by Ambasankar *et al*. (2009). The diets were formulated to contain BSFL produced from as a replacement for fishmeal. Three test diets were prepared, containing different inclusion levels of BSFL (20, 40 and 60% of the total protein). These inclusion levels equated to 74, 148 and 222 g BSFL per kg diet (Table 31). The control diet (BSFL0) contained approximately (50:50, w/w) of animal protein sources namely, fishmeal (FM, Triple Nine Fish Protein 999, Denmark) and a plant-based protein as the primary protein source in the diet. Soybean meal, feather meal, offal meal, wheat pollard, corn, corn starch, corn gluten, FM and BSF larvae were among the ingredients used in formulated feed. Ingredients were selected based on their availability in sufficient amounts, cost, and nutrient profiles. The level of inclusion of each ingredient in the feed and the nutrient composition of the experimental diets was formulated using the Microsoft Excel program developed by Halways Sdn. Bhd. Other ingredients such as dicalcium phosphate (DCP), limestone, vitamins and mineral premixes were added to the mixture to ensure that the final feed product meets the National Research Council recommended amount for seabass (NRC, 1993). During the feed preparation, wheat pollard and corn were ground to a finer texture, and all ingredients were sieved through a 2-mm sieve before weighing to prevent the extruder from becoming clogged due to foreign substances present in the materials. The weighed ingredients were mixed in a Hobart mixer (Hobart A-200, Troy, OH, 134 USA) to form a homogeneous mixture. Furthermore, the quantity of fish oil added to the diet was reduced inversely at higher levels of BSFL to prevent the high amount of Cfat in the replacement ingredient. A volume of water equivalent to 25% of the total ingredient weight was added to the mixture and thoroughly mixed by hand. Mixtures were extruded through a 3-mm dice to form pellets (SIMA Experimental

Extruder Model EXJ 320, Teck Seng Machinery, Kuala Lumpur) and oven-dried at 60^{o}C overnight. Pellets were manually sieved through a 2-mm mesh screen to remove powder from the mixture in the pellets. Upon sieving, the pellets were mixed in a mixture of fish oil and premix vitamin in a mixer (AICOH Mixer, Thailand). The diets were kept at -20^{o}C before the feeding trial.

Table 31: List of ingredients and nutrient composition of feed formulation.

	Diets			
Ingredients (g dry weight basis)	**BSFL0**	**BSFL20**	**BSFL40**	**BSFL60**
Peruvian Fishmeal	37	29.6	22.2	14.8
BSFL	0.0	7.4	14.8	22.2
Soybean meal	21.0	21.0	21.0	21.0
Poultry Feather meal	4.3	5.8	7.2	8.6
Poultry offal meal	5.5	6.3	7.0	7.7
Corn	6.7	5.0	3.5	2.1
Fish oil	6.6	6.0	5.4	4.7
Corn starch	6.0	6.0	6.0	6.0
Wheat pollard	2.0	2.0	2.0	2.0
Corn gluten meal	8.0	8.0	8.0	8.0
Mineral premix	1.2	1.2	1.2	1.2
Vitamin premix	1.2	1.2	1.2	1.2
DCP	0.2	0.2	0.2	0.2
Limestone	0.3	0.3	0.3	0.3
Total (g)	100	100	100	100
Chemical Composition (g/100g as it is basis)				
Dry Matter	98.94 ± 0.31	98.10 ± 0.32	98.91 ± 0.41	98.30 ± 0.45
Ash	10.91 ± 0.02	10.27 ± 0.16	10.29 ± 0.11	10.29 ± 0.14
Crude Protein	49.61 ± 0.15	49.04 ± 0.11	49.02 ± 0.15	49.52 ± 0.06
Crude Fat	8.48 ± 0.24	10.28 ± 0.21	10.60 ± 0.05	10.73 ± 0.05
Crude Fibre	2.33 ± 0.75	2.25 ± 0.12	2.16 ± 0.45	2.08 ± 0.64
Metabolisable Energy (MJ/kg) (by software)	20.11	20.57	21.03	21.49

BSFL0 = diet without BSFL meal (BSFLM) inclusion; BSFL20, BSFL40 and BSFL60 were 20%, 40% and 60% replacement level of FM with BSFLM, respectively.

6.2.2.2 Fish Feeding Trial and Facilities

The feeding trial was conducted at the Livestock Science Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor for a period of 3 months from December 2020 to March 2021. The University of Nottingham and MARDI Animal Ethics Committee approved the procedures and protocols used in the fish experiment following a local scientific review (Approved Number: 20200106/R/MAEC00074). The experimental units (tanks) with continuous aeration by air pumps were arranged in two rows with each row having ten tanks. The treatments (BSFL0, BSFL20, BSFL40, and BSFL60) were arranged in two rows, according to a random distribution pattern (Figure 41). A total of 300 Asian seabass juveniles with an initial weight of 46.77 ± 0.08 g/fish were randomly distributed in 20 tanks (300-L), with each tank containing fifteen fish. Each treatment was assigned to five replicated groups. The feeding trial was carried out for 90 days. Fish were weighed at the start of the trial and at 15-day intervals. The fish were initially given a control diet for two weeks prior to the start of the feeding experiment. The fish were fed by hand twice a day at 9:00 and 16:00. At the start of the 2-week trial, feed was provided at a rate of 2% of wet body weight/day through the 90-day feeding trial (Katya *et al.*, 2017). Uneaten feed was scooped out with a hand-held net one hour after feeding, cleaned with distilled water, dried in a 60^{o}C oven for 48 h, and weighed. Faecal samples were scooped out four hours after feeding to avoid contamination. Every two days, half of the tank's water was siphoned and replaced with well-aerated water from a water storage tank. The quality of water for each tank was monitored throughout the feeding trial to ensure adequate environmental conditions for the fish. Water temperature, dissolved oxygen (DO), total ammonia (NH_3), nitrite (NO_2), pH and temperature were measured once a week using YSI meter (Model 85, YSI Industries, Yellow Springs, OH, USA). The water quality parameters are shown in Table 32. These values were within the acceptable limits for fish culture as suggested by Boyd and Tucker, 2012. Throughout the experiment, a photoperiod of 12 h light: 12 h dark was maintained.

Table 32: Mean values ± standard error of the water quality parameters during the experimental trial (90 days)

Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Ammonia (mg/L)	Nitrite (mg/L)
26.88 ± 0.08	5.98 ± 0.03	7.67 ± 0.02	3.64 ± 0.09	0.12 ± 0.01



Figure 41: Twenty tanks arranged in two rows. Five replicates of each treatment were randomly distributed in rows.

6.2.2.3 Data Collection and Calculations of Growth Indices

The bulk weight of fish was measured every fortnight until the end of the 90-day trial period in order to determine the amount of feed given to fish and to monitor the growth performance of fish. The amount of feed given was changed every fifteen days based on the average collected weights. The amount of feed given was recorded daily and uneaten food was removed from the tank using a hand-held net and dried before its weight was recorded. The effects of the diets on fish were assessed using data on fish biomass, feed consumption, diet utilisation, and internal organ dissection. The growth performance parameters such as survival rate (SR, Formula 20), average daily gain (ADG, Formula 21), body weight gain (BWG, Formula 22), specific growth rate (SGR, Formula 23), feed intake (FI, Formula 24), and feed conversion ratio (FCR, Formula 25) were calculated according to Fawole *et al*. (2020).

At the end of the feeding trial, the bulk weight of the fish was measured after one day of starvation, and the average weight was calculated by dividing the bulk weight by the number of fish in each tank. Five fish from each tank were individually weighed, and their length was measured to calculate the condition factor (CF, Formula 26) in order to measure the growth performance. From each tank, three fish were decapitated and organs such as liver, gut and adipose tissue were harvested and weighed at 0.1 mg precision to determine the biological parameters such as hepatosomatic (HSI, Formula 27), viscerosomatic (VSI, Formula 28) and adiposomatic index (ASI, Formula 29) indexes, respectively. All formulae were calculated according to Renna *et al.* (2017) and Fawole *et al*. (2020).

Formula 20

a. $$Survival\ Rate\ (SR, \%) = \frac{Final\ number\ of\ fish\ intank}{Initial\ number\ of\ fish} \times 100$$

Formula 21

b. $Average\ Daily\ Gain\ \left(ADG, \frac{g}{day}\right)$

$$= \frac{Mean\ final\ weight\ -\ Mean\ initial\ weight}{Experiment\ length\ (days)}$$

Formula 22

c. $Body\ Weight\ Gain\ (BWG, g)\ = \frac{Final\ weight\ -\ Initial\ Weight}{Initial\ weight} \times 100$

Formula 23

d. $Specific\ Growth\ Rate\ \left(SGR, \frac{\%}{day}\right)$

$$= \frac{\{(Ln\ (Final\ Weight)\ - Ln\ (Initial\ weight)\}}{Duration\ in\ days} \times\ 100$$

Formula 24

e. $Feed\ Intake\ \left(FI, \frac{\frac{g}{fish}}{day}\right) = \frac{Total\ dry\ feed\ given\ (g)}{Number\ of\ fish}$

Formula 25

f. $Feed\ Conversion\ Ratio\ (FCR)\ = \frac{Total\ dry\ feedfed\ (g)}{Wet\ weight\ gain\ (g)}$

Formula 26

g. $Condition\ Factor\ \left(CF, \frac{g}{cm^3}\right)$

$$=\ Weight\ of\ fish\ (g)/(Length\ of\ fish)^3\ (cm)\ \times 100$$

Formula 27

h. $Hepatosomatic\ Index\ (HSI, \%)\ = \frac{Liver\ weight\ (g)}{Fish\ wet\ weight\ (g)} \times\ 100$

Formula 28

i. $$Viserosomatic\ Index\ (VSI, \%) = \frac{Gut\ weight\ (g)}{Fish\ wet\ weight\ (g)} \times 100$$

Formula 29

j. $$Adiposomatic\ Index\ (ASI, \%) = \frac{Adipose\ fat\ weight\ (g)}{Fish\ wet\ weight\ (g)} \times 100$$

6.2.3 Chemical Composition of Fish Fillet

Five fish from each tank were sliced and dried in the oven at 60°C (Binder, Germany) for 60 h, ground with a laboratory blender (Sumeet, India) and stored at 4°C for future analysis. The dried ground fillet was subjected to chemical analysis to determine the nutrient composition including DM, ash, CP, Cfat and fatty acid (FA) profiles. Each sample was tested in triplicate. Protocols were executed as described in Chapter 2. Protein content of fish fillets was calculated by multiplying N with 6.25 (Koeckel *et al.*, 2012). However, the procedure for measuring FA in fish samples was slightly modified from the protocol described in Section 2.2.5 of Chapter 2. The dried ground fish fillet was used instead of the fat sample due to the limited fat content in the fillet samples. A 0.5 g of dried fillet was incubated in a test tube containing 5 ml methanol for 12 h at room temperature. The mixture was then neutralised by adding 5 ml of 3N methanolic-HCl with an internal standard of heneicosanoic acid (C21:0, Sigma-Aldrich, USA) and vortexed. The following procedure was performed according to the protocol described in Chapter 2. Briefly, an internal standard consisting of 2 mg/ml (w/v) of C21:0 dissolved in methanol (Fisher Scientific, USA) was used to determine the amount of fatty acid. A total of 200 µl of C21:0 was added to each mixture, and the tubes were heated for one hour at 95°C before being cooled to room temperature. Subsequently, 8 ml of 0.88% NaCl (w/v) and 3 ml of n-hexane (Fisher Scientific, USA) were added to the mixture and vortexed thoroughly. The mixture was separated into three layers by

centrifugation at 3,000 g for 5 min (Kubota 2000, Interscience, Germany). The following steps were performed according to the procedure described in Chapter 2 (2.3.3). The peaks were identified by comparing the relative retention times of the FAME peak in the samples with those of the standard FA methyl ester (Supelco 37, Sigma-Aldrich, USA).

6.2.4 Serum Biochemical Parameters Analysis

Two fish per tank were lethally anaesthetised using 0.1% (100 mg/L) of clove oil (Davis *et al*., 2015). The fish were weighed, and the blood samples were collected from the caudal peduncle vein using a 1-mL heparinized syringe to collect approximately 500 µl of blood and placed in a 1.5-mL microfuge tube. The blood samples were centrifuged at 518 g for 10 min at 4°C (Zhou *et al*., 2017) using a refrigerated microcentrifuge (5810R, Eppendorf, European). Two layers were formed where the upper layer, which was a yellowish supernatant known as the serum was pipetted out and transferred to a new 1.5-mL tube. The serum tubes were stored at –80°C until future analysis. The serum samples were sent to Veterinary Laboratory Services Unit, Faculty of Veterinary Medicine, University Putra Malaysia for the analysis of biochemical metabolites, namely, alkaline phosphatase (ALP, U/L), alanine transaminase (ALT, U/L), aspartate aminotransferase (AST, U/L), total protein (TP, g/L), albumin (ALB, g/L), globulin (Glo, g/L), albumin:globulin (A:G Unit), glucose (Gluc, mmol/L), total bilirubin (TB, umol/L), cholesterol (Chol, mmol/L), triglyceride (TGL, mmol/L), low-density lipoprotein (LDL ,mmol/L), high-density lipoprotein (HDL, mmol/L) which were determined using an automatic biochemical analyser (BA 400, BioSystems Reagents and Instruments, Manila, Philippines).

6.2.5 Sensory Evaluation

Five fish per replicate were percussively slaughtered, removed of the scales, and filleted. Each fillet was wrapped in LDPE plastic bag, sealed, and chilled at 3˚C using blast chilling and kept overnight at this temperature. Organoleptic evaluation was conducted at the Food Science and Technology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI). The sensory analysis was conducted by utilizing 40 untrained panellists made up of MARDI staff, who were not allergic to fish and seafood. Each panellist was assigned a separate cubicle and instructed not to communicate with the other panellists during the sensory evaluation period. Five fish were sliced from both sides per replicate, yielding a total of ten fillets per replicate for sensory evaluation. One side of fish fillets were wrapped in an aluminium foil and steamed in a steamer for 5 minutes. Each panellist was served with four fillets in an aluminium foil representing four dietary treatments (BSFL0, BSFL20, BSFL40 and BSFL60). Each fillet was labelled with different numbers to hide its identity and panellists were asked to evaluate on five sensory parameters namely, colour, odour, texture, taste, and overall acceptability (Kulawik *et al*., 2016) on a 7-point hedonic scale, ranging from 1 (dislike very much) to 7 (like very much) (Lim *et al*., 2022).

6.2.6 Statistical Analysis

Data on the growth performance, serum metabolites, and chemical analysis of fillets were examined for normal distribution using the Shapiro-Wilk and sensory test data using the Kolmogorov Smirnov tests. Variables with a normal distribution and uniform variances were calculated by one-way analysis of variance (ANOVA) using the General Linear Model (GLM) procedure in Statistical Analysis System, SAS version 9.4 (2013, SAS Institute, Inc., Cary, NC, USA). The Tukey's honest significant difference (HSD) test was used when a significant treatment effect was observed in order to compare means between the treatments particularly for fatty acid profiles.

Differences were statistically significant when $p < 0.05$. All data were expressed as means ± standard error of the mean (SEM) in tables. The broken line model (Robbins *et al*. 1979) was used to determine the best dietary level of black soldier fly larval meal as a fish meal replacement in Asian seabass.

6.3 Results

6.3.1 Growth Performance and Feed Utilisation

The weight gain (WG) over 90 days of Asian seabass fed on the test diets containing BSFL as partial replacement for fishmeal (FM), is shown in Figure 42. The fish readily accepted the experimental diets, and their weight doubled during the feeding trial, from an average initial weight of 46.77 ± 0.08 g/fish to the average final weight of 106.83 ± 3.40 g/fish. The WG of fish did not differ significantly ($p > 0.0839$) across treatments, but it decreased as BSFLM levels increased in the diets. However, BSFL20 showed the best weight of 113.53 ± 5.68 g followed by BSFL0 (111.22 ± 2.89 g), and the lowest was in BSFL60-fed fish (98.77 ± 4.52 g) (Table 33). The final weight of fish reduced approximately 6.7% and 11.2% when the fish were fed with BSFL40 and BSFL60, respectively, compared to BSFL20. A broken line regression study of the average WG revealed that BSFL meal could replace 30% of FM without affecting seabass growth performance ($R^2 = 0.8049$) (Figure 43).

The survival rate of fish fed with BSFLM did not differ significantly ($p = 0.0905$) from the control (BSFL0), ranging from 70.7% (BSFL60) to 88.7% (BSFL20). Fish fed with BSFL20 had the highest survival rate (SR) compared to BSFL0 of 88.67 ± 5.58% and 81.33 ± 3.26%, respectively, while BSFL40 and BSFL60-fed fish had survival rates of 76% and 71%, respectively ($p = 0.0905$). Likewise, there were no significant effects of BSFLM inclusion on the final weight (FW), average daily gain (ADG), or any of the growth parameters, including specific growth rate (SGR), condition factor (CF), hepatosomatic index (HSI), viscerosomatic index (VSI), and adiposomatic index (ASI)

(Table 33). There was no difference in feed intake (FI) between the control and BSFLM inclusion diets (p = 0.5673). The FI of fish fed on BSFL0 was 91.85 ± 1.72 g/fish/day, slightly higher than the 89.11 ± 4.73 g/fish/day recorded in fish fed with BSFL60. The fish were observed to readily accept the test diets because all the feeds were consumed without any rejection or loss. The SGR of fish fed with BSFL diets was lower than that of fish reared on the control diet. High inclusion levels of BSFLM at 60% had a negative impact on the FCR. In comparison to the control diet (1.43 ± 0.05), the FCR improved when BSFL was added at 20% (1.27 ± 0.07) and declined at 60% inclusion level (1.74 ± 0.11) Fish that were fed with the BSFL20 diet were more likely to consume the feed during the feeding time compared to the fish in the other treatments.

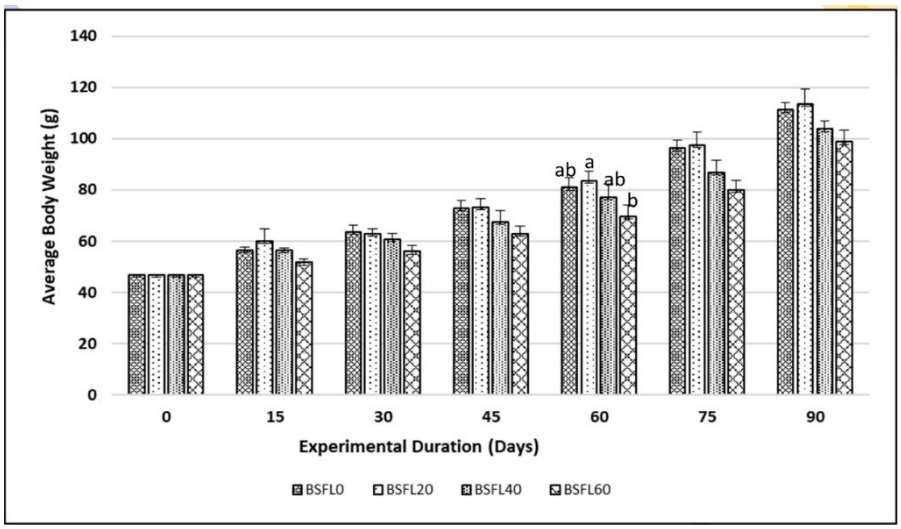


Figure 42: Fifteen days weight of Asian seabass, *Lates calcarifer* fed on different diets in the 90-day feeding trial.

Values are represented as mean ± standard error in grams of the mean of five fish replicates in each BSFL level. Values within the same period with different superscripts are significantly different (p < 0.05). BSFL0 = diet without BSFL meal (BSFLM) inclusion; BSFL20, BSFL40 and BSFL60 were 20%, 40% and 60% replacement levels of FM with BSFLM, respectively.

Table 33: Growth performance of Asian seabass fed on the control and test diets over a 90-day feeding trial.

	Diets				
Para-meter	**BSFL0**	**BSFL20**	**BSFL40**	**BSFL60**	***p*-value**
IW	46.74 ± 0.20	46.76 ± 0.17	46.76 ± 0.14	46.81 ± 0.20	0.9921
FW	111.22 ± 2.89	113.53 ± 5.68	103.79 ± 2.59	98.77 ± 4.52	0.0973
SR	81.33 ± 3.26	88.67 ± 5.58	76.00 ± 4.52	70.67 ± 3.40	0.0905
ADG	0.72 ± 0.03	0.74 ± 0.06	0.63 ± 0.03	0.58 ± 0.05	0.0835
WG	137.96 ± 6.37	142.77 ± 11.88	121.98 ± 6.20	111.06 ± 10.07	0.0839
SGR	0.96 ± 0.03	0.98 ± 0.06	0.88 ± 0.03	0.82 ± 0.05	0.0838
FI	91.85 ± 1.72	84.68 ± 5.00	85.58 ± 3.52	89.11 ± 4.73	0.5673
FCR	1.43 ± 0.05^{a}	1.27 ± 0.07^{a}	1.59 ± 0.14^{ab}	1.74 ± 0.11^{b}	0.0257
Body Indices					
CF	1.32 + 0.06	1.32 + 0.03	1.23 + 0.03	1.22 + 0.07	0.3936
GW	0.98 ± 0.05	1.14 ± 0.17	1.05 ± 0.12	1.30 ± 0.22	0.5294
HSI	0.19 ± 0.03	0.20 ± 0.02	0.21 ± 0.04	0.20 ± 0.02	0.9576
VSI	1.01 ± 0.03	1.09 ± 0.16	1.11 ± 0.13	1.38 ± 0.19	0.2759
ASI	1.57 ± 0.22	1.49 ± 0.19	2.01 ± 0.30	1.32 ± 0.20	0.1981

IW=initial weight (g), FW=final weight (g), SR=survival rate (%), ADG=average daily gain, WG=weight gain, SGR= specific growth rate (%/day), FI=feed intake (g/fish/day), FCR=food conversion ratio, CF=condition factor (g/cm^3), GW=gut weight (g), HSI=hepatosomatic index (%), VSI=viscerosomatic index (%), ASI=adiposomatic index (%).

BSFL0 = diet without BSFL meal (BSFLM) inclusion; BSFL20, BSFL40 and BSFL60 were 20%, 40% and 60% replacement levels of FM with BSFLM, respectively.

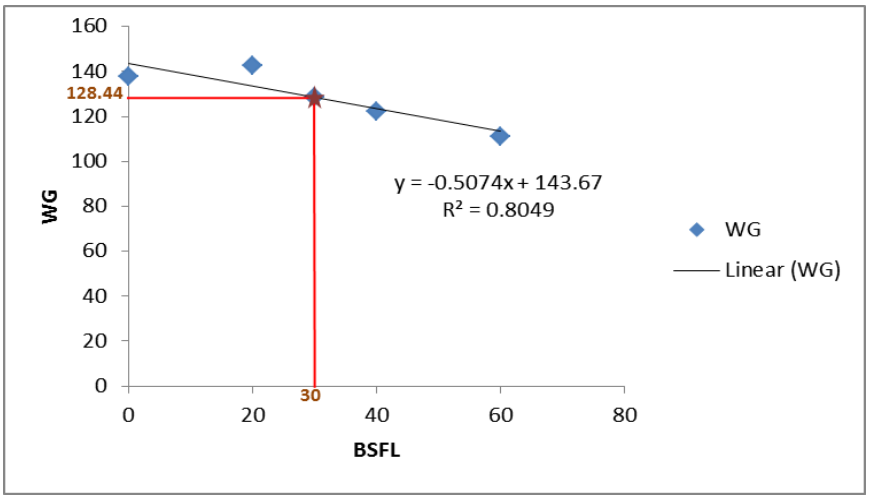


Figure 43: Broken line regression analysis of average weight gain (WG) of Asian seabass, *Lates calcarifer*-fed black soldier fly larval meal for 90 days.

6.3.2 Chemical Composition and Fatty Acid Profiles of Asian Seabass Fillets

The fillet composition of Asian seabass fed with the test diets is presented in Table 34. The replacement of FM with BSFLM had no significant effects ($p > 0.05$) on dry matter (DM), ash, CP, or Cfat of whole fish. The DM of fish ranged from 19.80 ± 0.49 (BSFL60) to 21.11 ± 0.80 g/100g (BSFL0) while the ash content ranged from 5.19 ± 0.05 (BSFL40) to 5.58 ± 0.13 g/100g DM (BSFL0). The CP was approximately 86 g/100g DM across all treatments, whereas the Cfat concentration ranged between 5.38 ± 0.22 g/100g DM in the control group (BSFL0) and 6.19 ± 0.45 g/100g DM in BSFL40.

On the contrary, the whole-body FA composition of the Asian seabass was significantly affected ($p = 0.0001$) by BSFLM inclusion (Table 35). The inclusion of BSFLM in the diets, caused an increase of lauric acid (C12:0) from 2.01% to 5.20% in the whole-body total FA whereas this FA could not be detected in fish on BSFL-free diet (BSFL0). Stearic acid (C18:0), which formed the most abundant saturated fatty acid (SFA), was found in higher concentrations in the fish fed with BSFLM than in the fish fed with BSFL0. The BSFL20-fed fish had the highest concentration of C18:0 which corresponded to 44.11 ± 0.94% of the total FA content. This concentration

decreased as the BSFLM inclusion levels increased and was lowest in fish fed on the control diet. Fish fed on BSFL60 had the highest concentration of linoleic acid (18:2n6c) of 16.62 ± 0.05% and was significantly different (p = 0.0060) from that fed with the control diet and two other diets with BSFL of BSFL20 and BSFL40. The highest concentration of eicosapentaenoic acid (EPA, C20:5n3) was observed in the fish that were fed with the BSFL0 diet. The EPA levels reduced with the inclusion of BSFLM, although the differences were not statistically significant (p = 0.5619). The lignoceric acid (C24:0) was significantly reduced linearly as the BSFLM was increased in the diets. Fish fed with BSFL20 had the highest levels of saturated fatty acids (SFA), at 80.44 ± 1.43%, while the fish fed without BSFL (BSFL0) had the lowest level of about 57.15 ± 2.31%. The monosaturated fatty acid (MUFA) was not detected in BSFL20-fed fish, which may be presence in a minute amount, whilst the total PUFA content of fish fillet showed a declining trend in the control group (21.36 ± 0.25%) to the BSFL60-fed fish (16.63 ± 4.89%).

Table 34: The nutrient composition of Asian seabass fillet fed on different dietary treatments of black soldier fly larvae meal during the 90-day feeding trial.

	Diets				
Nutrient Composition	**BSFL0**	**BSFL20**	**BSFL40**	**BSFL60**	***p*-value**
Dry Matter (g/100g)	21.11 ± 0.80	20.61 ± 0.19	20.29 ± 0.39	19.80 ± 0.49	0.3673
g/100g DM					
Ash	5.58 ± 0.13	5.35 ± 0.10	5.19 ± 0.05	5.26 ± 0.15	0.1219
Crude Protein	86.47 ± 0.99	86.88 ± 0.66	86.92 ± 0.19	86.31 ± 1.16	0.9406
Crude Fat	5.38 ± 0.22	5.62 ± 0.35	6.19 ± 0.45	5.91 ± 0.32	0.4045

Means ± standard error of the mean is not significantly different at $p > 0.05$.
BSFL0 = diet without BSFL meal (BSFLM) inclusion; BSFL20, BSFL40 and BSFL60 were 20%, 40% and 60% replacement levels of FM with BSFLM, respectively.

Table 35: The fatty acid profile of Asian seabass fillet fed on different dietary treatments of black soldier fly larvae meal during the 90-day feeding trial.

Fatty Acid (%, as it is) / Diets	**BSFL0**	**BSFL20**	**BSFL40**	**BSFL60**	***p* - value**
Lauric acid (C12:0)	ND	0.74 ± 0.26^{c}	3.04 ± 0.26^{b}	5.08 ± 0.52^{a}	0.0001
Myristic acid (C14:0)	2.54 ± 0.12^{c}	2.01 ± 0.62^{c}	4.20 ± 0.15^{b}	5.20 ± 0.21^{a}	<.0001
Palmitic (C16:0)	22.13 ± 0.76	26.55 ± 2.23	22.86 ± 0.97	21.40 ± 0.23	0.0519
Palmitoleic acid (C16:1)	3.43 ± 0.01	ND	ND	ND	
Stearic acid (C18:0)	23.36 ± 2.87^{b}	44.11 ± 0.94^{a}	30.04 ± 4.60^{a}	29.50 ± 4.60^{a}	0.0080
Cis-oleic acid (18:1n9c)	19.43 ± 1.88	ND	18.19 ± 4.22	17.74 ± 0.57	0.8298
Linoleic acid (18:2n6c)	14.72 ± 0.39^{b}	14.47 ± 0.19^{b}	14.32 ±0.26^{b}	16.62 ± 0.05^{a}	0.0060
Linolenic acid (C18:3n3)	1.57 ± 0.09^{a}	0.86 ± 0.11^{b}	0.84 ± 0.22^{b}	1.18 ± 0.05^{ab}	0.0055
Methy linolenate (C18:3n6)	0.92 ± 0.02	0.79 ± 0.05	0.98 ± 0.11	1.31 ± 0.47	0.3830
Arachidic acid (C20:0)	1.885 ± 0.00	ND	ND	ND	
Eicosatrienoic (C20:3n3)	0.68 ± 0.31	0.78 ± 0.06	0.72 ± 0.04	0.85 ± 0.06	0.8889
Arachidonic (C20:4n6)	1.39 ± 0.05	2.15 ± 0.60	2.26 ± 0.18	2.13 ± 0.51	0.0802
Eicosapentaenoic acid (C20:5n3)	3.14 ± 0.27	2.44 ± 0.70	2.64 ± 0.14	2.57 ± 0.17	0.5619
Behenic acid (C22:0)	1.19 ± 0.01	0.75 ± 0.13	1.63 ± 0.98	ND	0.7432
Lignoceric acid (C24:0)	7.27 ± 0.69^{a}	6.64 ± 0.21^{ab}	5.31 ± 0.43^{bc}	4.91 ± 0.31^{c}	0.0138
ΣSFA	57.15 ± 2.31	80.44 ± 1.43	63.59 ± 5.24	65.34 ± 5.11	
ΣMUFA	21.49 ± 2.56	-	18.19 ± 4.22	18.91 ± 0.69	
ΣPUFA	21.36 ± 0.25	19.61 ± 1.57	18.90 ± 0.87	16.63 ± 4.89	

^{abc}Means ± standard error of the mean in percentage (n=3) within row followed with different superscripts is significantly different at $p < 0.05$. ΣSFA=Total Saturated Fatty Acid, ΣMUFA=Total Monounsaturated Fatty Acid, ΣPUFA=Total Polyunsaturated Fatty Acid. ND=not detected. BSFL0 = diet without BSFL meal (BSFLM) inclusion; BSFL20, BSFL40 and BSFL60 were 20%, 40% and 60% replacement levels of FM with BSFLM, respectively.

6.3.3 Blood Serum Parameters of Asian Seabass

Table 36 shows the blood serum biochemicals of Asian seabass reared on the different treatments. There were no significant differences due to diet on any of the parameters measured ($p > 0.05$) except for globulin (Glo) concentration which was significantly higher ($p = 0.05$) in the BSFLM-fed fish than the control. The highest concentration of Glo of 34.14 ± 0.88 g/L was observed in BSFL40. Furthermore, the blood metabolites of fish raised on BSFLM diets did not vary from those of control fish (BSFL0). Although not significantly different, alkaline phosphatase (ALP) concentration increased from 69.20 ± 17.05 U/L in BSFL0 to 140.20 ± 31.72 U/L in BSFL60. Fish fed with BSFL60 had a higher aspartate aminotransferase (AST) concentration of 241.54 ± 59.43 U/L than those fed with a lower amount of BSFLM, while the fish fed with BSFL0 had 128.86 ± 39.32 U/L. The glucose (Gluc) concentration was lower in fish fed with BSFLM compared to the control group (BSFL0), but the differences were not significant between treatments ($p = 0.1698$). The alanine transaminase (ALT) concentration was lower in BSFL20 and BSFL40 than in the other treatments. The concentrations of triglyceride (TGL) and low-density lipoprotein (LDL) were highest in BSFL20 (1.99 ± 0.26 and 0.72 ± 0.16 mmol/L, respectively), followed by BSFL0 (1.86 ± 0.24 and 0.68 ± 0.02 mmol/L, respectively), and lowest in BSFL40 (1.68 ± 0.26 and 0.46 ± 0.13 mmol/L, respectively) and BSFL60 (1.69 ± 0.44 and 0.35 ± 0.10 mmol/L, respectively). The high-density lipoprotein (HDL) was higher in fish fed with BSFL20 (1.14 ± 0.07 mmol/L) and BSFL40 (1.20 ± 0.13 mmol/L) compared to the control (0.95 ± 0.07 mmol/L) and BSFL60 (0.92 ± 0.07 mmol/L). However, the differences between the concentrations were not significantly different ($p = 0.1056$).

Table 36: The blood serum biochemistry of Asian seabass reared on the different dietary treatments.

	Diets				
Parameter	**BSFL0**	**BSFL20**	**BSFL40**	**BSFL60**	***p*-value**
ALP	69.20 ± 17.05	72.40 ± 20.58	93.80 ± 20.56	140.20 ±31.72	0.1545
ALT	27.00 ± 8.51	15.2 ± 2.85	20.40 ± 4.20	30.00 ± 8.15	0.3888
AST	128.86 ±39.32	139.42 ± 38.40	117.18 ±46.44	241.54 ±59.43	0.2512
TP	36.16 ± 2.31	41.44 ± 1.45	41.78 ± 1.22	38.04 ± 3.19	0.2416
ALB	8.00 ± 1.24	7.78 ± 0.53	7.64 ± 0.41	6.72 ± 0.59	0.6594
Glo	28.16 ± 1.50^{b}	33.66 ± 1.04^{a}	34.14 ± 0.88^{a}	31.32 ± 2.70^{ab}	0.050
A:G	0.28 ± 0.04	0.22 ± 0.02	0.22 ± 0.02	0.22 ± 0.02	0.2835
Gluc	13.46 ± 1.54	11.18 ± 1.48	8.36 ± 0.58	9.76 ± 2.25	0.1698
TB	1.04 ± 0.22	1.22 ± 0.17	1.08 ± 0.10	1.10 ± 0.12	0.8711
Chol	4.01 ± 0.17	4.57 ± 0.42	4.25 ± 0.41	3.42 ± 0.35	0.1687
TGL	1.86 ± 0.24	1.99 ± 0.26	1.68 ± 0.26	1.69 ± 0.44	0.8778
LDL	0.68 ± 0.02	0.72 ± 0.16	0.46 ± 0.13	0.35 ± 0.10	0.1161
HDL	0.95 ± 0.07	1.14 ± 0.07	1.20 ± 0.13	0.92 ± 0.07	0.1056

Means ± standard error of the mean in the row showed that the comparison between treatments is not significantly different ($p > 0.05$).

Parameters: ALP=Alkaline Phosphatase (U/L), ALT=Alanine Transaminase (U/L), AST=Aspartate Aminotransferase (U/L), TP=total protein (g/L), ALB=albumin (g/L), Glo=Globulin (g/L), A:G=Albumin:Globulin (Unit), Gluc=Glucose (mmol/L), TB=Total Bilirubin (umol/L), Chol=Cholesterol (mmol/L), TGL=Triglyceride (mmol/L), LDL=Low-Density Lipoprotein (mmol/L), HDL=High-Density Lipoprotein (mmol/L).
BSFL0 = diet without BSFL meal (BSFLM) inclusion; BSFL20, BSFL40 and BSFL60 were 20%, 40% and 60% replacement levels of FM with BSFLM, respectively.

6.3.4 Sensory Evaluation of Asian Seabass Fillet

There was no significant association ($p > 0.05$) between the dietary inclusion levels of BSFLM and sensory properties, including colour, pleasant aroma, flavour, texture, and overall acceptability (Table 37). Across all the sensory acceptability parameters, fish reared on BSFL20 (5.03 ± 0.22) had the lowest overall score. Compared to the

control group (BSFL0) and other BSFL replacement groups, a 40% replacement of FM with BSFLM produced the highest colour intensity score of 6.08 ± 0.13 for steamed seabass assessed by visualisation for the whiteness of the fish fillet. Even though there were no statistically significant differences between the dietary groups, fish fed with BSFL20 had lower scores for aroma and taste (4.90 ± 0.22 and 4.73 ± 0.26, respectively) than fish fed with BSFLM at a 60% replacement rate of 5.45 ± 0.25 and 5.30 ± 0.24, respectively. The fillet texture of fish fed with BSFL0 was superior to that of fish raised on diets that contained BSFLM. In terms of overall approval by the evaluation panel, fish fed with BSFL60 scored the highest (5.48 ± 0.23), while BSFL20-fed fish scored the lowest (5.03 ± 0.22).

Table 37: The sensory properties of muscle fillets of Asian seabass fed with various levels of BSFLM diets during a 90-day feeding trial.

	Diets				
Parameters	**BSFL0**	**BSFL20**	**BSFL40**	**BSFL60**	***p*-value**
Colour	5.93 ± 0.13	5.65 ± 0.19	6.08 ± 0.13	5.90 ± 0.14	0.2325
Aroma	5.18 ± 0.19	4.90 ± 0.22	5.23 ± 0.21	5.45 ± 0.25	0.3766
Earth Taste	4.88 ± 0.24	4.73 ± 0.26	5.18 ± 0.25	5.30 ± 0.24	0.3259
Texture	5.78 ± 0.12	5.68 ± 0.15	5.50 ± 0.18	5.68 ± 0.18	0.6728
Overall Acceptability	5.30 ± 0.20	5.03 ± 0.22	5.40 ± 0.22	5.48 ± 0.23	0.4725

Means ± standard error of the mean is not significantly different at $p > 0.05$.
BSFL0 = diet without BSFL meal (BSFLM) inclusion; BSFL20, BSFL40 and BSFL60 were 20%, 40% and 60% replacement levels of FM with BSFLM, respectively.

6.4 Discussion

The nutrient composition of BSFL is directly influenced by the substrate on which they are reared. This may account for the variability in results when BSFL meal is included in fish diets (Fisher *et al*., 2020; English *et al*., 2021). For example, the BSFLM used in this feeding trial had a protein content of 49.50 g/100g DM compared to the BSFLM used by Bolton *et al*. (2021) at 36.8 g/100g DM and Devic *et al*. (2017) at 41.6 g/100g DM, which contributed to the varying results when the BSFL is incorporated into fish diets. Finke (2007) mentioned the importance of the larval stage used in aquafeed, as the presence of chitin in the older larvae (pupae or pre-pupal stages) can influence fish growth rate. The presence of chitin in BSFL at levels ranging from 1.6 to 7.3% (Sheppard *et al*., 1994; Diener *et al*., 2009) reduces nutrient digestibility by making it more difficult for nutrient absorption by the fish (Gasco *et al*., 2019a; Elesho *et al*., 2021), resulting in a decrease in fish growth (Cummins *et al*., 2017; Gasco *et al*., 2019a; Elesho *et al*., 2021). Many fish species are unable to digest chitin due to the lack of chitinase enzyme (Rust, 2002). The chitin concentration of BSFL may be affected by the type of feed substrate, which could subsequently impact the digestibility and growth of fish (Fisher *et al*., 2020). The bioavailability of nutrients in BSFL products may increase due to mechanical defatting at high pressure, requiring less digestion to absorb the amino acids and fatty acids (Roques *et al*., 2020). Belghit *et al*. (2019) replaced 100% of FM in their study by using 8-day-old seaweed-fed larvae. Early harvesting of larvae avoided the presence of chitin, which made the larvae more easily digestible.

The inclusion of BSFL meal in the fish diets did not affect the palatability of Asian seabass diets, as evidenced by the similar feed intake (FI) across treatments. Similar findings are reported on Atlantic salmon (Belghit *et al*., 2018) and rainbow trout (Renna *et al*., 2017; Dumas *et al*., 2018) fed with BSFL meal diets. One plausible explanation is that feed intake was unaffected by the increased inclusion level of BSFL because they need to consume more (of inferior quality) feed to meet their nutritional needs. However, it appeared that the fish had more difficulty digesting

the feed with a higher percentage of BSFL (60%) than those fed with a lower inclusion level (20%) of BSFL in the diet due to a lower body weight in BSFL60-fed seabass than in other treatments. Thus, replacing up to 40% of the FM with BSFLM in Asian seabass diets was acceptable, where the growth performance of BSFL40-fed fish was not significantly different from the control and BSFL20-fed fish. However, a broken line regression analysis of mean weight gain indicated that the appropriate amount of FM replacement was 30% for optimal growth of juvenile Asian seabass reared in freshwater without adverse effects on fish performance. In other study, Katya *et al*. (2017) discovered that BSFLM could replace up to 50% of FM without affecting growth or survival in seabass. But, they estimated that the optimal percentage of BSFLM that could replace FM in the fish diet was 28.4%, which is consistent with this study.

The BSFL meal (BSFLM) is considered an emerging protein source in aquafeed and thus, further research is required to optimise the percentage of BSFLM that can be used to replace FM in a fish diet without affecting fish growth, health, and consumer acceptance. Glencross and Rutherford (2011) advocated a threshold of 15% FM in barramundi (seabass) meals to avoid a lack of appetite and palatability. According to Burr *et al*. (2012), palatability, feed intake and nutrient absorption decreased when a high percentage of FM was replaced with BSFL meal in the fish diet. In the present study, fish fed on BSFLM experienced a 3 – 7.8% decrease in feed intake, but the conversion of feed to body weight was still effective. This was especially true for fish fed with BSFL20, which had greater feed conversion to body weight than the control group (BSFL0). The feed conversion ratio (FCR) of fish fed with BSLM20 improved by 11.2% when compared to BSFL0. This may have been due to improved nutrient digestibility, which improved feed conversion efficiency. The growth behaviour of the Asian seabass fed with increasing inclusion levels of BSFLM, makes it abundantly clear that BSFLM could be a good replacement for FM in the diet of carnivorous fish species. There were no discernible variations in the growth parameters between fish fed with a BSFL-free diet and those fed with diets that had the FM replaced by 20 to 60% of BSFLM. The weight gain (WG) and specific

growth rate (SGR) declined as the FM content in the diets decreased. However, the values were not significantly different from the control group.

Body indices are important parameters that are used to express fish growth (Xiao *et al*., 2018). In this study, there were no differences in the hepatosomatic index (HSI), viscerosomatic index (VSI) and adiposomatic index (ASI) of the fish fed with BSFLM-based diets with the control group. The small value of these indices in this study indicated no abnormal fat deposition as a result of feeding BSFLM, as well as liver and intestinal functions of Asian seabass were not affected. In contrast, higher VSI and HSI were found in carp (Zhou *et al*., 2017), Atlantic salmon (Lock *et al*., 2016; Belghit *et al*., 2018) and Siberian sturgeon (Caimi *et al*., 2020) than those given control diets. Excessive lipid deposition could hamper liver health and functions. This was consistent with the findings of Fawole *et al*. (2020) and Xiao *et al*. (2018), who observed that dietary BSFL meal had no influence on HSI and VSI in African catfish (*Clarias gariepinus*) and yellow catfish (*Peltobagrus fulvidraco*), respectively. The lowest survival rate (SR) was recorded in fish fed with BSFL60. BSFL60-fed fish may not receive adequate nutrients for growth at a higher BSFLM in diet due to the low digestibility of BSFLM. The survival rate of Asian seabass in the present study was lower than that of Katya *et al*. (2017) and other fish species (Xiao *et al*., 2018, Fawole *et al*., 2020). According to Venkatachalam *et al*. (2018), the survival rate of seabass was around 60 – 71% under two system aquacultures, such as an integrated mangrove-aquaculture system and open-aquaculture system. This indicates that the seabass survival rate is low, although not given BSFLM. Therefore, the low SR in this study is not attributable to BSFLM feeding, but this species is likely highly sensitive to any environment. Fawole *et al*. (2020) observed low survival rate at 60% in *Clarias gariepinus* when fed with 50% BSFL compared to the control at 80%. Almost all the fish that died in the present study were small due to competition for feed and cannibalism, similar to the observation by Fawole *et al*. (2020). Occasionally, two or three fish in a tank will appear stunted compared to the other fish, and they will be preyed upon by the other fish. Feed palatability is critical in determining optimal nutrient utilisation and decreasing economic losses in aquaculture. Low

palatability of BSFL60 meal may occur due to a decrease in FM in the diet, which results in low feed attractiveness to fish (Yuan *et al*., 2021). This is in accordance with Kroeckel *et al*. (2012) who recorded a significant decrease in diet palatability, feed intake, and growth performance of turbot at higher BSFLM inclusion levels in turbot (*Psetta maximus*) diets. It is difficult to eliminate the use of FM and fish oil in diets without affecting growth due to the nutritional needs of carnivorous fish. In addition, the high sensitivity of carnivorous species to dietary imbalances and antinutritional factors present in diets, caused the total replacement of FM may be problematic (St-Hilaire *et al*., 2007b).

With regards to the chemical composition of seabass fillet, high dietary inclusion levels of up to 60% of BSFLM did not affect the nutrient contents of fillet. This was consistent with Belghit *et al*. (2018) and Zhou *et al*. (2017), who found no significant effects of dietary BSFLM on Atlantic salmon and carp, respectively fed with BSFLM in the whole-body composition. The whole-body composition of juvenile turbot (*Psetta maxima*) revealed that the inclusion of BSFL meal did not influence body protein content. However, the body lipid content decreased with increasing BSFLM inclusion levels as reported by Kroeckel *et al*. (2012). Fawole *et al*. (2020) observed that the protein content of African catfish (*Clarias gariepinus*) fed with 50% BSFL was higher than in control, which indicated that BSFL meal could affect the protein levels in fish. In the present study, the insignificant differences in nutrient composition of body content between the treatments and control indicate that the BSFL meal does not induce excessive nutrient accumulation in Asian seabass. However, the protein content of Asian seabass fillets fed with BSFL reared on a combination of SG leaves, and milk-extracted coconut meat (MECM) in the present study was higher than in Asian seabass fed BSFL reared on SG alone which was between 56 and 67% DM (Katya *et al*., 2017). However, the ash and fat content of the fillet (5.2 – 5.6 g/100g and 5.4 – 6.2 g/100g DM, respectively) were lower than those reported by Katya *et al*. (2017), which was more than 17% DM. The Cfat and ash contents of Asian seabass were similar to those reported on yellow catfish, *Peltobagrus fulvidraco* (Xiao *et al*., 2018). Differences in fish nutrient composition

are influenced by the differences in diet preparation and BSFL nutrient composition. The protein concentration of the formulation diet and BSFL in the present study was higher than Katya *et al*. (2017). Additionally, the age of the fish used, ingredients in the feed, and fish conditions may have contributed to the differences observed between the studies (Yu *et al*., 2013).

Increasing levels of BSFLM in the diet led to a significant increase in SFA, such as lauric acid (C12:0) and stearic acid (C18:0), and a decrease in PUFA, such as linolenic acid (C18:3n3) and eicosapentaenoic acid, EPA (20:5n3) in the whole body of Asian seabass, similar to the results observed by Renna *et al*. (2017) and Mancini *et al*. (2018). Belghit *et al*. (2019) discovered a linear increase in lauric acid and EPA in the fish when BSFL was included in the diet. The presence of a high percentage of FA, such as lauric acid in BSFLM-fed fish improves the fish's intestinal health by boosting their immune system (Gasco *et al*., 2019b). However, this FA was not found in the BSFL0-fed Asian seabass, similar to the result observed in Jian carp fed with a non-BSFLM diet as a control (Zhou *et al*., 2017). Most fish are consumed for the long chain (and essential) PUFA they contain. With a reduction of FM in the diets of fish (a natural source of PUFA), and an increase in BSFL and other alternative ingredients rich in SFA and MUFA, there is a decline in PUFA and an increase in SFA in fish, which could have implications for cardiovascular health (Briggs *et al*., 2017). This situation will impact the consumption of BSFL-fed products (Purba *et al*., 2020). The type of lipids found in fish depends on water salinity (Rabeh *et al*., 2015) and water temperature (Bin *et al*., 2021), whereby long-chain PUFA are detected in considerable concentrations at low water temperatures (Bin *et al*., 2021). In freshwater fish, the SFA and unsaturated FA are used for energy or to synthesise long chain-PUFA, but marine fish requires them to sustain osmotic pressure (Rabeh *et al*., 2015).

Blood biochemistry analysis has been recognised as an invaluable tool in evaluating the effects of ingredient substitution in diet on fish health status (Fawole *et al*., 2020) and whether the fish is under chronic stress (Bahmani *et al*., 2001). The

present study found no differences in serum parameters when FM was replaced with BSFLM in the Asian seabass diet, which is consistent with previous studies on Nile-tilapia and Atlantic salmon (Ogunji *et al*., 2007; Belghit *et al*., 2019). There were no significant differences in blood levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and total protein (TP) between the treatments. However, the ALP enzyme levels were seen to increase in serum samples of Asian seabass fed with BSFLM, which contradicted the findings by Fawole *et al*. (2020) who stated that ALP levels decreased with increasing levels of BSFLM. Fawole *et al*. (2020) fed *H. illucens* to African catfish (*Clarius gariepinus*) but did not provide any information on the culture procedure and the feed substrates used for rearing the BSFL which made it difficult to make a direct comparison. The globulin (Glo) levels rise at increasing percentages of BSFLM in fish diets, particularly BSFL20 and BSFL40. However, there was a sudden decrease in Glo level in BSFL60, which was thought to be related to a stronger innate immune response of the fish (Wiegertje *et al*., 1996). Albumin, globulin, and protein in the blood plasma are required to maintain the normal physiological functions of fish physiology and health conditions of the fish (Kurkani, 2021). As opposed to Scherr *et al*. (2015), the present study found that Asian seabass had a relatively high cholesterol content, but it was lower than in Atlantic salmon (Belghit *et al*., 2019). The study showed that increasing the BSFLM levels in the fish diets from 40% to 60% reduces insignificantly the triglyceride (TGL) content in Asian seabass. This condition might be due to the presence of chitin, which has been linked to the hydrolysis of triglycerides, resulting in improved lipid utilisation in fish (Fawole *et al*., 2020). Glucose (Glu) is a marker of stress in fish (Polakof *et al*., 2012). The higher levels of BSFLM in the diets were found to decrease the concentration of glucose in the blood serum of fish. The incidence of death of Asian seabass in the current study was not attributable to any form of stress or infectious diseases linked to BSFLM consumption, as suggested by the glucose levels.

The sensory evaluation of Asian seabass fillets, in which BSFLM replaced 20 to 60% of the FM, showed comparable performance to the control diet. Studies have

reported that the inclusion of BSFLM in fish diets has no negative impact on the taste of fish and, in most cases, the taste is improved by the addition of BSFLM. Other parameters such as aroma, earth taste, texture and overall acceptability of fish fed BSFLM were not significantly different from the control as observed in the present study. A northern Italian survey indicated that 90% of the consumers will purchase fish that have been fed with insect meal (Mascuso *et al*., 2016). Sealey *et al*. (2011), reported that the BSF prepupae meal had no impact on the sensory quality of rainbow trout fillets and also in salmon (Belghit *et al*., 2019). Fish fillet colour influences a consumer's buying decision (Maciel *et al*., 2014), and when it deviates from the traditional white flesh colour, it loses marketability (Kin *et al*., 2010). However, Belghit *et al*. (2019) showed that 50% of the consumers described a stringy texture in fish products with a high BSFL meal inclusion rate, while another 20% expressed light flavour and salty taste for the low and moderate inclusion rates, respectively. Borgogno *et al*. (2017) reported that utilising insect meals in fish diets can significantly alter the FA composition of fillets and influence the sensory qualities of fish products, such as aroma and flavour. Nevertheless, it is unclear how the BSFL substrate influences the sensory parameters of the fish products and, ultimately, consumer acceptance. Therefore, future research should incorporate a sensory panel in the experimental approach to gain a better understanding of these relationships (English *et al*., 2021).

6.5 Conclusions

The present results show that the replacement of up to 60% of FM in the diet of Asian seabass (up to 222 g BSFLM/kg diet) did not show significant differences between the treatments, including the control (BSFL0) in terms of growth performance, serum biochemical parameters, fillet quality, and sensory test of the fish. Asian seabass fed on BSFL20 resulted in the highest body weight gain, growth rate, and feed conversion ratio (FCR) among the dietary treatments, including the control group. The fish fillet sample showed that Asian seabass contains high protein but low in fat. However, the quality of the fish fillet was not satisfactory due to the limitation of PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in seabass fillets. The sensory analysis showed no discernible differences between the control and BSFL-fed fish. Based on the current findings shown by the fish given BSFL20, the study concluded that the ideal replacement of FM in the Asian seabass diet is 20%, which equivalent to 74 g/kg. However, it can be suggested that up to 30% of BSFLM could be added to the diets of seabass without having any negative effects on fish performance, utilisation of feed, or its preference among consumers. The findings presented here determined that *Hermetia illucens* has proven to be a suitable substitute for fishmeal in Asian seabass, similar research can be conducted in other cultured species to develop a novel and sustainable source of nutrients for future aquafeed formulations.

CHAPTER 7

GENERAL DISCUSSION, LIMITATIONS, FUTURE RESEARCH AND CONCLUSIONS

7.1 General Discussion

Fishmeal (FM) has traditionally been the preferred protein source for aquafeed due to its high protein content, well-balanced amino acid profile, and excellent digestibility (Olsen and Hasan, 2012). Due to depleting wild fish stock (English *et al*., 2021), the production of FM became unsustainable, and the world supply of FM has decreased. This leads to an increase in the price of FM, which affects the profitability of the aquaculture sector (Tacon and Metian, 2008; Barosso *et al*., 2014). Therefore, there are ongoing research activities to find alternatives to FM that are cost effective with high protein and moderately high essential fatty acids contents. Previously, many animal-based protein sources have been used as a substitute for FM and later, research attention moved towards plant-based protein sources. These sources have also met with varying levels of success due to the imbalance of amino acids and the presence of anti-nutritional substances within plant materials that interfere with protein digestibility (Caimi *et al*., 2020). Recently, insects and insect meal have received increasing research attention as potential replacers for FM, in particularly, black soldier fly larvae (BSFL, *Hermetia illucens*) due to its high fat and protein content with a balanced amino acid composition (Henry et al., 2015; Liland *et al*., 2017; Meneguz *et al*., 2018; Nogales-Merida *et al*., 2018). However, the growth indices and nutritional composition of BSFL are significantly affected by the chemical compositions of the organic substrates (Barragan-Fonseca *et al*., 2017; Liu *et al*., 2017; Spranghers *et al*., 2017; Danieli *et al*., 2019; Scala *et al*., 2020). Therefore, it is important to know and standardise the nutritional value of the substrates to maximise and optimise the nutrient content of the BSFL to meet the dietary needs of a particular fish species.

In this study, the main aims were to evaluate selected underutilised plants and agro-industrial by-products as feed substrates for the production of BSFL with high protein and fatty acids content, followed by incorporating these BSFLs to substitute FM in aquafeed and enhance the growth of Asian seabass (*Lates calcarifer*). The aims of this study have been achieved by using a mixed substrate containing milk-extracted coconut meat (MECM) and *Sesbania grandiflora* (SG) at the ratio of 1:1 as feed for the BSFL production and the oven dried BSFL that harvested at 19 days after hatched were later used as an ingredient in fish feed. The current studied showed that high level inclusion of MECM-SG fed BSFL in the diet as high as 40% has no negative effects on fish performance, utilisation of feed, or preference of Asian seabass meat among consumers. The outcome of this study had contributed to reducing the amount of fishmeal used in fish feed and could potentially reduce the feed cost. Furthermore, BSFL is a sustainable protein source for aquafeed as its production uses locally available substrates and the production process is a simple and could be easily upscaled. This study shows that insect-derived products are a viable alternative in the food industry and have the potential to contribute to the traditional development of the aquafeed industry over the long term.

Summary of Findings

In Chapter 1, five (5) interrelated research questions were identified in order to test the hypothesis of the study, that is, partial substitution of FM with BSFLM in the diet of Asian seabass has no adverse effect on feed acceptance and performance.

RQ1. What is the impact of different feed substrates on the performance and nutrient composition of black soldier fly larvae (BSFL)? Is there an impact of feed substrate on the ability of BSFL to convert substrate meal into larval biomass, considering factors such as waste reduction, bioconversion, and protein conversion ratio?

As described in Chapters 2 and 3, nutritional quality of six available feedstuffs, two sources from underutilised plant species, *Moringa oleifera* (MO) and *Sesbania*

grandiflora (SG) and four agro-industrial by-products such soybean waste (SBW), wheat pollard (WP), rice bran (RB) and milk-extracted coconut meat (MECM) were determined, and their influence as rearing substrates were evaluated on the nutritional quality of BSFL. Based on protein content, MO, SG, and SBW were in high protein group, whereas WP and RB had moderate protein content, and MECM was low in protein content (Chapter 2). Such a comparative study is essential when deciding the suitable feed substrates for BSFL mass rearing. In this study, substrates with different protein content significantly influenced the nutrient composition of larvae (Chapter 3), which corroborated with previous results (Spranghers *et al*., 2017; Wang and Shelomi, 2017; Barragan-Fonseca *et al*., 2018, Lalander *et al*., 2019; Hopkins *et al*., 2021; Siddiqui *et al*., 2022), in particular, crude protein and fat that enable the replacement of other animal- or plant-derived protein sources by BSFL in aquafeed. The results indicate that through BSFL conversion, it was worthwhile to convert substrates with a low nutrient value such as MECM into a high protein and fat larvae biomass. The protein conversion efficiency in dry matter (PrCEDM) of BSFL was between 25 to 139%. The lower the CP of the feed substrate, the more efficient protein conversion by the larvae to meet their growth requirements. MECM-fed larvae had the highest PrCEDM due to the protein substrate was only 4 g/100g DM while the lowest was larvae fed with leaf meals, MO and SG. However, a great deal of emphasis is placed on the quality of protein and fat in terms of essential amino acids and fatty acids because they depend on the quality of the substrate material used to feed them. The EAA and essential FA required by the animals were provided by the diet, which they could not synthesise. BSFL faces the same issue; the feed substrate must supply these nutrients. The essential content of AA and FA in feed substrates with a low nutrient value is typically low, reflected in the larvae grown on, particularly MECM. Thus, to ensure the long-term viability of BSFL in the animal feed industry, a substantial amount of effort and investment must be devoted to developing this replacement material equivalent to FM regarding nutrient quality, digestibility, and cost of production.

Bioconversion efficiency and the time taken to reach the maturity of the BSFL are influenced by the substrate type and the rearing conditions. Proteins, fats, digestible carbohydrates, and maybe a portion of hemicelluloses, as well as anti-nutritional factors, are believed to positively impact larval performance (Fojas *et al*., 1982; Makkar and Becker, 1996; Barragán-Fonseca *et al*., 2018; Gold *et al*., 2020). The utilisation of an underutilised plant species, MO and SG, as larvae feed has provided an excellent chance to promote this leaf while containing high-quality nutrients. BSFL play a great role in organic waste management (Czekała *et al*., 2020; Purkayastha and Sarkar, 2021). In the present study, BSFL was found to reduce the volume of single feed substrate (MO, SG, SBW, WP, RB and MECM) from 54 to 75%, while on mixed feed substrates (MECM:SG) was between 45 and 70%. But what draws attention is the total dry weight of protein produced from the larvae's conversion efficiency of each feed substrate. This is because the efficiency of converting the substrate consumed into larval biomass was not more than 23%, which means that only a small volume of the feed substrate consumed was converted into the weight of larvae. Larvae were small creatures, and only about 22 – 33% (depending on feed substrate and larval age) of their total mass was composed of dry matter. BSFL farming necessitates a huge quantity of organic waste as a feeding substrate for large-scale production. Using BSFL as a viable alternative to FM as a source of protein will require a significant investment of time and effort. Rearing BSFL was challenging due to the many factors that influence their development and the quality of the larvae, which caused concern about the yield at the same time. Therefore, identifying nutritionally valuable, readily available organic waste streams in adequate volume is a prerequisite for the success of BSFL mass rearing.

RQ2. Which feed substrate is optimum for larval production? When is the best time to harvest larvae, considering the development and nutrient composition factors, such as protein, fat and total larval weight?

This study was performed to produce BSFL using underutilised plants and agro-industrial by-products for FM replacement in Asian seabass. BSFL converts leaf meals and low-nutrient organic wastes into high protein and fat larval biomass which can be used in animal feed (Makkar et al., 2014; Józefiak et al., 2016; Dortmans et al., 2017). Different substrates affecting larvae quality and development time (Chapter 3). The harvesting age for BSFL is difficult to determine due to the short life cycle of 45 days under optimal conditions, whereby the larval stage is only 18 days (Ferrarezi et al., 2016). The nutrient makeup of BSFL changes as it develops through various stages of growth (Liu et al., 2017). It was recommended that the BSFL be harvested when it was in the white larvae stage, considering that the larvae protein was still high. The presence of chitin in the prepupae stage, which acts as an anti-nutritive factor, can affect the meal's digestibility and, as a result, the growth of the fish. Early harvesting resulted in a lower larval yield than later harvesting. Late harvesting led to a rise in the cost of producing larvae, as more substrate was used throughout their development and other facilities. Farmers, feed manufacturers, and industries sometimes fail to consider the time of harvest, which further affects the quality of the products and leads to variations in the BSFLM nutrient composition (Pretorius, 2011), thus affecting the selling price. Determining the optimum time to harvest larvae is important to minimise major loss of nutrients during larval development, especially the protein value, which decreases with larval growth (Liu *et al*., 2017). However, on an industrial scale, harvesting BSFL at the late larvae stage, such as the five instars, is recommended to increase the yearly product yield and nutritional values of BSFL (Wong *et al*., 2019). Most previous research focused on the ability of BSFL to self-harvest as they migrate away from the wet feeding medium and pupate on dry surfaces which facilitates larvae separation and lessens operational work (Diener *et al*., 2011). Nonetheless, the current industry practice still employs the manual sieving method to separate the larvae from the feeding medium (Caruso *et al*., 2014). The criteria for selecting the day for harvesting is crucial because BSFL must be produced in sufficient quantities to replace FM (millions of metric tonnes annually). These factors will put BSFL operators/breeders in a tough situation. Thus,

based on the optimal nutrient composition and performance of the larvae in terms of the highest mean dry weight, CP, and Cfat, day 17 was selected as the best age of BSFL harvest for all feed substrates in the present study (Chapter 3). Although the protein content of larvae was highest at an early age (Liu *et al*., 2017; Beniers and Graham, 2019), factors such as biomass yield and the amount of protein produced by the larvae were taken into consideration in selecting the day for harvesting larvae.

One of the most frequent criticisms of the use of insects to consume substrate and the resultant BSF conversion of biomatter is the absurdity of feeding the larvae when the feed can be given to livestock directly. Whilst this is true in relation to feed that is given directly to livestock, the same cannot be applied to all feed livestock situations. Livestock performance is correlated with dietary quality; hence any decline in diet quality will seriously impact the growth rate, and nutritional quality of those animals in receipt of the new formulation. It is essential to identify a possible substrate carefully. The BSF bioconversion can still have a role to play in the provision of protein-rich feed sources. This is important for low-nutrient organic waste and unsuitable feedstuff, which the BSFL bioconversion can improve to produce a high-quality feed for animals rather than giving them directly to the animals. These organic wastes undergo BSFL's intermediate stage to boost their nutrient content through larvae biomass production. In the context of the sustainability of substrates, feeding the BSF larvae with organic waste is perfectly reasonable, considering the long-term availability and cost of available substrate materials. According to Gold *et al*. (2021), the selection of BSFL feed substrates can be based on four criteria: process performance, availability and cost, product safety, and recovery hierarchy. The efficacy of a biowaste treatment process influences treatment time, growth and larvae composition and residual levels per unit of biowaste, as well as treatment product costs and profits (Gold *et al*., 2021). Although rearing BSFL is technically feasible, the larvae are currently produced on a small scale with low yield and efficiency to cater to niche markets. The BSFL sector is still insufficient in terms of volume to successfully compete with FM as the

primary protein source. Mass production is still many years away (Lu *et al*., 2022). In this situation, mechanisation is one of the key issues in the growth of this industry particularly during harvesting larvae and preparing for the feed substrate. The availability and price of BSFL continue to be insufficient to compete with FM and soybean meal, resulting in BSFL not being widely used in aquafeed, particularly for carnivorous fish that require a high protein diet. However, the potential of BSFL as a feed source has been recognised and used by early adopters who use it in limited quantities. Following the increase in the price of FM, feed manufacturers need to find alternative ingredients to reduce the overall price of aquafeed. The high price and unreliable (sometimes inferior) quality of currently available BSFL have been prohibitive to increased uptake of this ingredient as a replacer for FM.

For any feed ingredient, the nutritional value must be consistent. It is impossible to formulate aquafeeds if the nutrient quality of an ingredient varies from batch to batch. The type of substrate on which larvae are reared is critical for the nutritional value of the larvae, not only concerning the amount and quality of protein, purposely in essential AA, but also to the presence of essential fatty acids and long-chain PUFA. As such, the feed substrates used to feed BSFL should be carefully selected based on their ability to enrich the larvae with EPA and DHA, especially when they are produced to be used in aquafeed (English *et al*., 2021). RB appeared to be the best of the six employed feed substrates for larval production based on the performance and nutrient composition of the larvae produced. However, RB is a conventional ingredient in other animal feed, and the production of larvae on RB is rather expensive due to its high cost in the market. According to Smetana *et al*. (2019), wastes with high process performance may already have prior applications, such as animal feed. Production of animal feed through BSFL conversion may not result in cost-effective treatment products. Unfortunately, the frequent presence of fungal growth and contamination in RB the substrate poses a risk to the fish in the future. The incidence of fungal contamination has prompted the exclusion of RB as BSFL substrate in the present study. However, future experiments may need to be carried out to identify this fungus and its effects on larvae and fish growth.

RQ3. Is using a combination of feed substrates superior to using a single substrate for larval development and nutrient composition? What is the impact on the larval ability to convert feed substrate into biomass?

RQ4. Following on from question 3, what is the optimum mix of different substrates on larval development and nutritional value?

It is recommended to mix two or three single substrates to generate a complete substrate in terms of nutrient composition to compensate for nutrient deficiencies in any substrate (Bonelli *et al*., 2020). Mixing nutritionally distinct single streams may improve BSF performance (Rehman *et al*., 2017a; 2017b). As a result of the enhanced digestibility and palatability, larvae will consume more nutrients from the mixed feed with improved nutrient composition, hence growing into better individuals in terms of performance and nutritional values (Brits, 2017). In this study, a combination feed substrate consisting of equal amounts of SG, and MECM resulted in the improved performance of BSFL in converting substrate to larval biomass compared to their controls (Chapter 4). This study selected a combination of MECM and SG at a proportion of 1:1 (L4 feed substrate) to replace FM in the Asian seabass diet. Larvae on a mixed feed substrate were harvested on day 19, considering the protein content and other growth parameters in larvae reached their maximum levels. It was harvested two days later than those on the single feed substrates of MECM and SG (day 17). Although larvae on single feed substrate harvested on day 17, MECM-fed larvae developed into prepupae on day 28. Lalander *et al*. (2019) reported that larvae raised on fruits and vegetables required nearly 30 days to reach the pupal stage. However, when the feed substrate was combined with abattoir waste, the larvae reached the prepupal stage in only 12 days. Combining substrates is preferable because the larvae can be harvested more quickly, making the process more cost-efficient. Additionally, the nutrient value of the larvae can be improved without relying solely on a single substrate source, which usually lack certain nutrients. The mixed feed substrates could increase the

performance and nutrient composition, particularly the protein content of larvae, compared to single substrate-fed larvae (Lim *et al*., 2019; Li *et al*., 2022). Better bioconversion by the larvae and higher protein conversion efficiency of feed substrate achieved using improved-tasting or more palatable substrates where it is easier for the larvae to digest the substrate. The findings in the present study indicate that the protein levels in the mixed feed substrates with suitable fat content should be prioritised in order to improve the performance of larvae on the low-nutrient feed substrate. Substrate containing 12.4% protein was sufficient for larvae growth (Nyakeri *et al*., 2019). As demonstrated by RB-fed larvae and L4-fed larvae, a CP and Cfat ratio of feed substrate of around 1:1 was likely to be more adequate. The CP and Cfat of RB were 12.2% and 11.9%, and L4 was 14.1% and 17.8%, respectively. Therefore, this study suggests that the protein content of around 15% was sufficient to produce high-protein larvae and support larval growth.

RQ5. Can black soldier fly larval meal (BSFLM) replace fish meal in the Asian seabass diet? What is the ideal percentage of BSFLM replacement in the diet that does not affect the growth and development of Asian seabass?

The BSFL produced from 50% MECM and 50% SG (MECM:SG, 1:1, L4) has been used to replace FM in the Asian seabass (*Lates calcarifer*) diet (Chapter 5). Numerous studies on the use of BSFL in aquafeed have been done. Different amounts of BSFL incorporation have been noted in research (Borgogno *et al*., 2017; Xiao *et al*., 2018; Cardinaletti *et al*., 2019; Mohd-Zulkifli *et al*., 2019), with some stating that BSFL completely replaced FM (Bondari and Sheppard, 1981; Zhou *et al*., 2017; Belghit *et al*., 2019). The use of larvae at different stages gives different results. The use of larvae that are 8 days old gives the best results, which can replace 100% FM in Atlantic salmon. The use of larvae at a very early stage prevented the maturation of BSFL into prepupae, which would have resulted in higher amounts of chitin content which affected fish performance (Belghit *et al*., 2019). All the growth and body indices were not significantly different between treatments (BSFL20, BSFL40 and

BSFL60) and control (BSFL0), except for the feed conversion ratio. The present study suggests that up to 40% of FM replacement, equivalent to 148 g/kg BSFLM, may be included in the Asian seabass diets without negatively affecting fish performance and utilisation of feed or fish product preference by consumers. In many instances, when levels of BSFL inclusion reached more than 50%, negative effects on fish growth parameters were seen (St-Hilaire *et al*., 2007b; Rana *et al*., 2015; Katya *et al*. 2017; Devic *et al*., 2017; Cardinaletti *et al*., 2019; Mohd-Zulkifli *et al*., 2019). This is most likely due to the high chitin content and the inability of fish to utilise insect-based diets as a source of nutrients. This study shows that the nutrient composition of seabass fillets contained high protein but low fat. The high saturated fatty acid (SFA) content of BSFL-fed fish, particularly lauric acid (C12:0), should be noted and in line with previous studies (St-Hilaire *et al*., 2007a; Spranghers *et al*., 2017; Meneguz et al., 2018). Furthermore, the omega-3 content of fish was found to be low in fish-fed BSFL (St-Hilaire *et al*., 2007a). The high levels of SFA found in the larvae reared on mixed feed substrate (MECM and SG) in this study, which ranged from 75 to 88%, influenced the fatty acid (FA) profile of the seabass. However, the presence of this FA should not be interpreted as entirely negative. Lauric acid has a broad spectrum of antimicrobial activity against enveloped viruses, as well as harmful bacteria and protozoa (Ramos *et al*., 2009; Matsue *et al*., 2019), and it is essential for animal growth performance and gut health (Schiavone *et al*., 2018). However, the quality of the BSFL produced could be enriched by changing the fatty acid composition of the BSFL, particularly by manipulating the composition of substrates, such as microalgae, seaweed, and fish offal (Surendra et al. 2020)

The insufficiency of protein in the diet of Asian seabass is most likely due to the overestimation of BSFL protein. Insects, particularly BSF, contain nitrogen from protein and non-protein sources like chitin. In the present study, a conversion factor of 6.25 was used to determine the crude protein content of BSFL (Spranghers *et al*., 2017; Ewald *et al*., 2020). This factor has been considered high due to non-protein nitrogen (NPN) (Janssen *et al*., 2017; Nery *et al*., 2018). It has been reported that the NPN in *Hermetia illucens* (BSFL) is between 16 and 26%, and in Tenebrio

molitor, between 12 and 23%. Separating NPN from protein nitrogen was necessary to get accurate crude protein content and avoid overestimated values using the standard conversion factor. The new conversion factors of 4.76 and 5.60 for whole larvae and insects were more accurate for protein estimation (Janssen *et al*., 2017). The protein value appeared to be more than 24% less than the amount reported using the conversion factor of 6.25 when a new conversion factor of 4.76 was used (Jansen et al., 2017). Utilising the correct nitrogen conversion factor is essential when formulating a diet because it enables feedstuffs to reflect their protein content more accurately (Nery *et al*., 2018). The substantial difference between the actual value of protein and the overestimated value has resulted in the fish receiving a very small amount of protein than it should get. The conversion factor of 6.25 may be useful for various substrates in future research; however, the conversion factor of 4.76 will be more appropriate for the BSFL. Additionally, the chitin presence in BSFL must also measure to prevent this deficiency in aquafeed (Janssen *et al*., 2017).

Insect meals, such as BSFL, are an alternative source of protein that has been reported to have a variety of beneficial effects on both the production and health of animals (Zozo *et al*., 2022). For instance, looking for sustainable and alternative protein sources for animal production in the animal feed industry has become necessary. There are several considerations to commercialise the BSFL as FM replacer. Currently, the price of BSFL remains quite high compared to FM and other animal-based protein sources, creating a critical limitation to evaluating its feasibility as an alternative feed and deterring the insect-rearing sector. Establishing a facility for the rearing of insects, as well as providing a feeding substrate that can produce high-quality larvae and yield, should be prioritised in accordance with the particular environmental conditions. Insects should have a diverse or similar potential market as FM due to their capability to be employed as feed in aquaculture and livestock, also in the pet industry (van Huis, 2013). In addition, the quality of BSFL should be consistent in terms of nutritional composition and palatability comparable to FM. This is important because BSFL was evaluated as a

potential replacement for high proteinaceous feedstuff and must have a protein content that is either not drastically different or comparable, for BSFL to be an effective replacement for FM. The low quality of BSFL has a significant and negative impact on the treatment's effectiveness due to the low content of amino acids and fatty acids in larvae. For the time being, most of the high-quality BSFL FA derived from organic waste could not compete with FM in terms of PUFA content and digestibility, mainly with a higher inclusion of BSFLM in aquafeed is challenging. With the quality of BSFL produced in this study, BSFL can partially replace FM in the Asian seabass diet. It is possible that the BSFLM was suitable for use in omnivorous or herbivorous freshwater fish species, where nutrient requirements are lower than in marine and freshwater carnivorous fish. This would have the additional benefit of lowering the effect of BSFL on fish growth. Carnivorous fish species require high qualities of proteins and lipids to grow (Hasan, 2001). The most important factor in the preservation of BSFL is the continuous supply of feed sources to ensure the sustainability of BSFL in aquafeed. BSFL products must achieve maximal production while preserving nutritional quality for the desired species to be a reliable protein supply. High output and quality can help BSFL products match and outcompete FM and fish oil in the nutritional content, environment, economic and social sustainability (Smetana *et al*., 2019; Fisher *et al*., 2020).

7.2 Limitations

The availability of feeding substrate is one of the primary obstacles to the growth of the BSF industry. The selection of feed substrate for rearing BSFL is crucial for food safety as stipulated by the European Union (EU) legislation (2017/893), which prohibits the use of human and animal excreta, slaughterhouse and rendering-derived products, and food waste to prevent the spread of zoonotic diseases within the production facilities (Espinoso *et al*., 2020). Ethically this Act provides protection for the consumers but the feed resources to produce BSFL thus become limited. This situation constrains the feed resources for the sustainable production of BSFL.

In addition, insects are also allowed to be used as feed ingredients in poultry and pig diets (EU 2021/1372). Selection of suitable organic materials for feeding the larvae should consider the product's safety (Van der FelsKlerx *et al*., 2018) and the community's perspective toward insect-derived foods. This is because the larvae processed as feed ingredients in livestock and aquaculture feeds will ultimately end up as food for human consumption. Moreover, the feed substrates used in the production of BSFL affect their quality, product odour and taste (Wang and Shelomi, 2020). Unfortunately, the lack of specific and consistent regulations regarding the inclusion of insects in fish feed products in Malaysia or ASEAN countries in general is delaying the growth of the insect farming industry. Feeding BSFL with human or animal waste can be inappropriate and controversial to the community as it contradicts health regulations for human consumption. Food and feed safety guidelines are crucial in the insect-rearing industry, which if available, must be adhered to by the insect producers. The safety of feed or food on supermarket shelves is the responsibility of the relevant entrepreneurs or manufacturers.

Direct extrapolation of laboratory scale or one-off studies may not provide valid information for the widespread use of insect-based ingredients in the animal feed processing industry. The protein concentration of larval biomass is lower than FM, whereby the economic value of animal feeds is based on their protein content. The protein rich BSFL biomass observed in this study is anticipated to be less cost-effective than FM. Most studies are conducted on a bench or a lab set-up which could change the progress of BSF in industrial-scale production (Surendra *et al*., 2020). Future applications may be hampered due to the limited availability of data on an industrial scale. An extrapolation of the data may not behave linearly to the laboratory-scale studies. Unlike the traditional methods that use larvae as the starting material, the use of eggs in this study is more in line with industrial practices. The parameters of BSFL lifespan may shift from the laboratory to a mass production set-up (Sorensen *et al*., 2012). Any extrapolation of laboratory-scale data to mass-scale production will profoundly impact BSF output and performance. Any data collection done on a significant scale by an insect producing company

becomes the company's property and a trade secret that is not disclosed to the public in any way. It is challenging to achieve uniformity in the nutrient composition of the larvae because this 'mini-livestock' is significantly impacted by the environment as well as the continually changing nutrient content of the feed substrate (Wang *et al.*, 2017; English *et al.*, 2021; Salam *et al*., 2022; Siddiqui *et al.*, 2022). Changes in the chemical composition and larval growth performance were clearly noticeable in this study because sampling was done at different time intervals throughout larval development on the same feed substrate.

The use of BSFL as a protein replacement for FM looks promising, and additional research may assist in the sustainable growth of the aquaculture sector as the demand for seafood continues to increase. To ensure the long-term sustainability of BSFL resources and maintain a level of competitiveness with FM, a constant feed source of BSFL is highly crucial. It is necessary to perform a dietary manipulation of BSFL to generate high-quality BSFL that is on par with FM in terms of the quality of proteins and fatty acids it contains. Although the production cost of BSFL in the local production scenarios is still relatively high due to the requirement of a huge quantity of feed material, most of the BSFL industries operating in Malaysia are self-funded (Raman *et al*., 2022). The large volume and big-scale operation of BSFL rearing in other countries decreased the price compared to the BSFL in Malaysia. In addition, the availability of feed resources that are utilised as a substrate for rearing the larvae needs to be ensured in order to guarantee that BSFL will continue to function as an alternative source of protein in fish feed. For example, utilising the current best practices for food waste segregation is one of the more tangible ways that food waste can be managed to ensure a sustainable and continuous big-volume production of BSFL. Maintaining a consistent nutritional quality of BSFL is a real challenge in the BSFL industry. More efforts must be provided to expand the BSFL industry to reach the same level of FM popularity as a feed ingredient. This is necessary so that the operators and breeders of insects as animal feed have a choice and can be assured that this alternative protein source is an ideal replacement for FM.

7.3 Conclusions

The aquaculture sector, which contributes to animal protein sources, fatty acids, and micronutrients, needs to increase the production of marine resources to ensure the sustainability of the industry. However, over the past few decades, the increase in the price of the main ingredients in aquafeed, which are FM and soybean meal (SBM), has put a lot of pressure on the industry to grow. The search for alternative ingredients, namely insects, has given great hope to the sustainability of aquaculture due to its high protein content and amino acid levels equivalent to FM and SBM. The black soldier fly larvae (BSFL) are one of the most likely insects to be used as an alternative source of protein in aquafeed. BSFL requires extensive research because it has the potential to replace traditional protein sources. It has recently been demonstrated as a potential alternative protein source for several fish species, albeit the results are highly variable depending on the study's circumstances and the source of the BSFL product employed. Understanding the rearing and processing of BSFL to generate a protein-rich meal is crucial since these processes greatly impact the nutritional quality of BSFL meal. In addition to overall protein content, it is essential to consider the amino acid profile and the level of essential fatty acids present in the novel food. Although BSFL can convert a wide range of organic materials, including manures, food waste, agro-industrial by-products and leaves, a suitable feed substrate should be selected to yield BSFL with desirable nutritional content based on the nutritional requirements of the fish species. The conversion of the six feed substrates, SG, MO, SBW, WP, RB, and MECM, by BSFL, produced biomass with high protein and fat contents, with some amino acids that were comparable to or higher than those in FM and SBW. An optimised feed substrate consisting of a combination of SG and MECM at a ratio of 1:1 (50% SG and 50% MECM), harvested at day 19, was used as a substitute for FM. Substitution of FM using BSFL in the diet of Asian seabass at 20%, 40% and 60% was a possibility, as suggested by the superior fish performance observed in the present study. The inclusion of BSFL meal (BSFLM) in the diet did not influence the growth performance, whole-body of nutritional composition, serum biochemical

parameters, or sensory tests of Asian seabass. The feed conversion ratio was found to be significantly different between treatments, with the BSFL20 treatment having the lowest ratio. According to the findings in the present study, up to 40% of BSFLM, equivalent to 148 g/kg, may be incorporated into Asian seabass diets without adversely affecting fish performance or utilisation of feed. The levels of BSFL inclusion in the Asian seabass diet were determined by partial replacement of up to 40% FM. Similar research might be conducted on other cultured carnivorous species to develop a novel and sustainable source of nutrients for future aquafeed formulations.

7.4 Future Research

Future research on the nutritional content of BSFL should examine the relationships between the feeding substrates and the abiotic factors surrounding the rearing process, such as temperature, moisture, oxygen, and pH. Additionally, it is important to investigate the effects of larvae and feed densities on the bioconversion efficiency of BSFL, particularly in an upscaled or industrial production system. A better knowledge of the interaction of these factors with the nutritional quality of BSFL would enable the optimisation of mass rearing of BSFL for incorporation into fish feed.

Setting up sanitary processes for bio-waste and controlling diseases, pesticides, and heavy metals are crucial to achieving a safe insect meal as a dietary feed element. It is important to ensure that insects are produced in a healthy environment and that the sanitary quality of the substrate is taken into account in order to remain free from pathogenic. The importance of investigating heavy metals and mycotoxins for the safety of insect products should be emphasised. Insects may be exposed to chemical substances, such as toxins and heavy metals, through contaminated food or water (Marone, 2016). BSFL could accumulate a variety of heavy metals from the feed. The accumulation of mercury, lead, and arsenic from seaweed by BSFL increased linearly, increasing seaweed concentration in the feed from 10% to 100%. (Biancarosa *et al*., 2018). Furthermore, at the beginning of BSFL farming, the substrate is susceptible to fungal infection. Mycotoxins, which are metabolic by-products of fungi such as *penicillium*, *fusarium*, and *aspergillus*, are harmful in different ways to humans and animals, but mycotoxins not accumulated in BSFL (Purschke *et al*., 2017). However, to ensure the safety of the food and feed, the BSFL should be tested for the presence of contaminants before any commercial-scale BSFL rearing is done. If more information about BSFL's chemical safety becomes available, legislation could be updated to reflect these discoveries, and organic waste streams that appear to be safe but are currently underutilised could be upgraded and used as insect feed.

APPENDICES

APPENDIX 1

Preparation of Neutral Detergent Solution (NDS)

18.61 g of EDTA (Disodium ethylenediaminetetraacetate) and 6.18 g of Sodium Borate decahydrate ($Na_2B4O_7.10H_2O$) were added in a beaker containing 500 mL of distilled water and heated until dissolved. After that, 30 g of Sodium Lauryl Sulphate ($C_{12}H_{25}OSO_3Na$), 10 ml of Triethylene glycol ($C_6H_{14}O_4$) and 4.56 g of Disodium Hydrogenphosphate (Na_2HPO_4) was added to the beaker. Water was then made up to 1 L and heated until all chemicals dissolved. The pH was adjusted between 6.95-7.05.

APPENDIX 2

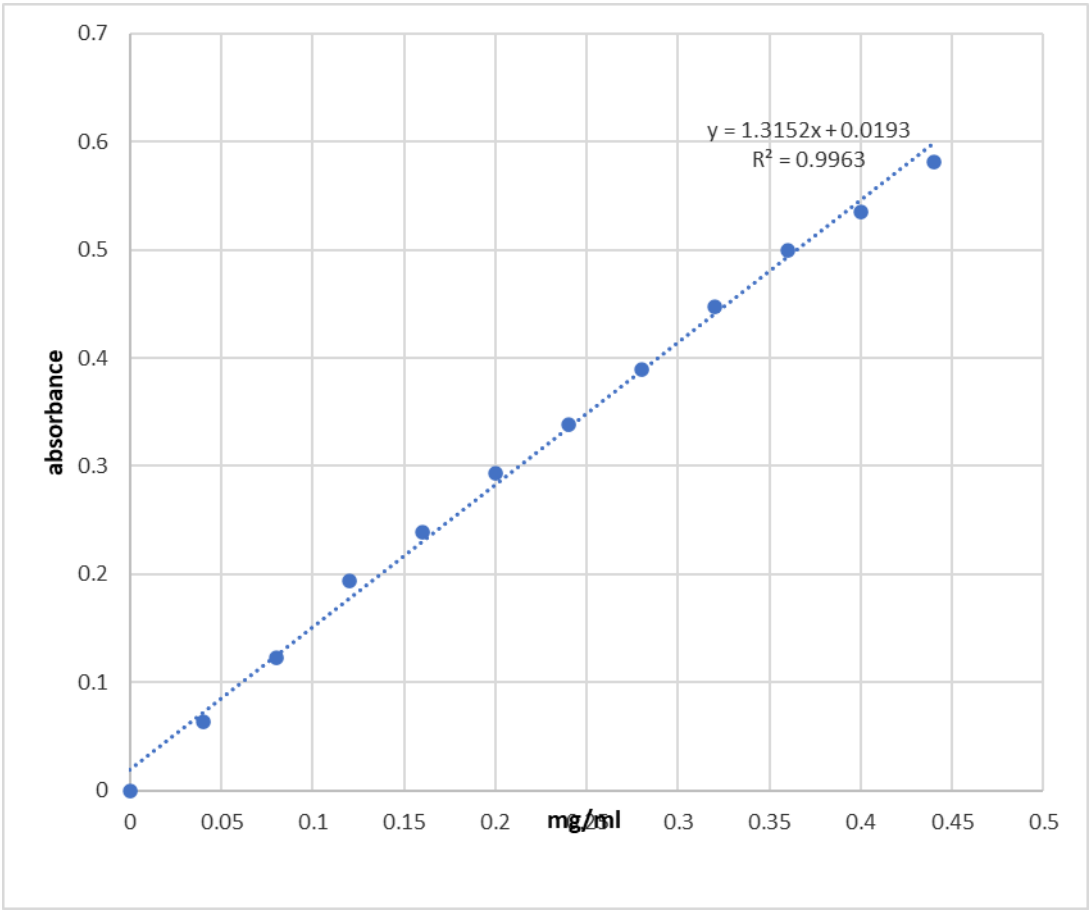
Preparation of Acid Detergent Solution (ADS)

0.5 g Sulfuric acid (H_2SO_4) with CTAB

49.04 g of concentrate H_2SO_4 was added into 1 L volumetric flask containing 400 ml distilled water. 20 g of CTAB was added and the volume was made up with distilled until 1 L.

APPENDIX 3

Standard curve for measuring chromium.



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