Effect of different dietary amino acid profile on growth and chemical composition of Black soldier fly (*Hermetia illucens* L.) larvae

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Abstract

With increased focus on food security worldwide, including alternative proteins in manufactured animal feed and human diets is an increasingly popular topic among diverse groups. Black soldier fly (BSF) (*Hermetia illucens* L.) larvae have gained significant attention in recent decades. The larvae provide good-quality proteins; however, the rearing procedures and rearing substrate for this species vary considerably. It is essential because the body composition of these insects is highly dependent on the rearing substrate in terms of all essential nutrients and other substances, such as heavy metal residue. The current study looks at the dietary amino acid influence on the body composition of BSF larvae. This research aimed to find out whether the BSF larvae require a list of essential amino acids as the monogastric animals do and whether they need an ideal amino acid profile for their efficient production. Answering these questions can shine a light on the mass production efficiency of this species of insect and aid the production of BSF with a relatively constant nutritional profile so they can be reliably incorporated as feed ingredients.

Earlier literature indicates that the growth of BSF larvae is affected by ambient conditions such as temperature and culturing substrates. The chemical composition of the substrates is directly related to the content of the larvae, and amino acid requirements, being the foundation of the proteins, are important to understand. Two distinct diets were formulated and presented in two treatments in the current study. Ten replicates of 50-week-old larvae in each treatment were established and reared for 14 days. The larvae and their feeding substrates were then sent for chemical, including amino acid, analysis. My findings suggest that BSF larvae can synthesise a relatively stable amino acid profile regardless of the amino acid composition of the diet. However, for optimal growth performance, balanced diets are essential. There are only a few amino acids that are most likely essential for BSF larvae, namely valine, leucine, lysine, and taurine.

Acknowledgment

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Sincerely,

Marryana

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Chapter 1. General introduction

With increasing protein consumption worldwide, it becomes challenging to meet traditional farming requirements on land and in water (Sogari et al., 2019). de Souza-Vilela et al. (2019) stated that the forecast increase in meat consumption from 2019 to 2050 is approximately 40%. Furthermore, over 30% of the land used for growing crops is currently producing livestock and poultry feed to meet the meat demand. With the increasing human population, urbanisation, and diet shift towards animal protein due to economic development, the current food supply, especially protein, is at risk of shortage in the near future (Fukase & Martin, 2020).

Protein is essential for tissue accumulation and replacement in plants, animals, microbes, and for enzymes, as well as for maintaining the functionality of the body . Amino acids are the smallest building units for proteins; amino acids are the building blocks of proteins tied together by amid bound, also known as polypeptide bounds (Lopez & Mohiuddin, 2023). Animal protein is usually considered high quality due to its similarity to human amino acid profile and good digestibility. Animal sources of calories and protein are primarily contributed by meat, milk, and eggs. There are advantages of conventional animal products, such as essential macro-micronutrient content and income generation at both household and national levels. However, there are also disadvantages associated with conventional farming of production animals, especially in contrast to insects. The feed in the production animal's diet may contain human food sources, such as soybean meal in poultry, which eventually competes for food security with humans. Moreover, warm-blooded production animals are less efficient for converting feed into human-edible food. In addition, the concentration of protozoa and archaea in the rumen of major meat production ruminant animals produces a significant amount of greenhouse gases, mainly methane, contributing to global warming.

Since the 1970s, insects have been proposed as an alternative feed for meat-producing animals to increase food production efficiency (Finke, 2002; Sogari et al., 2019). Since 2018, the European Commission has promoted the development of alternative feed ingredients for production animals for self-sufficiency in protein demand and ultimately to relax the growth in demand for feed material that competes directly with human food security (IPIFF, 2020).

Several studies suggested that insects have good nutritive value for production animals (Dabbou et al., 2018; de Souza-Vilela et al., 2019; Khusro et al., 2012; Sogari et al., 2019) Khusro et al. (2012) also confirmed that the insect can be fed to broiler chickens as safely as the conventional feeds. This levels off the demand for soybean meal in broiler chicken production and lowers the overall commercial farming cost. Furthermore, because of the protein-rich nature of selected insect species, Khusro and colleagues (2012) also recommended that the chosen insects could provide reliable and sustainable high-quality protein for broilers with an appropriate breeding method. Furthermore, if the selected insects are fed organic waste, they can contribute to the agriculture industries other than poultry due to the high efficiency of their feed conversion. In this instance, the insect feed for production animals creates a positive food chain, contributing to excellent sustainability (Khusro et al., 2012).

Despite around 2,000 species of insects being consumed by Asian, African, and Latin American populations historically, it was not until 1975 that the insects were suggested to have value in contributing towards world food security (Chakravorty et al., 2016; Ghosh et al., 2017; Rumpold & Schlüter, 2015). Many edible insects contain high levels of crude protein and many essential amino acids for humans, plus insects have high levels of polyunsaturated fatty acids (up to 70%), and some insect species also contain high levels of vitamins and minerals (Belluco et al., 2013; Kouřimská & Adámková, 2016).

In addition to their nutritive values, including insects in the human diet could be a positive solution to releasing resource competition pressure on animal production for human use. Insects require little space to farm (Lalander et al., 2019). They can live on organic waste and have a high feed conversion ratio, indicating a good potential for bioconversion (Halloran et al., 2016; Oonincx & de Boer, 2012). Halloran et al. (2016) also suggested that carbon dioxide production in insects is highly species-dependent, and methane production also depends on the external environment, which indicates that there is a possibility to manipulate the farming environment in reducing greenhouse gas emissions by focusing on insects farming for the alternative food source.

Vantomme and Halloran (2013) also suggested that farming insects for animal feed and human consumption provides livelihood and social benefits. They argue that insects are easily harvested from the wild, and technical and capital investments are minimal to start rearing. Due to the abundance in the wild, the poorest human populations could directly benefit from insects as food and cash conversion. Furthermore, insect by-products could provide life-changing opportunities to increase economic well-being among people experiencing poverty.

Using insects for animal feed and human consumption could benefit global sustainability in the production of animal farming and human food security in many developing countries. Insects play a significant role in reducing pollutants and increasing bioconversions. Using insects for feed and food is ecologically friendly and promotes sustainability. On top of that, insect farming can increase the livelihood among people with low incomes and ultimately achieve social benefits. The current study uses black soldier fly (*Hermetia illucens* L.) larvae as the subject to closely study the factors that influence their amino acid profile associated with the rearing substrate. Understanding the amino acid profile in black soldier fly larvae and the influencing factors in the substrate in terms of amino acid is crucial in the aspects of rearing good quality feed, increasing stability of production, and improving rearing techniques in the commercial environment as it was denoted an enormous range for protein content in the black solider fly larvae (Lu et al., 2022).

Chapter 2. Literature review

2.1 The use of black soldier fly and its larvae

The world human population is predicted to exceed 9.5 billion by 2050 (Chia et al., 2018b) and 10.9 billion by 2100 (Maja & Ayano, 2021). The rapid growth in the human population has put tremendous pressure on the conventional food industries (Maja & Ayano, 2021). Conventional horticulture and agriculture have posed multiple challenges to the local, regional, and global natural resources and social welfare. Many studies (Broeckx et al., 2021; Chia et al., 2020; Singh & Kumari, 2019; Tanga et al., 2021) have argued the urgent need for an alternative protein and energy source to replace conventional protein ingredients in animal feed, such as fishmeal and soybean meal (Chia et al., 2020). Research for alternative feed ingredients can not only assure the food supply but also come as a potential solution to environmental issues (Broeckx et al., 2021).

With the growing population, croplands, and livestock numbers, organic waste management has become a key challenge for many developing countries. Chia et al. (2018b) illustrated a case study in Nairobi, the capital of Kenya, where organic waste weight was 62% of the total waste that the city generates daily. Most of these organic wastes are of low feeding value to livestock animals. Buratti et al. (2015) pointed out that organic wastes cause an environmental burden, not only to developing countries but also to developed countries. Cho et al (2020) sum up that organic waste, especially food waste due to population growth, threatens the ecosystem and human health. Furthermore, Chia et al (2018b) also pointed out the importance of recovering nutrients from organic wastes, as it is related to many ecological and social issues.

In the wild, many animals, including fish and birds, consume insects as part of their regular diet (Sogari et al., 2019). Insects have gained increased interest for their outstanding sustainability and efficiency in biomass conversion (Makkar, 2018; Sogari et al., 2019). Black Soldier Fly (BSF) (*Hermetia illucens,* Diptera: Stratiomyidae) has been extensively studied as it is a non-pest species with excellent waste conversion abilities (Broeckx et al., 2021).

Bonelli et al. (2019) suggested that the conformation and pH levels in three different gut compartments of the black soldier fly to make the insect well adapted to live on the secondary metabolites, including organic wastes from animal and plant origins. Gao et al. (2019) suggested that black soldier flies are potentially good candidates for maise straw pre-treatment for biofertiliser. Zhang et al. (2022) pointed out that black soldier fly larvae can also treat cyanobacteria, one of the organic pollutants, due to their heavy feed ingestion and high bioconversion efficiency to utilise those as nutrient sources for biomass accumulation. Elieh-Ali-Komi & Hamblin (2016) and De Smet et al. (2018) pointed out that chitin from black soldier fly larvae is another important bioproduct that can be utilised in many industries.

BSF can survive in a wide range of substrates; however, because they are converting the waste biomasses to their body mass, their nutritional values and growth parameters mainly depend on the feed substrate they have been given (Broeckx et al., 2021; Shumo et al., 2019b; Tschirner & Simon, 2015). To utilise BSFs in mass production for animal feed, nutritional values and their growth indicators should be examined to provide thorough information (Chia et al., 2018b; Chia et al., 2020; Cho et al., 2020).

2.2 Black soldier fly life cycle

The black soldier fly is a synanthropic, saprophagous species (Hoc et al., 2019; Marshall et al., 2015). It is widespread worldwide in warm zoogeographic regions (Marshall et al., 2015). Black soldier fly larvae live and feed in moist decaying organic matter in the wild conditions (Diclaro II & Kaufman, 2009); while the adult flies are believed to only drink water and are free from transmitting diseases (Abd El-Hack et al., 2020). The insect's larva has chewing mouthparts, whereas the adult fly has non-functional, sponge-liked mouthparts (Lievens et al., 2021). These sponge-like mouthparts allow the adult flies to drink water but no other feed forms (Tomberlin et al., 2002).

Black soldier fly has five stages, from an egg to an adult fly, as illustrated in Figure 1. The eggs hatch approximately four days after oviposition. The larval stage lasts approximately 13-18 days, with ecdysis between instars. Five instars are observed before the larva becomes a pre-pupa (De Smet et al., 2018; Soetemans et al., 2020). At the pre-pupa stage, it changes colour from light to dark brown and eventually turns black. During this phase, the insect empties its digestive tract and migrates to a cool, dry place to pupate. Pupation can vary significantly in length, ranging from 10 days to months, depending on ambient conditions. After emerging, the adult flies live for 5 to 8 days to mate and oviposit (Soetemans et al., 2020). Although the female flies can lay eggs without mating (Liu et al., 2020), these eggs will not be viable.

2.3 Factors influencing BSF larval growth and survival

2.3.1 Temperature

Cold-blooded insects are highly dependent on the temperature (Régnière et al., 2012). Ambient temperature affects the survival, rate of development of an insect, as well as fecundity. The range of temperatures an insect can survive is much broader than the temperature to conduct normal activities (Ratte, 1985). It is generally recognised that the survival rate is much lower at both ends of the temperature scale, and survival is the highest at its optimal temperature under the constant temperature regime. Development rate, as well as fecundity rate, are following the same pattern. Under the fluctuating temperature regime, development at each stage varies depending on the temperature changes (Ludwig, 1928).

Chia et al. (2018a) found that egg eclosion significantly differed between 15°C and 35°C and occurred after 14 and 2.6 days, respectively. Eggs incubated at 10°C and 42°C completely ceased to grow. Fazli Qomi et al. (2021) showed that the Hatching percentage for BSF was the best at 30°C (80%), where it was only 50% at 20°C. Furthermore, Shumo et al. (2019a) found that the time required for larvae to reach the prepupal stage decreased with temperature rise and was least at 30°C. Fazli Qomi et al. (2021) discovered similar patterns and recorded that BSF larvae growth was lowest at 20 °C and highest at 30°C. Prepupal weight shows a peak between 25°C to 30°C (Shumo et al., 2019a; Tomberlin et al., 2009). In addition, the prepupal survival rate was highest at 25°C and 30°C, lowest at 20°C and 35°C (Fazli Qomi et al., 2021) and none of the larvae developed to the prepupal stage at 40°C et al., 2018a).

Figure 1. Life cycle of black soldier fly (De Smet et al. 2018).

Pupal development was also closely associated with ambient temperature (Tomberlin et al., 2009). However, it was not closely associated with sex nor the sex-temperature interaction. Chia et al. (2018a) recorded that the temperature affected pupal development significantly. Shumo et al. (2019a) found that pupal development was shortest at 30°C and longest at 15°C. The development time was gradually decreased with a constant increase in temperature and increased once the temperature was over 30°C. Fazli Qomi et al. (2021) found that the optimal pupal growth rate was obtained at a slightly higher temperature (35°C). This might be due to the diet difference that the larvae were reared on (chicken feed in Fazli Qomi et al. (2021); brewer spent grain in Shumo et al. (2019b). According to a study by Fazli Qomi et al. (2021), the survival rate for BSF pupal was highest at 30°C (77%) and lowest at 20°C (61%). The authors also found that increasing temperature in the lower domain from 20°C to 25°C increased the survival rate to 67%, but increasing the temperature in the upper domain from 30°C to 35°C increased the mortality rate, and only 65% of pupae survived.

Tomberlin et al. (2009) noted that at 27°C, 83.2-91.8% of larvae survived to adulthood, while 74.2-96.7% survived to adulthood at 30°C. At a slightly lower temperature, the minimal survival rate was higher. However, the overall survival was better at 30°C. Although their findings suggest that the temperature effect is insignificant, the adults reared at 27°C lived longer than those reared at 30°C. The trade-off is that the larval development is slightly lower at 27°C. Having said that, temperature can significantly affect longevity in adult BSF regardless of their sex (Chia et al., 2018a). They found that adult BSF performs better in the intermediate temperature range than at the upper limits.

Furthermore, pre-oviposition in female adult BSF is affected by temperature greatly. At 20°C, the period was as long as 16 days, but only five days when the temperature rises to 35°C. The best fecundity rate was recorded at 30°C, and both lower (15°C) and higher (37°C) temperatures result in a low fecundity rate (Chia et al., 2018a). Shumo et al. (2019a) suggest that rearing substrates and temperature affect adult longevity and fecundity. The higher the temperature, the lower the longevity was, regardless of the diet. The maximum longevity was achieved when adults were kept at 15°C. With an increase in temperature, longevity is reduced, and the reduction rate reached almost half at 35°C compared with those at 15°C. Oviposition is also closely associated with temperature, increasing with temperature to a maximum of 30°C. When the temperature reached 35°C, fewer eggs were laid by females (27-30% less depending on the rearing substrate)(Chia et al., 2018a). Fazli Qomi et al. (2021) obtained similar results – the higher the temperature, the shorter the lifespan in adult BSF. However, fecundity was not considered by these authors.

In conclusion, temperature is a crucial environmental factor for survival in all stages of the BSF. As mentioned above, many other factors may have an interactional effect on BSF development in different stages. It is well known that insects are highly dependent on ambient temperature, as the background chemical reactions and metabolism are also highly dependent on the temperature. The critical temperature range changes with the change in the life stages of the insects; in such cases, larvae prefer a higher ambient temperature than adults and different temperatures are required for different life activities (oviposition at 30°C, while adults live longer at the cooler condition). Differences in the studies are due to the experiment design, colony source and adaptation of the experimental conditions.

2.3.2 Feeding substrate and its effect

For industrial use of black soldier flies, survival rate, larval weight, development time, bio-conversion ratio, and nutritive values are important parameters to monitor for production efficiency. Review results have shown that survival rate is closely associated with substrate composition at a given rearing density. Different composition of substrate given to the BSF results in different insect performance. By identifying the optimal feed composition, mass production of BSFs for animal feed could elevate the production performance. Many studies have investigated the feeding substrate in relation to the growth performance of BSFL.

2.3.2.1 On survival

Figure 2 illustrates the study conducted by Broeckx and colleagues (2021. The results show an average of 86% overall survival rate. Fruit puree offered the best survival rate, while tomato leaves yielded none, and the grain middling 1 offered a moderate survival rate under the same ambient conditions (Broeckx et al., 2021).

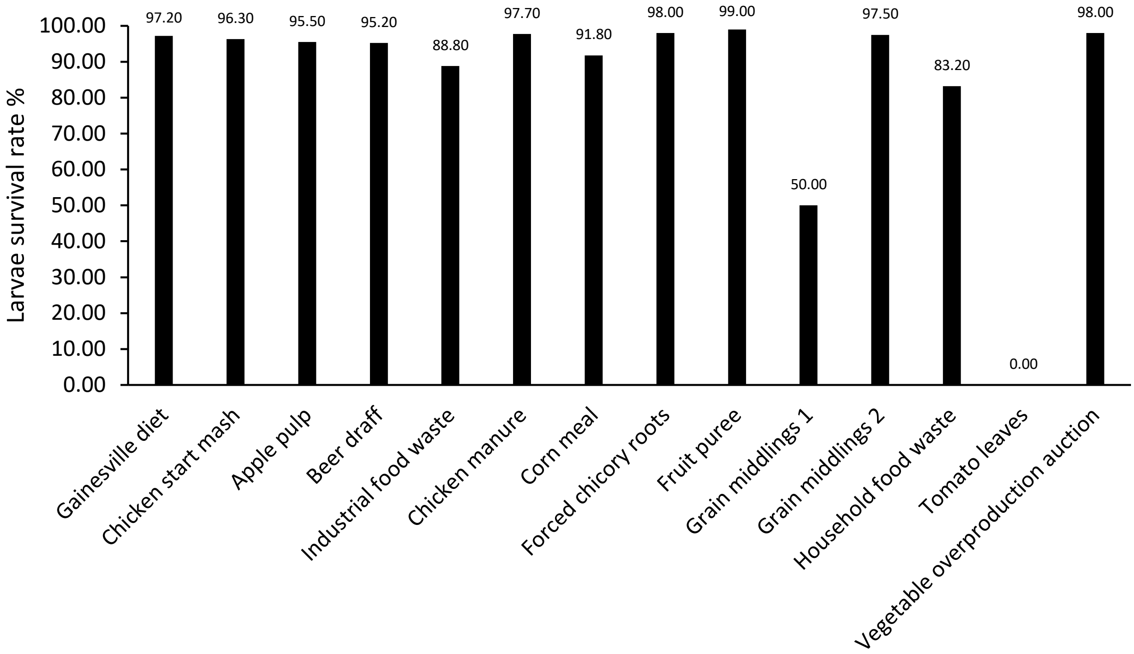

Figure 2. Black Soldier Fly larvae survival rate reared on different substrates (Broeckx et al., 2021).

Ewald et al. (2020) showed a greater spectrum of survival rates. In their study, food waste offered a similar survival rate to Broeckx et al. (2021) at 89.1%. Figure 3 shows the effect of substrates on survival in one of the two trials by Ewald et al. (2020); fresh mussels show the best survival rate, which is similar in performance to food waste. Romano et al. (2022) studied sweet potato, spent coffee, and dough fed to BSF larvae. Their result shows the highest survival achieved with spent coffee and the lowest survival rate on the dough (Figure 4).

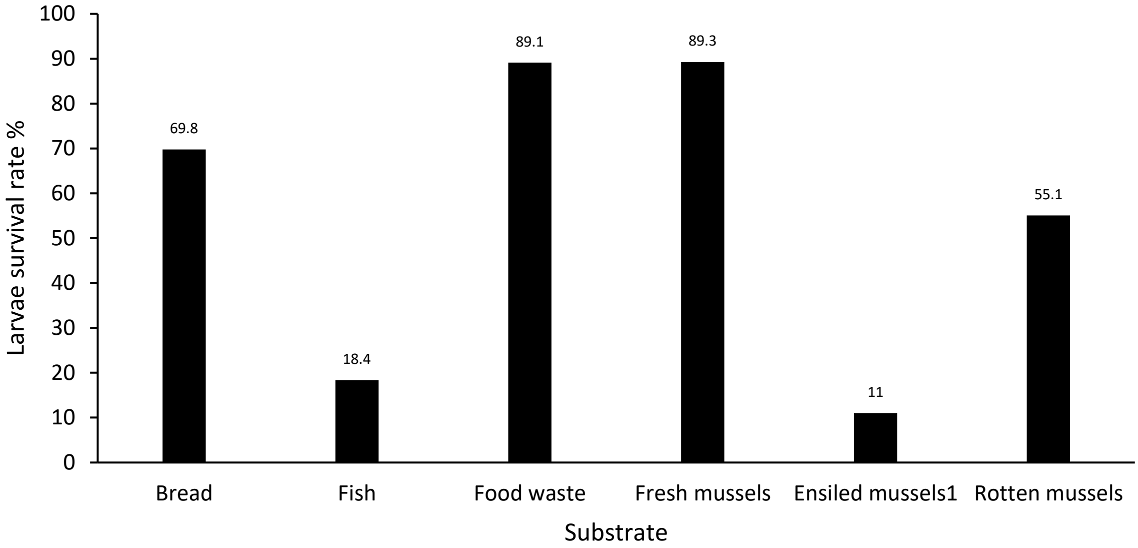

Figure 3. Larvae survival rate on different categories of substrates (Ewald et al., 2020).

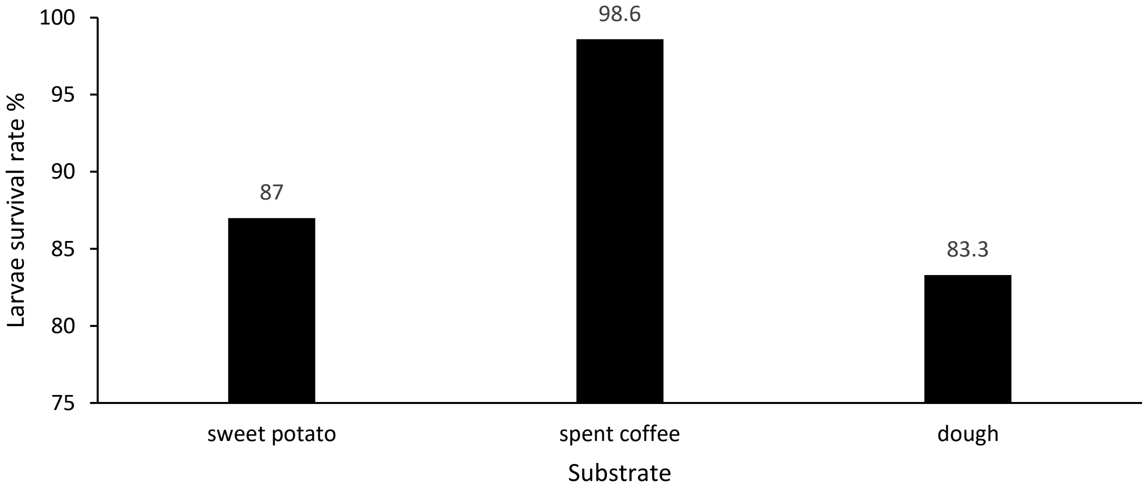

Figure 4. Larvae survival rate on different substrate (Romano et al., 2022).

2.3.2.2 On larval weight

Larval weight is another critical parameter in the mass production of BSF. The purpose of rearing black soldier fly larvae is for the yield, or the larvae harvest weight, similar to any other primary production procedure; the higher the larvae weight, the better the production yield, hence a more efficient production output given a constant input. As shown in Table 1, a wide spectrum of larval weights has been recorded. These differences are due to varied handling procedures and rearing conditions, and the most significant is the feeding substrate. Although black soldier flies can survive and flourish in a wide range of ambient environments, the optimal feeding substrate and ambient conditions can offer advantages in the final biomass yield.

Table 1. Summary of larval weight affected by different substrates from various authors.

Substrate	Larval age (days)	Fresh larval weight (mg/ind.)	Author
Gainesville diet	15	83.8±13.8	Broeckx et al. (2021)
Chicken start mash	14	148.4±21.8	
Apple pulp	15	38.3±4.4	
Beer draff	14	130.9±19.6	
Industrial food waste	16	176.4±15.3	
Chicken manure	15	134.9±11.8	
Corn meal	16	110.4+13.2	
Forced chicory roots	15	46.7±4.9	
Fruit puree	16	80.1±8.0	
Grain middling 1	16	98.5±26.3	
Grain middling 2	15	78.0±12.5	
Household food waste	15	65.3±13.9	
Tomato leaves	8	3.1±0.0	
Vegetable Overproduction auction	14	88.1±5.8	
Bread	19	137±7	Ewald et al. (2020)
Fish	19	89±18	
Food waste	19	191±19	
Fresh mussels	19	235±15	
Ensiled mussels*	19	25	
Rotten mussels	19	106±29	
Bread and mussels (10%)	19	133±39	
Bread and mussels (20%)	19	181±10	
Bread and mussels (30%)	19	168±2	
Bread and mussels (40%)	19	138±21	
Bread and mussels (50%)	19	131±20	
Chicken feed	prepupae	219.8±7.8	Spranghers et al. (2017)
Digestate	prepupae	90.8±3.6	
Vegetable waste	prepupae	140.3±4.4	
Restaurant waste	prepupae	154.1±5.1	

Substrate	Life stage	Fresh weight (g/ind.)	Author
Balanced	prepupae	290.4±7.2	Tschirner & Simon (2015)
Protein rich	prepupae	270.7±14.28	
Fibre rich	prepupae	34.8±4.48	
Chicken feed	prepupae	0.30±0.29	Adebayo et al. (2021)
Brewery waste	prepupae	0.28±0.00	
Food remains	prepupae	0.2±0.24	
Fruit waste	prepupae	0.12±0.02	

*Larval weight was estimated on a small sample (n<10 larvae) due to poor growth.

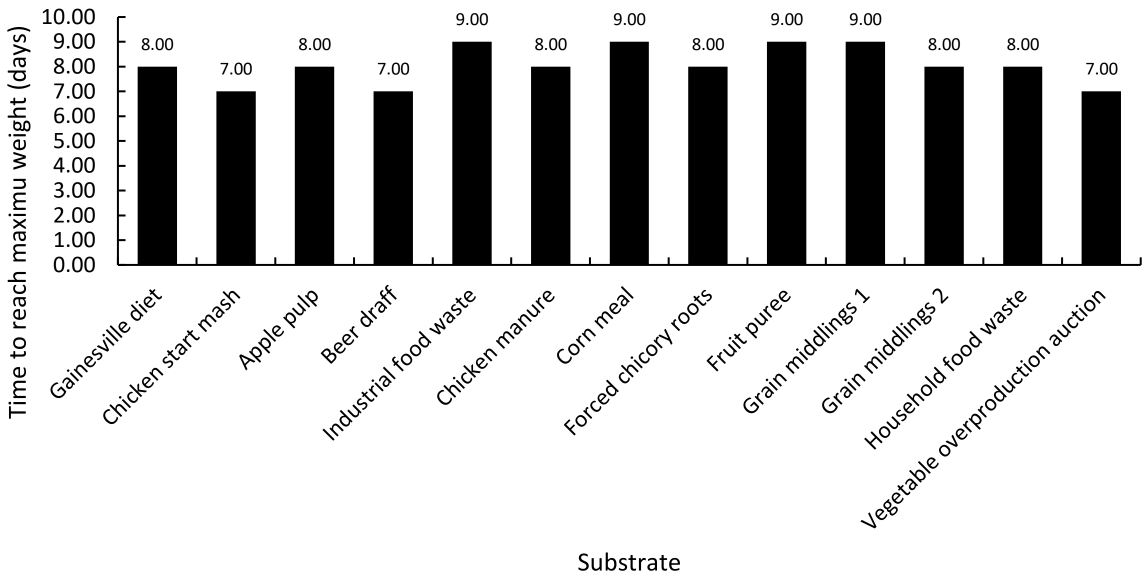

Figure 5. Growth time of BSF larvae on various substrates (Broeckx et al., 2021).

2.3.2.3 On development time

In Broeckx et al. (2021), larvae only survived one day in tomato leaves. Hence, this data is discarded in the current review as it does not have a significant meaning in the study. The remaining results are shown in Figure 5, which is the time needed to reach the maximum weight for the larvae.

Chia et al. (2018b) show that the substrates with different supplements result in varying growth times. Larvae, pre-pupal and total development time were affected by the diet supplied to the larvae, while the pupal development time was not affected (Figure 6). The longest time (42.6 days) needed to reach the pupal stage was by Nguyen et al. (2013) with larvae fed with fish, and the shortest day was 12.3 days, as recorded by Spranghers et al. (2017). Data from Nguyen et al. (2013) is shown in Figure 7.

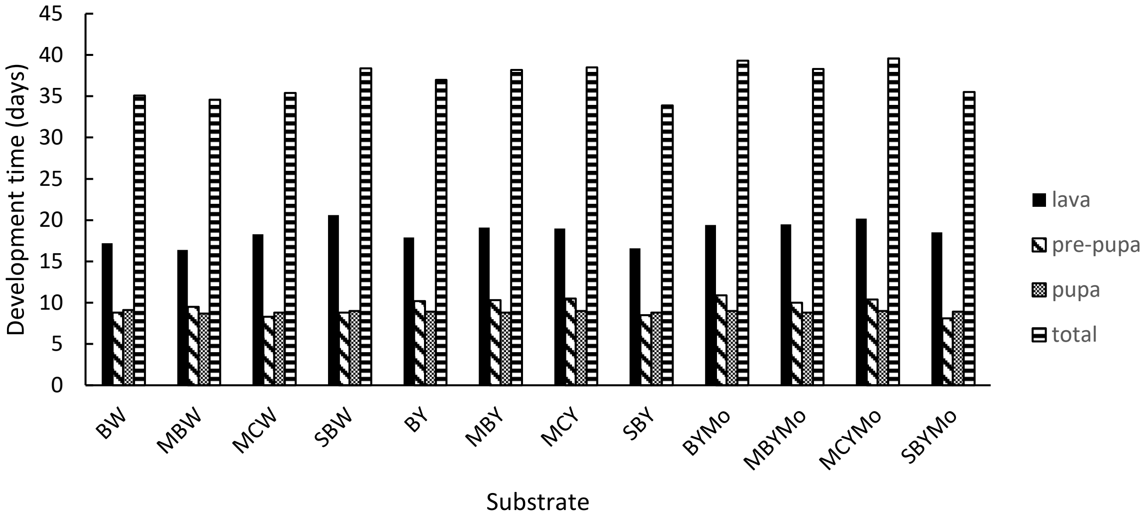

Figure 6. Effect of substrate composition on development time required for BSF stages before adult. BW=Barley with water; MBW=Malted barley with water; MCW=Malted corn-starch with water; SBW=Sorghum and barley with water; BY=Barley with brewer's yeast; MBY=Malted barley with brewer's yeast; MCY=Malted corn-starch with brewer's yeast; SBY=Sorghum and barley with brewer's yeast; BYMo=Barley with brewer's yeast and molasses; MBYMo=Malted barley with brewer's yeast and molasses; MCYMo=Malted corn-starch with brewer's yeast and molasses and SBYMo=Sorghum and barley with molasses (Chia et al., 2018b).

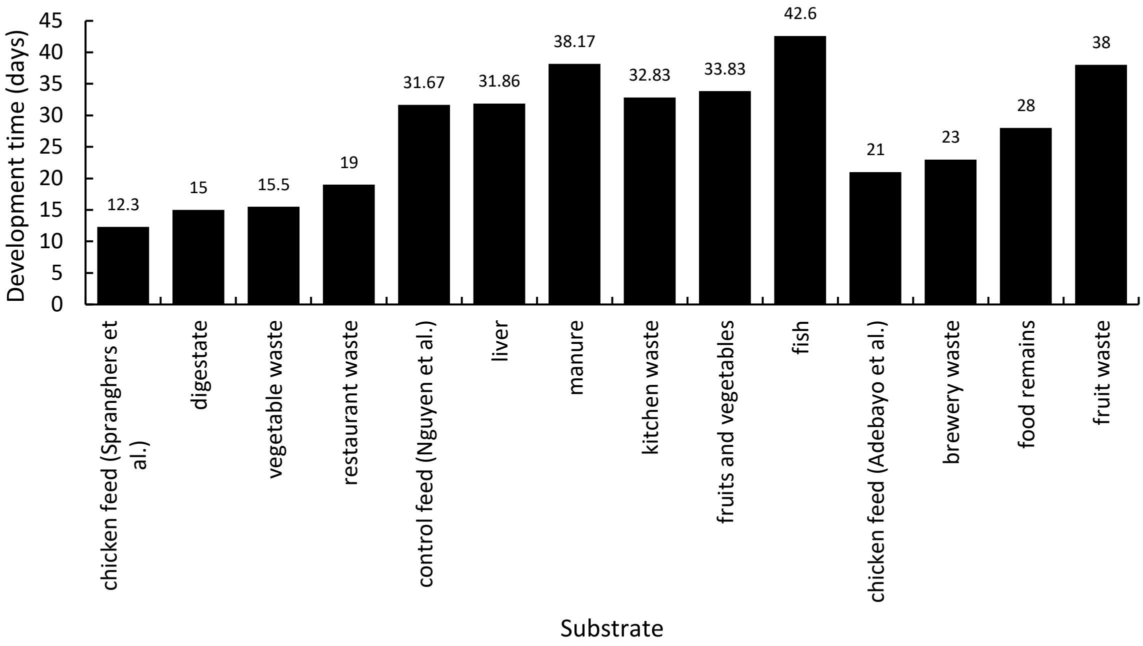

Figure 7. Summary of BSF larvae development time to reach pupal in days on different rearing substrates (Nguyen et al., 2013).

2.3.2.4 On bioconversion efficiency

Some studies have compared bio-conversion ratios between different substrates (Figure 8). Ewald et al. (2020) show a bio-conversion ratio of less than 1% for fermented mussels, but this was estimated in less than ten larvae due to poor growth. Tomato leaves have shown the same result; hence, these substrates are discarded in the present study. The highest conversion ratio is 55.8%, was in Romano et al. (2022) for dough. The majority of substrates show a bio-conversion ratio of less than 20% (Romano et al., 2022), while industrial food waste (Broeckx et al., 2021), general food waste (Ewald et al., 2020), sweet potato, spent coffee and dough (Romano et al., 2022) are above the average.

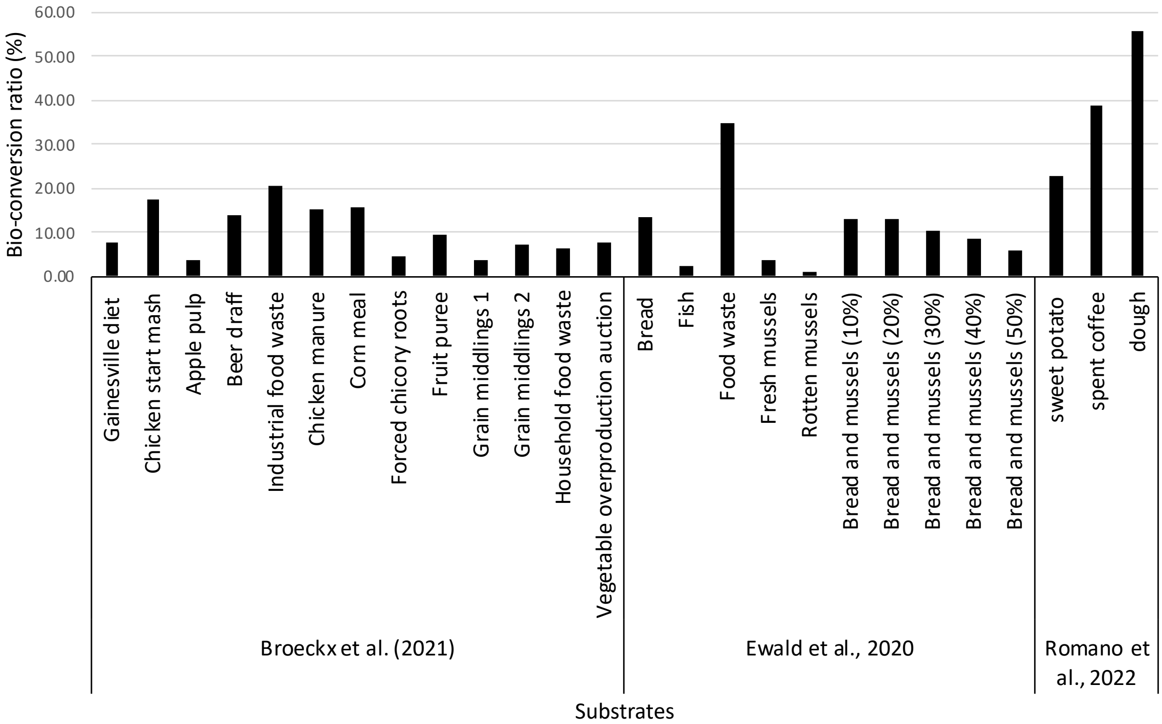

Figure 8. Substrate effects on bio-conversion ratio percentage in BSF larvae.

2.3.3 Chemical compositions of substrate and it influence on Black Soldier Fly Larvae

2.3.3.1 Chemical composition of substrate and it influence on larvae growth

Figure 9 shows the results of a trial conducted by Ewald et al. (2020) which looked at the fatty acid influences, in which EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) were closely monitored; 10% mussels in the diet with bread contributed to the best larval survival rate.

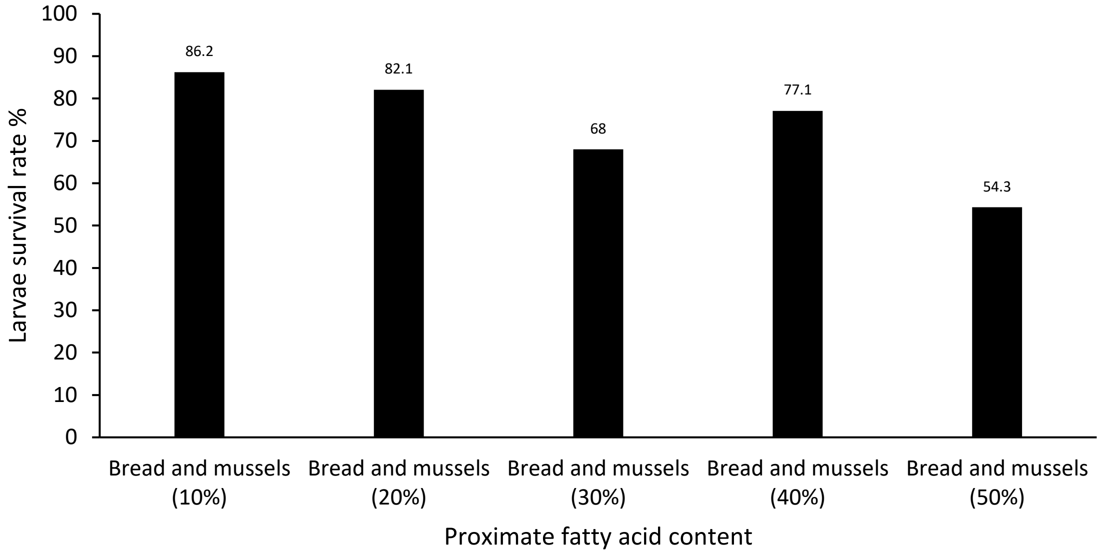

Figure 9. Influence of fatty acid content on larvae survival (Ewald et al., 2020).

Furthermore, Tschirner and Simon (2015) looked at the relationship between the properties of the ration and the survival rate of the larvae. Their study examined three different rations, in which the control diet (nutritionally balanced), protein-rich ration and fibre-rich ration showed varied survival rates (Figure 10).

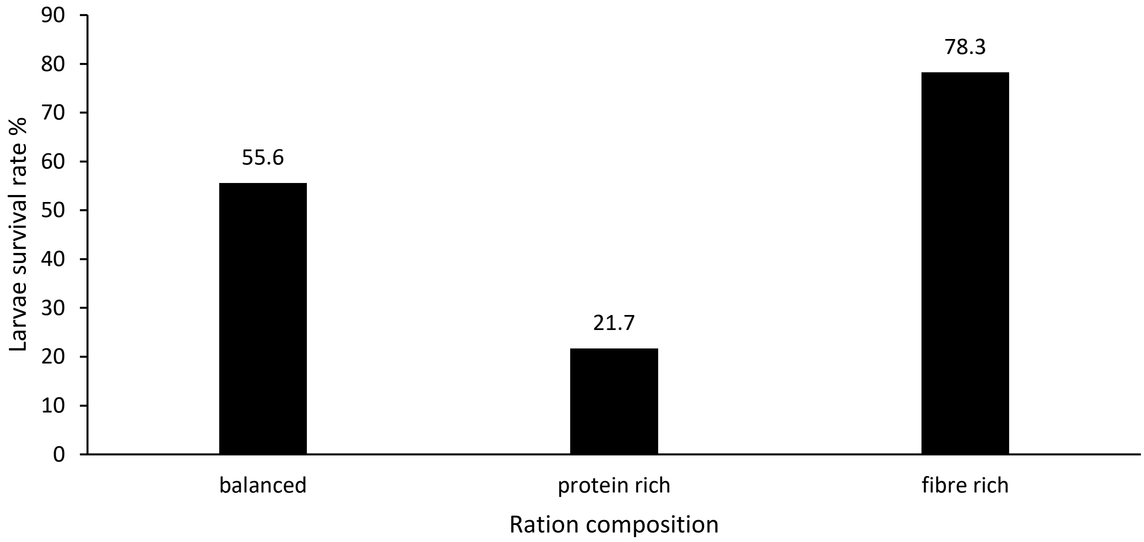

Figure 10. Larval survival rate on different ration compositions (Tschirner & Simon, 2015)

BSF larvae growth parameters can be significantly affected by the nutrient composition. Many reviewed articles have suggested that the composition of the larvae could also be affected by the substrate. Protein, fat, fibre, moisture, carbohydrates, and minerals are all important to the survival, weight gain, development time and bioconversion ratios in the larvae. Moreover, black soldier fly larvae can survive in a wide range of low-nutrient-value organic waste such as food waste, agri-industry by-products, animal waste and meat with no or minimum pre-treatment. This is an advantage in mass-rearing facilities as wastes are easily obtained from up-stream in the processing chain and converted to high-nutrient-value body mass to feed animals (Broeckx et al., 2021; Permana & Ramadhani Eka Putra, 2018). Surendra et al. (2020) sum up that the co-treatment of organic wastes with the larvae and synergistic microorganisms can potentially elevate the bioconversion efficiency. Depending on the aim of the larvae usage, feeding regimes, rearing density and nutrient profile of the substrates can be segregated. Higher density and lower feeding rate are the best approaches for higher bio-conversion efficiency (Surendra et al., 2020), while a higher feeding rate (95-163mg/larva/day) and relatively balanced nutrient profile are recommended for larvae biomass productivity (Parra Paz et al., 2015). As Broeckx et al. (2021) pointed out, there are different rearing conditions, such as light, temperature, and humidity, between different labs, making it difficult to compare the larval growth for many parameters. However, studies have provided concurrent statements that the BSFs are tolerant to a wide range of conditions and can adjust and thrive to grow in many different organic substrates (Barragán-Fonsec, 2018; English et al., 2021; Nguyen et al., 2013; Surendra et al., 2020).

Protein is the main limiting factor for larvae development to prepupae, and it is proposed to pre-treat the larvae with an appropriate starter diet before rearing them on organic waste to reduce the cultivation period (Pliantiangtam et al., 2021). Chia et al. (2020) recorded low fat accumulation in larvae fed with high crude protein content. Chia et al. (2018b) and Tschirner & Simon (2015) pointed out that protein to net energy balance is the crucial component for maximum production of BSF larvae; they suggested that 44.6% is preferable, and any excessive protein content in the diet may hinder the development of larvae. Grease in the substrate leads to longer cultivation time for the larvae, as it is difficult for black soldier flies to digest (Spranghers et al., 2017). This is concurs with Nguyen et al. (2013). Although fat could be an important factor in the diet for larvae growth, energy-dense substrates could also lead to higher synthesis of fatty acid and fat accumulation in the larvae. Nguyen and colleagues (2013) noted that the energy-, fat-, and protein-balanced substrate could offer the best growth performance in the BSF larvae, such substrates including animal liver and kitchen wastes. Ramzy et al. (2022) pointed out that high amounts of lignin and unbalanced nutrients in the substrate could be deleterious for larvae development time; however, higher fibre content resulted in higher conversion efficiency in lauric acid by 79.76% and palmitoleic acid (omega 7) by 65.05%. Li et al. (2022) suggested that dietary C18:0, C18:3n-3 and C18:2n-6 are the drivers of pre-pupae rate in the larvae, while C16:0 controls body weight.

In the reviewed studies, there was limited focus on the moisture and mineral content of diets, but it was shown that calcium may play a role in larval survival rate (Broeckx et al., 2021). Other minerals can be important in the larvae for animal feed values (Adebayo et al., 2021), but few studies have looked closely at the mineral content of the diet and its effects on the larvae growth. Lievens et al. (2021) mentioned a few chemical substances that can accumulate in the larvae when fed with contaminated substrates, such as metals, mycotoxins, pesticides, pharmaceutical compounds and persistent organic pollutants. Cho et al. (2020) looked at the microplastic contamination and salinity effect on the growth performance of black soldier fly larvae. Their findings indicate that survival was not affected by microplastic contamination. On the other hand, higher salinity substrate resulted in lower larval weight and pupation ratio.

Broeckx et al. (2021) suspected the low survival rate in the larvae fed on grain middling 1 diet and tomato leaves was due to relatively high calcium content, 2.29g/100g DM and 8.14g/100g, respectively. This high calcium content is inversely proportional to the survival rate. Other than the mineral content, there was no outstanding nutrient content that these two substrates were different from other diets in the study. Having said that, Tschirner and Simon (2015) illustrated that a protein-rich diet resulted in the lowest survival rate in the study (21.7%). However, the calcium content in this protein-rich substrate was the lowest (0.82g/kg DM) among all three investigated diets. Ewald et al. (2020) did not provide detailed substrate composition in the study. Instead, only larvae growth performance has been recorded. Their results show that with an increase in blue mussel inclusion in the diet, a lower survival rate has been observed. The only exception was including 40% mussel in the diet; the survival rate has increased to 77.1%, compared to 30% mussel inclusion. Blue mussel is known to have high protein content (Nørgaard et al., 2015), higher inclusion means the diet is richer in protein. This result concurs with Tschirner and Simon (2015). Romano et al. (2022) have acquired similar survival rates in the sweet potato, spent coffee, and dough. In them, spent coffee outperformed the other two, reaching as high as 98.6% larvae survival. Spent coffee is a readily available organic waste that can be easily obtained from local coffee shops. It is known to have a relatively high indigestible fibre content. In Romano et al. (2022), the cellulose content is 4.61% compared with 2.17% in sweet potato and 0.83% in dough. The reasonable survival rate on spent coffee agrees with Tschirner and Simon (2015); they showed the same result when the BSF larvae were reared on the fibre-rich substrate (15.6% crude fibre in dry matter), the survival rate was the highest in the study (78.3%).

While fibre-rich substrates suggest a promising survival rate, studies have found that the larval weight is not the best, most likely because fibre contains fewer nutrients and requires more time and energy to digest (Broeckx et al., 2021; Romano et al., 2022). Broeckx et al. (2021) suggested that the heaviest larval weight is produced on industrial food waste substrate, where the crude protein content was 18.5g/100g DM In their study, those survived larvae that grew over 100mg were supplied with substrates that contained crude protein between 13.1g/100g DM to 20.4g/100g DM; the only exception was for corn meal, which has a relatively high gross energy 300 kcal/kg DM. Spranghers et al. (2017) also pointed out that the lowest larval weight in their study was related to the highest fibre-content substrate, the biogas digestate (386g/kg DM), which had the highest crude protein content among all studied substrates. They explain that moisture also plays a crucial role in the larvae development. Similarly, Tschirner and Simon (2015) recorded that the lowest larval weight in the fibre-rich diet was only 34.8mg on average. Pliantiangtam et al. (2021) observed that balanced rations achieve the desirable larval weight; high protein content may result in waste of feed input and loss of larvae yield. Adebayo et al. (2021) used substrates with similar crud protein content and found that harvested larvae had similar protein composition in their biomass.

Bio-conversion efficiency is the by-product of larval weight gain, and food reduction is a key factor in the calculations. Food reduction is positively correlated with larval weight, hence the bio-conversion efficiency in Broeckx et al. (2021). Although Ewald et al. (2020) have not included the food reduction rate in the data, the bioconversion on a dry matter basis shows a similar pattern as in Broeckx et al. (2021). Romano et al. (2022) have obtained similar results, where the heaviest larvae and the highest bioconversion and food reduction happened in the dough substrate; however, the percentage of pre-pupae in the dough treatment was lower. The authors were not able to resolve this and advised further investigation. Romano et al. (2022) also indicated that the desirable protein-to-carbohydrate ratio makes the dough easier to digest and offers more nutrients for the larvae's weight gain.

While considering bio-conversion efficiency, development time is another key parameter in rearing black soldier fly larvae for feed. Scala et al. (2020) suggest that the rearing substrate significantly affects the larvae development time. Ramzy et al. (2022) pointed out that the development time is closely associated with fibre content in the diet; the higher the diet fibre, the longer development time is required. However, their results show that with 75% olive pomace residue included in the diet, development time (927 days) was shorter than in larvae reared on pig (36 days), quail (30 days), sheep (42 days) and horse (42 days) manure. Broeckx et al. (2021) noted that despite larvae reared on industrial food waste being the heaviest among all studied substrates, the larvae still did not reach the potential maximum weight at the pupa stage, indicating that the development time for this substrate is longer than others. Chia et al. (2018b) found that the larval development time in their study was the shortest for sorghum and barley treated with brewer’s yeast. It is worth noting that other substrates in the study treated with brewer’s yeast produced relatively longer development time to pupation. On the contrary, substrates treated with water showed an overall shorter development time, except for sorghum and barley. This indicates that moisture in the substrate is an important factor in promoting larvae development until pupation.

In comparison, Spranghers et al. (2017) indicate that the highest moisture provided in the substrate did not result in the shortest development time (15.5 days). This might be due to the substantial shortage of crude protein and ether extract in the diet. Tschirner and Simon (2015) show that a balanced diet offered the shortest development time (7 days), while the other unbalanced substrates took longer (15 days). In Nguyen et al. (2013), fish and liver substrates offered the shortest development time to prepupae, but it took longer for the larvae to emerge as adults. Larvae reared on fish took the longest (55 days) to emerge as adult flies. The missing nutrient in fish is carbohydrates, but they have relatively high protein content and the highest fat content. This shows that protein and fat content in the substrate are advantageous in rearing BSF larvae, but carbohydrates are crucial in adult fly development. This result suggests that in rearing BSFs at the industrial scale, brooding stock and production stock need to be reared separately for best productivity.

2.3.3.2 Amino acid composition of larvae and substrate

Insects have chemical compositions in their tissues similar to other animals, and their metabolic processes are also the same (Behmer, 2008). Like other animals, Behmer (2008) stated that insects can synthesise some nutrients, but most of the nutrients are derived from their rearing substrate. Nutrients, including amino acids not synthesised by an organism, are the essential nutrients that must be supplied via rearing substrates.

Amino acids are the foundation of proteins used for tissue structure and production of enzymes and receptor molecules. In addition to their use in large protein molecules, individual amino acids also serve important roles, such as in morphogenesis and neurotransmitters (Behmer, 2008). There are 200 amino acids identified from biological materials; of these, 20 have been found to contribute to building proteins. Among these 20 amino acids relevant to protein construction, 10 are essential for insects, as shown in Table 2 below. Regardless of the essentialness of the amino acids, the vast majority come from the dietary content. Behmer (2008) pointed out that lacking any single amino acid can affect the growth performance of insects, and to some extent, non-essential amino acids may be needed. Though denoted as non-essential, a balanced diet of essential and non-essential amino acid profiles is the key to optimum growth performance. Interestingly, Behmer (2008) recorded histidine in both groups due to its essentialness in some insects but not in others, while Oibiokpa et al. (2018) mentioned that histidine and arginine are semi-essential amino acids as they are not synthesised in sufficient amounts in order to promote human growth. Table 3, retrieved from Fischer et al. (2021), clearly states that typical essential and non-essential amino acids in BSF larvae can sustain ordinary livestock requirements.

Table 2. Amino acids usually found in insects (Behmer, 2008).

Essential	Non-essential
Arginine	Alanine
Histidine*	Asparagine
Isoleucine	Aspartate
Leucine	Cystine
Lysine	Glutamate
Methionine	Glycine
Phenylalanine	Histidine*
Threonine	Proline
Tryptophan	Serine
Valine	Tyrosine

*Semi-essential amino acid depends on the insect species. For some insect it is essential, while not essential for the others.

Table 3. Essential and non-essential amino acid found in black soldier fly (Fischer et al., 2021).

Essential	Non-essential
Histidine	Alanine
Isoleucine	Arginine
Leucine	Aspartic acid
Lysine	Cysteine
Methionine	Glutamic acid
Phenylalanine	Glycine
Threonine	Serine
Tryptophan	Proline
Valine	Taurine
	Tyrosine

Giannetto et al. (2020) found that BSF has an essential amino acid profile close to that of the fish, regardless of the life stage of the larvae. Fischer et al. (2021) suggested that the nutritional value, especially the amino acid profile, is affected by the substrate in which the larvae were cultured. When the substrate had higher crude protein content in their experiment, the larvae had higher amino acid content, suggesting that it is crucial to supply a reasonable quality feed to ensure the production of good quality insects as feed ingredients (Fischer et al., 2021). On the other hand, El-Dakar et al. (2021) had a different result, and their study showed that the substrate composition does not affect the amino acid profile in BSF larvae despite the tested substrate having different essential amino acid profiles. Fitriana et al. (2022) further extended that the different compositions of the substrate may have a more significant impact on the non-essential amino acids than on the essential group.

Essential amino acids are similar across all animals, with some extensions due to species differences, such as in birds, cats and ferrets (Hou & Wu, 2018). Essential amino acids are important not only in their absolute amount in the artificially formulated diets but also in their proportion to lysine; the proper proportioned diet is known as the ideal balance; the composition in this ideally balanced diet for each amino acid essentially matches the animal's dietary requirements (Liao et al., 2015; Wu, 2014). Ideal dietary amino acids are usually denoted proportional to lysine as a reference compound. This is not only because lysine is usually the first few limiting factors in monogastric animal diet, especially for swine and poultry, but also offers a standard reference for feed nutritional composition in monogastric animals and hence gives the universal understanding of the dietary requirement for each amino acid in different animal species (Hou & Wu, 2018; Leinonen et al., 2019; Wu, 2014).

2.4 Research Objectives

Based on the preceding literature review, the current study aimed to investigate how the amino acid composition of the diet influences the amino acid composition of black soldier fly larvae and their growth. Furthermore, the current study tests if the ideal amino acid concept in monogastric animals applies to insects; for example, amino acid/essential amino acid should be present in the right ratio to each other using lysine as the basis, as it is a widely adopted approach in researching monogastric animal nutrition profile. The current study is one of the foremost studies, if not the first, to examine the amino acid concept in black soldier fly larvae and its impact on their growth. The hypothesis is that the insects require not only dietary essential amino acids in the rearing substrate but also that the diet's ideally balanced amino acid profile positively impacts the growth and development of the BSF larvae. This study could fill the current knowledge gap in understanding the aimed impacts and offer insights into the stable production of black soldier fly larvae as an alternative source of protein. The current research utilised the proximate composition of the diets and the growth parameters such as weight and life stage to illustrate the objectives above.

Chapter 3. Materials and Methods

3.1 The colony

The larvae used in experiment 1 are the descendants of the colony described in Liu et al. (2018), hereafter referred to as the Massey colony. This colony were firstly derived from the wild population from three different locations at Thames, Coromandel Peninsula (37°03′S, 175°52′E), Pukenui, Northland (34°82′S, 173°15′E), and Palmerston North, Manawatu (40°36′ S, 175°64′E). They were primarily maintained in the BioPod Plus (Prota Culture Ltd.) for one year, then transferred to Broll and kept in a glasshouse of the Plant Growth Unit at Massey University. The insects were acquired when they were 10 days old. The larvae were transferred from the Plant Growth Unit to the Ecology lab at Massey University, Palmerston North. The larvae were fed broll from hatching until the start of the experiment. All larvae remained in the transferring container for one night in an incubator with a temperature set at 28℃. The next day, 20 replicates of 50 larvae each were selected randomly within the larval pool. Each replicate was then placed in a 400ml plastic container with holes in the lid for ventilation.

The second experiment used a different colony from a commercial live insect company (iNZect Direct, Masterton), hereafter referred to as a commercial colony. These insects were around a week old and had a lower average body weight than the Massey colony in the first experiment. They arrived in paper cups with broll. They were also placed in the incubator overnight at 28℃. Replicates numbers, larvae selection method and larvae numbers per replicates were the same as in experiment 1. The rearing containers were smaller (200ml) in the second experiment.

3.2 Diet

Two diets were formulated and used in both experiments. Both diets contained 30% broll and 53% maize meal but differed in their main protein sources. In one diet (SPI), the soybean isolate was used, and in the other diet (AA), a blend of synthetic amino acids (lysine, threonine, methionine, and tryptophan) was used to provide a similar amount of crude protein (Table 4). The SPI diet closely matched the BSF larvae's essential amino acid balance, and the AA diet did not (Table 5). The term "diet" is used here to describe the mixture of the dry ingredients, and the "feed" is the mixture of the diet and water. The diets were fed to the larvae mixed with water, as the feed structure is important for the larvae. A small experiment was conducted to determine the optimal diet-to-water ratio (D: W) for feed structure (Appendix 1.). The optimal D: W ratio was 1:1 for the AA diet and 1:1.5 for the SPI diet, as it had a higher water-holding capacity.

Table 4. Ingredient composition of the experimental diets.

Ingredients (% of total diet composition)	SPI	AA
Broll	30	30
Maize	53	53
Soy Protein Isolate	15	0
Lysine	2	10.5
Threonine	0	5
Methionine	0	1
Tryptophan	0	0.5

3.3 Experimental design

The larvae were kept in an incubator to maintain a constant temperature during the two experiments. The incubator was set to 28℃ one day before the start of the trial. A cup of water was supplied to adjust the moisture evaporation in the feed. A thermometer was placed in the incubator to track any temperature changes.

In Experiment 1, on the first day, 20 jars sized 400ml were filled with a 25g diet and 50 larvae each. Water was mixed with the diet according to the ratio stated in the diet section. Ten jars were allocated to each dietary treatment. The net weight of the jar, diet, water, larvae and the total weight of the rearing system (jar + feed + larvae) were recorded at the start and end of the experiment. Larvae were provided new food every five days, and the weight of newly added feed was recorded. Water was available ad libitum. Experiment 1 was terminated on day 15. On the last day of the experiment, the total weight of the feeding system (jar + larvae + frass) and the individual weight of the larvae were recorded. The larvae in experiment 1 were further split into four categories based on survival (dead or alive) and developmental stage. Numbers of larvae in each category were counted and used to calculate the mortality and pre-pupae rates.

Table 5. Amino acid balance (amino acid/lysine) for black soldier fly (BSF) and the experimental diets, as well as the diet to BSF ratio (limiting amino acids)

	Amino acid/Lysine ratio			Diet / BSF	
Amino Acid	BSF[1]	SPI	AA	SPI	AA
Aspartic acid	1.46	1.43	0.53	0.98	0.36
Threonine	0.65	0.65	0.83	1.00	1.28
Serine	0.69	0.84	0.33	1.22	0.48
Glutamic acid	1.55	3.42	1.38	2.20	0.89
Proline	0.92	1.33	0.56	1.44	0.61
Glycine	0.98	0.80	0.33	0.82	0.33
Alanine	1.06	1.08	0.46	1.01	0.43
Valine	1.07	0.86	0.35	0.81	0.32
Isoleucine	0.73	0.63	0.24	0.86	0.33
Leucine	1.12	1.70	0.70	1.51	0.63
Tyrosine	1.10	0.62	0.24	**0.56**	**0.22**
Phenylalanine	0.66	0.82	0.32	1.24	0.48
Histidine	0.54	0.52	0.21	0.96	0.39
Lysine	1.00	1.00	1.00	1.00	1.00
Arginine	0.85	0.99	0.37	1.16	0.43
Cysteine	0.11	0.30	0.12	2.62	1.10
Methionine	0.32	0.30	0.27	0.94	0.84
Tryptophane	0.15	0.14	0.10	0.88	0.62
Phenylalanine + Tyrosine	1.76	1.43	0.56	0.81	0.32
Methionine + Cysteine	0.43	0.60	0.25	1.38	0.58

[1] BSF balance means of four previous analyses conducted at Massey University. SPI and AA calculated value based on the chemical composition of the ingredient.

In Experiment 2, a similar set-up was used as in Experiment 1, but the feeding procedure was altered. Instead of feeding five days apart, larvae in experiment 2 were fed at two-day intervals. Experiment 2 was terminated on day 14. Larvae in the AA treatment in this experiment were washed and sieved to retrieve larvae because they lost substantial weight substantially, which is impractical to separate by sieving alone. This second experiment was carried out primarily to confirm the data in experiment 1, as there were distinctive differences in the harvesting weight and life stage of larvae between the two diets, and it is observed that AA samples are much drier than the SPI samples, to rule out any other influencing factors apart from diet difference, experiment 2 have carefully maintained the samples at the optimal moisture content for each diet.

There are some specific terminologies used in this research, where larvae counted at the beginning and end of the experiment refer to juvenile black soldier fly larvae at or younger than fifth instar, and pre-pupae counted at the end of the experiment refer to juvenile black soldier fly larvae at or older than sixth instar.

3.4 Calculations

The following calculations on an individual basis were done for each replicate in both experiments:

Starting liveweight (SLW) = total larval weight / 50 larvae in each replicate
Final liveweight (FLW) = total insect weight / the number of insects at the end of the trial in each replicate
Average daily gain (ADG) = (FLW – SLW)/experiment duration in days (14 days in trial 1, 15 days in trial 2)
Daily feed intake (DFI) = total diet weight/experiment duration in days (14 days in trial 1, 15 days in trial 2)
Bioconversion =ADG/ DFI

For the amino acid composition (mg/100mg) in the larvae:
Sum of amino acids (SAA) = Sum of all amino acid concentrations (in mg/100mg fresh weight)
Amino acid fraction (%AA in SAA) = each amino acid concentration /SAA*100
Amino acid to lysine ratio (AA/lysine) = amino acid /lysine

3.5 Chemical analyses

Samples of the two diets and larvae/prepupae in experiment 1 were submitted for chemical analysis at the Massey University Nutrition Laboratory, Palmerston North, New Zealand. For larvae harvested from experiment 1, both treatments were pooled to 1 sample (10 replicates) due to the low biomass yield in AA treatment. Two grams of larvae were randomly selected from each sample to pool for the SPI sample to match the AA treatment yield. Diets and larvae/prepupae in experiment 1 were tested for proximate chemical composition and amino acid composition. Larvae from experiment 2 were heavy enough to pool both treatments to

5 samples (2 replicates per sample) for amino acid composition analysis. The samples were freeze-dried and ground before the chemical analysis.

The diet and larvae were analysed for dry matter, ash, crude protein, fat and gross energy. Stable amino acids and methionine/cysteine were determined in the diets and the larvae. Amino acid profiles in the black soldier fly larvae were determined for both trials. Ten original samples were randomly blended into five groups in each treatment for both trials, one exception was for AA treatment in experiment 1 where there was insufficient material for chemical analysis; therefore, the ten replicates were blended into one sample for amino acid profile analysis.

The following methods were used for the chemical analysis:
Gross energy (GE) of the trial diets was determined by combusting the sample completely in a bomb calorimeter (AC-350, LECO Corporation, St. Joseph, MI, USA). Other chemicals were analysed according to the method of AOAC (2005): dry matter (AOAC 925.10 and 930.16); crude protein (AOAC 968.06, Dumas method); fat (AOAC 991.36, Soxtec method); Neutral Detergent Fibre (NDF, AOAC 2002.04); amino acid profile (acid stable: HCl hydrolysis followed by RP HPLC separation using AccQ Tag derivatization, AOAC 994.12); cysteine/methionine (performic acid oxidation, AOAC 994.12).

3.6 Statistical data analysis

A general linear model with diet and experiment and their interaction as a fixed effect was fitted to SLW, FLW, ADG, DFI and bioconversion. For the amino acid analysis, as there was no replicate for the AA diet in experiment 1, only three diet groups were analysed. The groups were: experiment 1 SPI diet (EXP1 SPI), experiment 2 SPI diet (EXP2 SPI), and experiment 2 AA diet (EXP2 AA). A general linear model with diet group as a fixed effect was fitted to the AA data. Contrasts were used to compare the colony effect (EXP1 SPI vs EXP2 SPI) and the diet effect (EXP2 SPI vs EXP2 AA). Both analyses were done using Proc GLM in SAS (SAS® software, version 9.4, 2016, SAS Institute Inc., Cary, NC, USA).

Chapter 4. Results

4.1 Diet analysis

The chemical composition of both diets is presented in Table 6. The diets have similar levels of dry matter, ash, crude protein, fat, neutral detergent fibre and gross energy. The main differences between the diets were in their amino acid composition.

Table 6. Proximate chemical composition of the diets.

%	AA Diet	SPI Diet
Dry Matter	88.5	87.6
Ash	2.1	2.6
Nitrogen	4.0	3.6
Crude Protein	25.0	22.5
Fat	2.4	2.2
NDF	17.2	16.0
GE kJ/g	16.5	16.8
AMINO ACIDS %		
Aspartic Acid	0.58	2.05
Threonine	5.31	0.72
Serine	0.35	0.98
Glutamic Acid	1.48	3.95
Proline	0.58	1.21
Glycine	0.35	0.87
Alanine	0.47	0.95
Valine	0.34	0.92
Isoleucine	0.23	0.76
Leucine	0.64	1.56
Tyrosine	0.26	0.72
Phenylalanine	0.36	0.99
Histidine	0.18	0.43
Lysine	8.04	2.26
Arginine	0.47	1.39
Taurine	0.02	ND
Cysteine	0.15	0.30
Methionine	0.99	0.34
Sum AA*	**20.8**	**20.4**

*SAA = sum of amino acid

4.2 Results for experiment 1

The data in Table 7 show that there was significant difference in BSF larvae performance between two tested diets in Experiment 1.

Final weights of BSF larvae reared on SPI diet were significantly heavier than insects reared on AA diet. It is also evident that the average daily weight gain of larvae on SPI diet was significantly higher than those on AA diet (Table 7). In addition, there was more diet offered to the larvae in the AA treatment, consequently the bioconversion ratio was significantly higher in the SPI treatment. The rate of larvae reaching pre-pupae, and the mortality of the larvae are also higher and lower, respectively, in SPI diet treatment comparing to AA treatment.

Table 7. SAS results for data analysis based on experiment 1. Result is expressed on a per larvae basis.

	LSMEANS		SE	P-value
	AA	SPI		
Liveweight Start (mg/larvae)	54.4	54.4	0.65	1.00
Liveweight End (mg/larvae)	31.3	101.9	2.20	< 0.0001
Daily Weight Gain (mg/d)	-1.6	3.4	0.18	< 0.0001
Diet offered (mg/d)	50.5	47.4	0.23	< 0.0001
Bioconversion (mg/mg x 100)	-3.3	7.1	0.37	< 0.0001
Prepupae (logit)[1]	-3.13	1.04	0.246	< 0.0001
Prepupae (%)[2]	4.2	74.0		
Mortality (logit)	-1.04	-3.21	0.255	< 0.0001
Mortality (%)	26.1	3.9		

[1] value on the logit scale, P-value for Chi-Square test

[2] % values are back-transformed logit values.

4.3 Results for experiment 1 and experiment 2

The growth data from both studies were also analysed together with a 2x2 factorial design (experiment and diet). The experiment effect represents the different colony used in each experiment. The diet treatments were the same in both experiments. The results are presented in Table 8.

The analysis for two colonies with two diet treatment each, showed that different colonies had significant ($p<0.05$) difference in all monitored growth performance variables. End liveweight and average daily gain were significantly affected by the type of diet. The interaction effect of colony and diet was significant for end liveweight, average daily gain and daily feed intake; all treatments in two colonies have performed significantly different for liveweight at the end of the experiment and the similar effect was found for the average daily gain. On the other hand, feed intake was affected by the colony but not by the diet.

The differences in the amino acid in larvae between colonies and diets are presented in Table 9, 10 and 11. Table 9 shows the amino acid content in the fresh larvae, Table 10 expressed each amino acid amount as a percentage of the sum of all amino acids, Table 11 is the ratio of the amino acid to lysine.

The amino acid content in the fresh larvae was different between both colonies (Table 9), only glutamic acid (P=0.081), alanine (P=0.826) and cysteine (P=0.08) were similar. It is worth to note that cysteine was the only amino acid which had a more significant diet effect (p=0.023) than the colony effect.

While a little more than half of the amino acid in fresh larvae had similar contents between diets in colony 2 (Table 9), aspartic acid (p=0.012), valine(p=0.007), isoleucine (p=0.047), tyrosine (p<0.0001), phenylalanine (p=0.0002), histidine (p<0.0001), arginine (p=0.0004), and cysteine (p=0.023) were significantly different in the fresh larvae reared on different diets.

Table 8. Growth performance for BSF larvae from two colonies fed two different diets. Results are expressed on per replicate of 50 larvae basis.

Colony (Experiment)	**Col1**	**Col1**	**Col2**	**Col2**		**p value**		
Diet	**AA**	**SPI**	**AA**	**SPI**	**SE**	**col**	**diet**	**col*diet**
LWS(g/50larvae)	2.72	2.72	1.07	1.01	0.038	<.0001	0.44	0.44
LWE(g/50larvae)	1.56^{b}	4.98^{c}	0.79^{a}	9.02^{d}	0.217	<.0001	<.0001	<.0001
ADG	-0.083^{b}	0.161^{c}	-0.019^{a}	0.534^{d}	0.0155	<.0001	<.0001	<.0001
DFI	2.53^{b}	2.37^{a}	2.59^{b}	2.71^{c}	0.039	<.0001	0.74	0.001

LWS: liveweight at the start of the experiment, LWE: liveweight at the end of the experiment, ADG: average daily gain per, DFI: daily feed intake, col: colony.

^{a,b,c,d} Treat values are significantly different from each other represented in different superscripts. (LSD, $p<0.05$)

Table 9. Amino acid composition of the fresh BSF larvae (%)

Colony (Experiment)	**Colony1**	**Colony2**	**Colony2**		**Contrast p-value**	
Diet	**SPI**	**AA**	**SPI**	**SE**	**SPICol1 vs SPICol2**	**SPICol2 vs AACol2**
Aspartic Acid	1.58^{c}	1.04^{a}	1.32^{b}	0.047	0.0023	0.0012
Threonine	0.70^{b}	0.52^{a}	0.57^{a}	0.025	0.0032	0.2136
Serine	0.75^{b}	0.54^{a}	0.59^{a}	0.021	0.0002	0.1144
Glutamic Acid	1.30^{a}	1.31^{a}	1.16^{a}	0.052	0.0805	0.0785
Proline	1.13^{b}	0.94^{a}	0.97^{a}	0.035	0.0071	0.5369
Glycine	1.16^{b}	0.89^{a}	0.82^{a}	0.035	<.0001	0.2119
Alanine	1.08^{a}	1.20^{a}	1.09^{a}	0.044	0.8261	0.0979
Valine	1.23^{c}	0.86^{a}	1.02^{b}	0.036	0.0015	0.0071
Isoleucine	0.81^{c}	0.56^{a}	0.64^{b}	0.025	0.0005	0.047
Leucine	1.26^{b}	0.92^{a}	1.01^{a}	0.040	0.0009	0.1414
Tyrosine	1.22^{c}	0.72^{a}	1.09^{b}	0.026	0.0036	<.0001
Phenylalanine	0.78^{c}	0.49^{a}	0.66^{b}	0.023	0.004	0.0002
Histidine	0.58^{c}	0.34^{a}	0.49^{b}	0.014	0.001	<.0001
Lysine	1.05^{b}	0.82^{a}	0.90^{a}	0.043	0.0244	0.1635
Arginine	0.91^{c}	0.60^{a}	0.76^{b}	0.025	0.0009	0.0004
Cysteine	0.15^{b}	0.15^{b}	0.14^{a}	0.005	0.08	0.0225
Methionine	0.49^{b}	0.38^{a}	0.38^{a}	0.025	0.0073	0.9766
SAA*	16.16^{b}	12.27^{a}	13.61^{a}	0.487	0.0030	0.0745

^{a,b,c} Treat values are significantly different from each other represented in different superscripts. (LSD, $p<0.05$)

*SAA = sum of the total amino acid

Table 10. Larvae amino acid composition as a % to the sum of the amino acid as % in total amino acid.

Colony (Experiment)	**Colony1**	**Colony2**	**Colony2**		**Contrast**	
amino acid	**SPI**	**AA**	**SPI**	**SE**	**SPICol1 vs SPICol2**	**SPICol2 vs AACol2**
Aspartic Acid	9.75^{b}	8.51^{a}	9.70^{b}	0.094	0.7528	<.0001
Threonine	4.30^{b}	4.23^{ab}	4.16^{a}	0.042	0.0331	0.3022
Serine	4.61^{b}	4.40^{ab}	4.33^{a}	0.069	0.0145	0.4688
Glutamic Acid	8.07^{a}	10.64^{c}	8.55^{b}	0.125	0.0193	<.0001
Proline	6.96^{a}	7.64^{c}	7.10^{b}	0.093	0.3069	0.0015
Glycine	7.15^{b}	7.22^{b}	6.04^{a}	0.074	<.0001	<.0001
Alanine	6.66^{a}	9.79^{c}	8.02^{b}	0.066	<.0001	<.0001
Valine	7.58^{b}	6.99^{a}	7.49^{b}	0.051	0.2349	<.0001
Isoleucine	5.00^{c}	4.59^{a}	4.71^{b}	0.027	<.0001	0.0064
Leucine	7.78^{b}	7.51^{a}	7.43^{a}	0.030	<.0001	0.0634
Tyrosine	7.58^{b}	5.91^{a}	7.99^{c}	0.087	0.0055	<.0001
Phenylalanine	4.81^{b}	4.02^{a}	4.86^{b}	0.035	0.2747	<.0001
Histidine	3.60^{b}	2.80^{a}	3.62^{b}	0.024	0.4748	<.0001
Lysine	6.51^{a}	6.57^{a}	6.60^{a}	0.117	0.5879	0.8404
Arginine	5.65^{b}	4.87^{a}	5.60^{b}	0.064	0.6155	<.0001
Cysteine	0.93^{a}	1.26^{b}	1.00^{a}	0.030	0.1032	<.0001
Methionine	3.06^{a}	3.06^{a}	2.78^{a}	0.142	0.197	0.193

^{a,b,c} Treat values are significantly different from each other represented in different superscripts. (LSD, p<0.05)

The amino acid proportion in total amino acids were mostly the same between colonies (Table 10), except threonine (p=0.033), serine (p=0.015), glutamic acid (p=0.019), glycine (p<0.0001), alanine (p<0.0001), isoleucine (p<0.0001), leucine (p<0.0001), and tyrosine (p=0.006) were significantly different.

Diet effects were mostly significant different in colony 2 (Table 10), with the exceptions of threonine (p=0.3), serine (p=0.45), leucine (p=0.06), lysine (p=0.84), and methionine (p=0.19).

Table 11. Larvae amino acid composition as ratio to lysine

Colony (Experiment)	**Colony1**	**Colony2**	**Colony2**		**Contrast**	
amino acid	**SPI**	**AA**	**SPI**	**SE**	**SPICol1 vs SPICol2**	**SPICol2 vs AACol2**
Aspartic Acid	1.50^{b}	1.30^{a}	1.47^{b}	0.028	0.5143	0.0012
Threonine	0.66^{b}	0.64^{a}	0.63^{a}	0.008	0.0185	0.2422
Serine	0.71^{b}	0.67^{a}	0.66^{a}	0.018	0.0646	0.5187
Glutamic Acid	1.24^{a}	1.62^{b}	1.29^{a}	0.025	0.1486	<.0001
Proline	1.07^{a}	1.17^{b}	1.08^{a}	0.030	0.868	0.0557
Glycine	1.10^{b}	1.10^{b}	0.92^{a}	0.022	<.0001	<.0001
Alanine	1.02^{a}	1.49^{c}	1.21^{b}	0.026	0.0002	<.0001
Valine	1.16^{b}	1.07^{a}	1.14^{b}	0.021	0.3431	0.037
Isoleucine	0.77^{b}	0.70^{a}	0.71^{a}	0.010	0.002	0.3274
Leucine	1.20^{b}	1.15^{ab}	1.13^{a}	0.017	0.0128	0.3935
Tyrosine	1.16^{b}	0.90^{a}	1.21^{b}	0.028	0.2486	<.0001
Phenylalanine	0.74^{b}	0.61^{a}	0.74^{b}	0.010	0.9051	<.0001
Histidine	0.55^{b}	0.43^{a}	0.55^{b}	0.011	0.8111	<.0001
Lysine	1	1	1	-	-	-
Arginine	0.87^{b}	0.75^{a}	0.85^{b}	0.019	0.4844	0.0021
Cysteine	0.14^{a}	0.19^{b}	0.15^{a}	0.007	0.3592	0.0013
Methionine	0.47^{a}	0.47^{a}	0.42^{a}	0.024	0.1834	0.2083

^{a,b,c} Treat values are significantly different from each other represented in different superscripts. (LSD, p<0.05)

The ratio to lysine for each amino acids in BSF larvae tended to be similar in different colonies (Table 11), only threonine (p=0.0185), glycine (p<0.0001), alanine (p=0.0002), and leucine (p=0.0128) were different.

The majority of amino acids ratio to lysine were significantly different between diets (Table 11), no significant difference was found for threonine (p=0.2422), serine (p=0.5187), proline (p=0.0557), isoleucine (p=0.3274), leucine (p=0.3935), and methionine (p=0.2083).

Chapter 5. Discussion

The main purpose of this research was to seek evidence to answer whether the BSF larvae require a set of essential amino acids in their diet and whether they require an ideal amino acid profile as other production animals do. While the experiment was run for this primary purpose, other parameters have been tested along the line, such as the diets' growth parameters and proximate composition.

Studies have looked at the growth performance, such as the weight and length of time to reach prepupal stage in BSF larvae reared on different substrates (Adebayo et al., 2021; Meneguz et al., 2018; Nguyen et al., 2015), survival rate (Broeckx et al., 2021; Chia et al., 2018b; Cho et al., 2020; Fischer et al., 2021; Jucker et al., 2017, 2020), development time required to reach a certain liveweight based on different feed stuff (Chia et al., 2018b; Jucker et al., 2017), and bio-conversion rate on different feed types (Ewald et al., 2020; Liu et al., 2018; Zhang et al., 2022).

Among the available accessed literature, most of the studies have focused on the proximate composition of the diets and their effect on larvae growth performance and three (El-Dakar et al., 2021; Fischer et al., 2021 and Spranghers et al., 2017) have studied the detailed amino acid composition and profile of the tested diets. These three studies will be analysed in detail with the current study later in this chapter. The significance of diet effect in the growth parameters of BSF larvae is widely accepted. Chia et al. (2018b) suggested that the diet's protein/net energy balance can significantly affect the growth of BSF larvae, and the optimal protein/net energy balance can optimise the production of larvae biomass. Beniers and Graham (2019) later proved that dietary protein, in general, can affect both fresh and dry larval weight proportionally. This agrees with Chia et al. (2018b), who demonstrated that fecundity and adult longevity are affected by the rearing substrate of the larvae. Fuso et al. (2021), the authors have discovered that the protein content in BSF larvae was positively correlated with the protein provided in the diet and the fibre content. However, an earlier study has warned that exceeding a critical amount of fibre can induce a reduction of adipose tissues in the larvae due to bulkiness-caused malnutrition (Tschirner and Simon, 2015). Later studies have drawn attention to the lipid content of BSF larvae to understand the proximate, fatty acid composition in the larvae. The findings of Ewald et al. (2020) suggest that replacing fish oil with BSF larvae fat has many limitations, but using BSF larvae fat as a substitute for vegetable oil in the plant and food sector can have some good opportunities. These authors also highlighted that BSF larvae fat is also a source of biofuel.

The current study looked at the effect of amino acid profile in the diet on the growth performance of BSF larvae in terms of feed intake and average daily gain and the effect of such diets on the composition of the amino acid profile in larvae. Current found that the amino acid profile differences in diets significantly affected the growth performance of larvae, where growth performance in SPI treatment (where the diet matched the essential amino acid balance of the BSF larvae) was better than in AA treatment in the Massey colony. This outcome is in agreement with many previous studies (Beniers & Graham, 2019; Bertinetti et al., 2019; Chia et al., 2018b; Fuso et al., 2021; Gobbi et al., 2013; Meneguz et al., 2018; Tomberlin et al., 2002). Growth performance was also significantly affected by the source of the colony. Amino acid composition in fresh larvae showed limited differences between different colonies fed with the same diet, apart from glycine (Table 9); because this is the test run on in two different colonies with the same diet composition, the diet must not be the causative factor, the reasons for the significant difference in glycine remains uncertain from this study. The amino acid composition of fresh larvae fed different diets showed that only tyrosine and histidine differed (Table 9), and the amino acid proportion to total amino acid content was generally the same between colonies (Table 10). In contrast, the effect of diet composition on the same colony had a greater impact (Table 10). For each amino acid, the ratio to lysine showed that the colony effect was not as significant as the diet effect on the larvae amino acid profile (Table 11).

In monogastric animals, including essential amino acids in their diet rations is a vital topic to consider and is crucial to production performance. The most common limiting amino acids in monogastric animals, such as poultry and swine, are methionine and lysine, respectively, with these limiting factors affecting the overall generation of protein in the animal, ultimately leading to reduced production if overlooked in the diet formulation (Farkhoy et al., 2012; van Milgen & Dourmad, 2015). Extensive studies have looked at diet rations in terms of balanced amino acids to promote animal survival, maintenance, reproduction, lactation, and health.

The term “functional amino acid” has been brought to the table to establish the importance of these amino acids regardless of the traditional essential and non-essential categories (Zhang et al., 2021). With emerging environmental concerns raised against the swine industry, farmers tend to reduce the crude protein supply in their diet to lower nitrogenous waste, but this approach can result in lower production yields (Zhang et al., 2021). For this reason, utilisation of synthetic amino acids provided in the diet with lower crude protein to create a balanced diet is viable (Lindberg, 2023).

To extend the ideal amino acid balance in a diet, it is of utmost importance to the overall health, growth and production performance of swine and poultry industries as amino acids are the foundation and precursor of many physiological functions in mammals and birds (Farkhoy et al., 2012; van Milgen & Dourmad, 2015). The first limiting amino acid species is the primary consideration when blending a diet with an ideal amino acid balance. Studies have shown that the first limiting amino acid is usually lysine in swine (van Milgen & Dourmad, 2015) and methionine in Broiler chicken (Farkhoy et al., 2012) and laying hens (He et al., 2021). National Research Council have published recommended amino acid inclusion levels in swine (National Research Council, 2012) and poultry (National Research Council, 1994) feed for optimal utilisation. Extensive studies have been done to determine ideally balanced diets according to amino acids and their impact on protein production. As black soldier fly larvae are a potentially viable alternative protein source in animal diets, it is worthwhile to consider their protein content, amino acid profile, and protein production.

The current study, using the SPI diet, which matched the essential amino acid balance of the BSF larvae and the AA diet, which did not, showed that the AA diet led to weight loss in the BSF larvae, while the daily weight gain for SPI treatment was significantly higher (Table 7). Larvae reared in the SPI treatment were generally more prominent than those from the AA treatment. Table 4 (section 3.2) shows that the SPI diet comprised soy protein isolates but did not include the amino acids threonine, methionine, and tryptophan. Although AA treatment was enriched in amino acids, larvae reared on this diet did not gain weight as well as those in the SPI diet. This raises the importance of the amino acid balance in BSF larvae-rearing substrates.

n the current study, the profile of amino acids not only had significance in contributing protein content to BSF larvae, but the ratio of amino acids was also important in contributing to the growth of the larvae. Beniers and Graham (2019) suggested that even though carbohydrate concentration in the diet has a negligible effect on larvae growth, the ratio of carbohydrates to dietary protein in the rearing substrate is important, as informed by Cammack and Tomberlin (2017).

Figure 11a shows the amino acid compositions of diets used in the current study and Spranghers et al. (2017), for comparison purposes, each amino acid content was converted based on its ratio to lysine. According to Spranghers et al. (2017), glutamic acid was exceptionally high in restaurant waste, aspartic acid was the most abundant amino acid in vegetable waste, chicken feed had the highest amount of leucine, and digestate was relatively balanced with all the amino acids. In the current study, however, both diets had lower amino acid concentrations than Spranghers et al. (2017). The AA diet had a higher content of lysine and serine, while the other amino acids were at a minimal level; the SPI diet had higher content than AA diets in almost all amino acids, with glutamic acid being the highest, followed by lysine and aspartic acid. In contrast to Figure 11a, Figure 11b illustrates the amino acid profile in the BSF larvae reared on different diets; the amino acid content was also converted to their ratio to lysine. As shown in Figure 11b, the content of each amino acid is comparable across different diets, suggesting that BSF larvae can utilise amino acids in the diet to formulate their own bodily amino acid profiles with minimal restrictions. This result agrees with Lalander et al. (2019), who found that the amino acid profile in the diet leads to a slight variation of the amino acid profile in larvae. Cystine in Figure 11 shows a proportional effect in larvae, and in diet, all diets in Figure 11 have supplied little cystine, which resulted in cystine being the lowest amino acid concentration in the larvae for all corresponding diets. This suggests cysteine may be an essential amino acid for BSF larvae development.

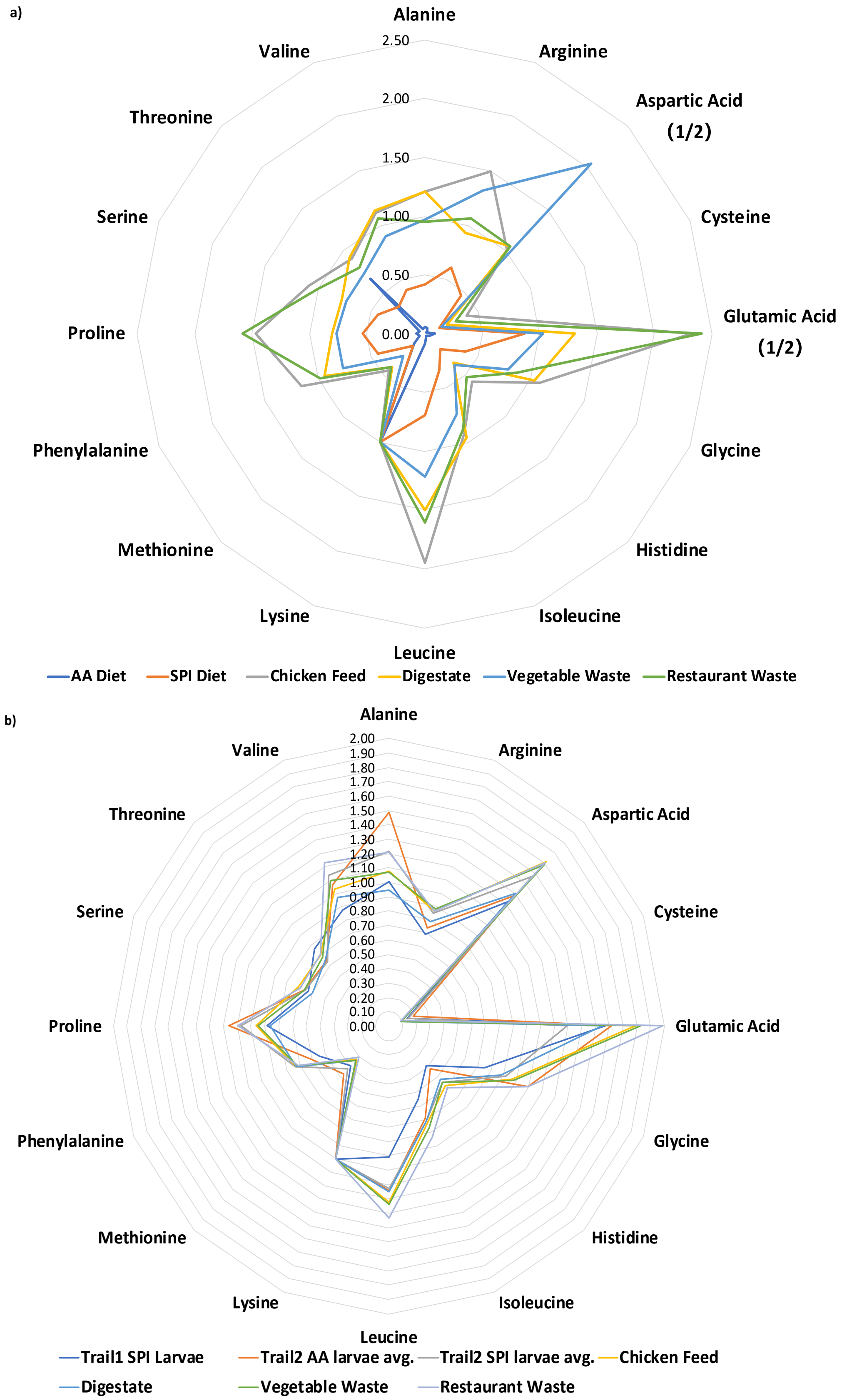

Figure 11. Amino acid ratio to lysine comparison in the diet (a) and BSF larvae (b). Include current study data for AA diet and SPI diet, and data for chicken feed, digestate, vegetable waste and restaurant waste are from Spranghers et al. (2017)

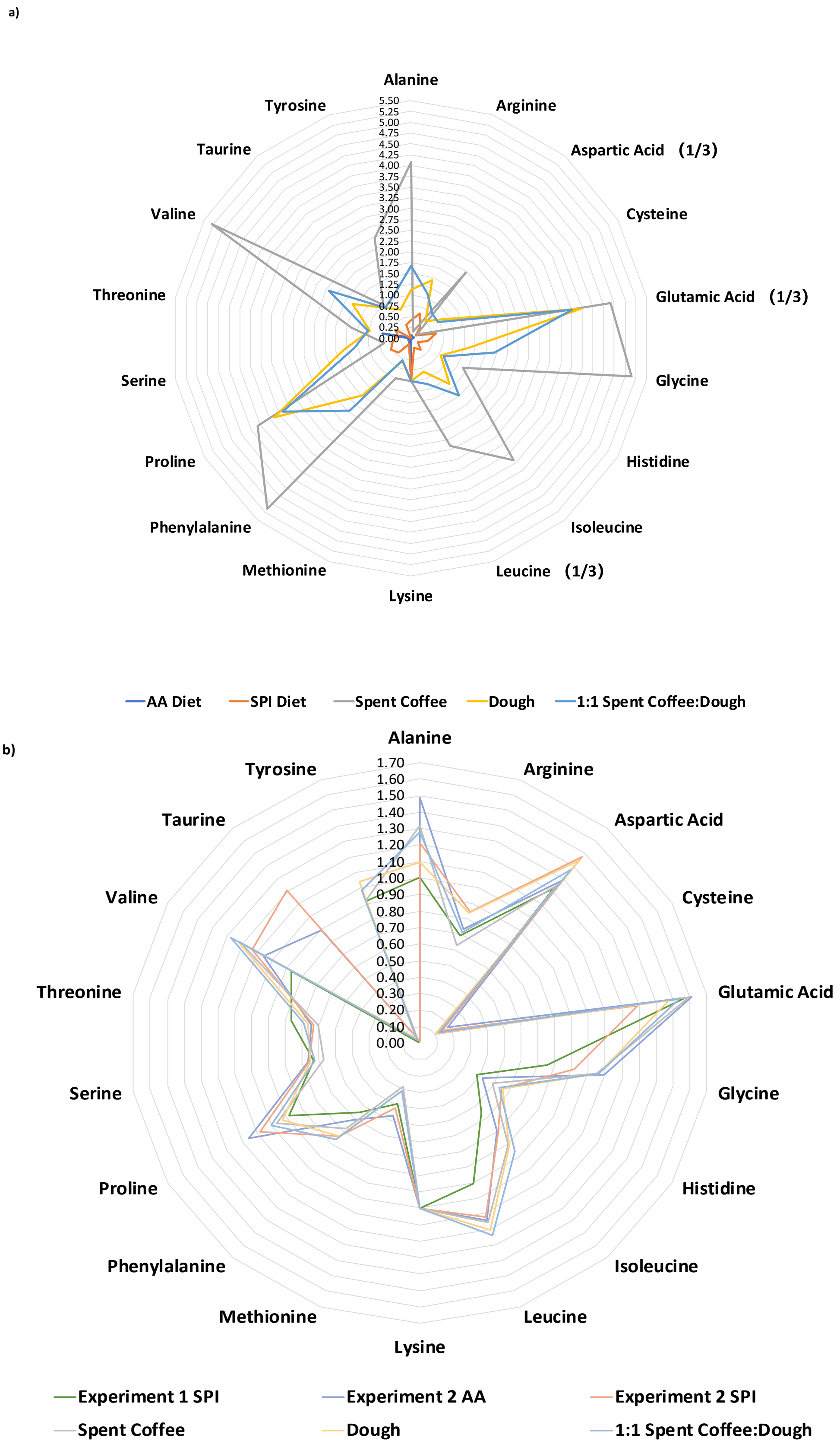

Figure 12. Amino acid ratio to lysine comparison in the diet (a) and in BSF larvae (b). includes current study data for AA diet and SPI diet, and data for spent coffee, dough, and 1:1 spent coffee: dough are from Fischer et al. (2021).

Figure 12a,b shows the comparison of amino acid profiles in diets and BSF larvae in the current study and Fischer et al. (2021), each amino acid has been converted to the ratio to lysine for a standard comparison. Figure 12a shows that while spent coffee offers more of almost every amino acid among five different diets, larvae fed with spent coffee did not appear to generate the most body amino acid contents. In contrast, the AA diet had the lowest content of almost all listed amino acids except threonine and lysine, but larvae reared on AA (Figure 12b) had reached amino acid to lysine ratios like those reared on the other diets. Cystine followed a pattern similar to that in Figure 12 and Figure 11, which proves that cystine is essential in the development of BSF larvae. It is worth noting that both input and output amounts of taurine and tyrosine in the diet and the larvae in our study (AA and SPI diet) resulted in deficient concentrations, while in Fischer et al. (2021) these two amino acids in larvae exceeded the amount that was supplied in the diet. This could indicate that taurine and tyrosine may be potential candidates for being essential or semi-essential to the BSF larvae development. Giannetto et al. (2020) showed that taurine is a species- and life-stage-specific amino acid, and its concentration in insects varies greatly. Though variation is evident, Giannetto et al. (2020) also reported that BSF's V instar larvae and prepupae contain high amounts of taurine compared to other life stages. El-Dakar et al. (2021) also provided supporting evidence for taurine being an essential dietary amino acid in BSF larvae.

Figure 13a,b compares the data from the current study and from El-Dakar et al. (2021). Data in El-Dakar et al. (2021) generally agree with Spranghers et al. (2017) and Fischer et al. (2021) in that BSF larvae can manipulate different amino acid profiles in diets to a relatively similar profile in their biomass. Interestingly, El-Dakar et al. (2021) did not include cysteine and taurine in their larvae amino acid composition analysis results. Hence, it is discarded when constructing Figure 13. In contrast to Fischer et al. (2021), El-Dakar et al. (2021) show that tyrosine might not be essential or semi-essential to the development of BSF larvae. Figures 11, 12 and 13 illustrate that black solider fly larvae can synthesise a relatively similar amino acid profile regardless of the rearing substrate's amount and amino acid profile.

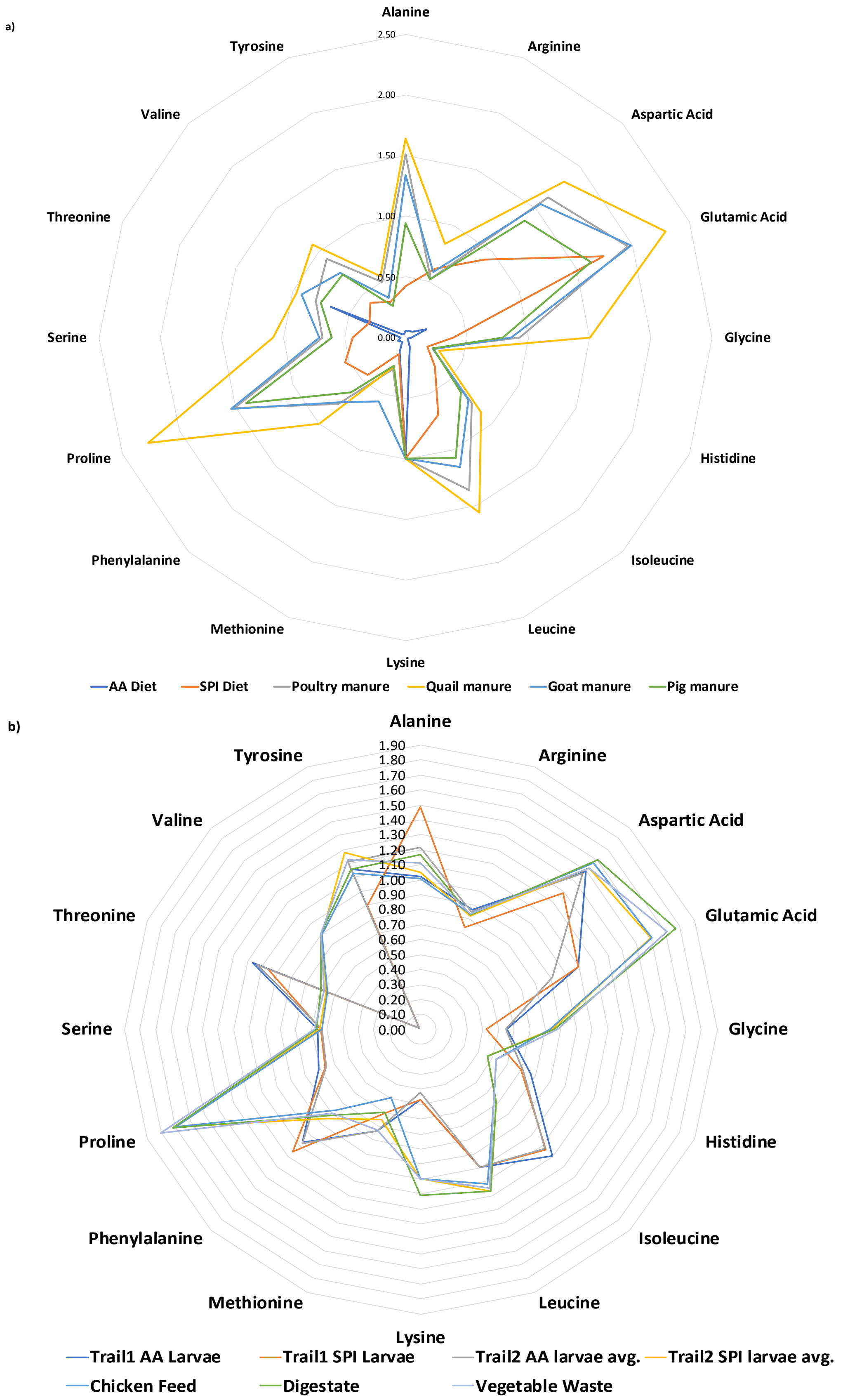

Figure 13. Amino acid ratio to lysine comparison in the diet (a) and in BSF larvae (b). Include current study data for AA diet and SPI diet, data for poultry manure, quail manure, goat manure and pig manure are from El-Dakar et al. (2021).

Note that in Figures 11a, 12a, 12b, and 13b, a few amino acids had a ratio to lysine at or very close to zero; for instance, the AA diet in Figure 12a had almost every amino acid close to zero except threonine, that is, because the ratio to the lysine of this amino acid was less than 0.5. A similar situation also applies to cysteine in SPI diet (0.13) and spent coffee (0.15); arginine in spent coffee (0.15); and histidine (0.19), methionine (0.15), and taurine (0) in SPI diet. With relatively compact amino acid ratios to lysine in the larvae, the scale of the graph axes was deliciated to 0.1. However, there were amino acids with a ratio to lysine less than this, especially taurine in experiment 1 SPI (0.01), spent coffee (0.03), dough (0.03), and 1:1 spent coffee: dough (0.03); and tyrosine in experiment 2 AA (0.01) and experiment 2 SPI (0.01). To mitigate this enormous difference between the aspartic acid and glutamic acid in Figure 11a and the rest of the amino acids, and between aspartic acid, glutamic acid, and leucine in Figure 12a with the rest of the amino acids, they have been divided by 2 and 3 in Figure 11a and Figure 12a, respectively. Figure 13a shows that tyrosine for SPI diet-reared larvae in experiment 2 from the current study has its ratio to lysine less than 0.1 (tyrosine=0.01), which may be due to the storage effect, as discussed before.

Current study has revealed that black soldier fly (*Hermetia illucens* L.) can synthesise a relatively stable amino acid profile regardless of the amino acid profile in the rearing substrate in terms of ratio to lysine. This is consistent with Lalander et al. (2019) and El-Dakar et al. (2021). Liu et al. (2017) suggested that BSF larvae can synthesise a broad spectrum of essential amino acids during their entire life span. Although lysine has been the most limiting factor in plant-based materials, it is at a relatively high concentration throughout the life cycle of the BSF (Liu et al., 2017). This is partially contrary to our study, which showed that glutamic acid is the most common amino acid in the prepupa stage. However, because this study has not collected amino acid data for larvae earlier than prepupae, we cannot compare it with Liu et al. (2017) for this aspect. However, lysine was the relatively abundant amino acid in our study. Liu and colleagues (2017) also found that the highest concentration for every amino acid appears at the early larval stage between 4-6 days of age; after this period, the concentration of the amino acid reduces until it reaches prepupation, then the concentration levels off and remains the same till the emergence of adult flies. Liu et al. (2017) also found that many amino acids in the post-mortem adults were relatively higher than in other stages of life history, corresponding with a high crude protein concentration in this sample group.

Moreover, Oibiokpa et al. (2018) state that the nutritional values of insects vary significantly between species and also within one species; for example, the metamorphosis states, the source of the insect (colony) and the diet that the insects have been reared or ingested can also cause the variation of the nutritional factors. Kröncke and Benning (2023) also pointed out that they studied mealworm larvae (*Tenebrio molitor L.*) for their great potential for essential amino acids. These authors also found that the feed conversion ratio increases as the protein concentration decreases in the mealworm-rearing substrate. Our result in the current study agrees with the findings of Kröncke and Benning (2023). Kröncke and Benning (2023) found 14 amino acids in the mealworm larvae, with alanine, aspartic acid, glycine, leucine, and tyrosine being the most abundant amino acids, and histidine and phenylalanine being less common. Eighteen amino acids were detected in our study, with glutamic acid being the most abundant amino acid, followed by aspartic acid, lysine, alanine, and tyrosine. The least common amino acids were cysteine, isoleucine, and taurine, which were almost undetectable in the proximate composition analysis. With different amino acid content compared with mealworm larvae, BSF larvae contain more amino acid species, and the concentration of each amino acid species varies, too.

pointed out that BSF prepupae had no significant difference in protein content and amino acid profile among different rearing substrates. Their study has focused on bird and mammal manure as the rearing substrate. Their study showed that probiotics give poultry manure the best weight return relative to BSF larvae biomass development. The authors also found that between goat and quail manure, larvae reared on quail manure took longer to reach pre-pupation because quail manure contains more protein than goat manure. Based on this finding, El-Dakar et al. (2021) extended that a balanced diet for BSF larvae is crucial for their development. This idea was first proposed by Simon et al. (2011), who pointed out that the higher protein content relative to carbohydrates can increase the survival rate and development time for *Ophyra* species, which also led to the conclusion that the balance of protein and carbohydrate in diet can elevate the growth performance of *Ophyra* species. El-Dakar et al. (2021) also found that BSF larvae reared on pig and poultry manure had no significant difference in amino acid profile; however, the amino acid profile of larvae reared on quail manure was significantly different from pig, poultry and goat manure, while larvae reared on goat manure were different from that from pig and poultry manure. El-Dakar et al.

(2021) also stated that the essential amino acid content was higher in BSF larvae reared on different manures than that of fishmeal and soybean for the same animal species, which indicates that BSF larvae are a good source of protein and essential amino acids for production animals.

Fuso et al. (2021) fed fruit and vegetable by-products to black soldier fly larvae on a seasonal basis. The Fuso et al. (2021) analysed dietary protein as one category. Fuso et al. (2021) found that amino acids valine and leucine can be affected by higher levels of lipids and proteins and lower levels of carbohydrates in the diet and suggested that valine and leucine are essential amino acids to the development of BSF larvae. In addition to valine and leucine, lysine content is affected by the concentration of carbohydrates and lipids and may also be an essential amino acid for BSF larvae. Fuso and colleagues' study (2021) concluded that the diet's protein-to-fibre ratio must be well-balanced to obtain a sustainable amount of lysine in the BSF prepupae.

Table 12 compares amino acid utilisation in the current study and data from previous analyses done at Massey University as the reference for BSF larvae's balanced amino acid profile. The current study shows that both colonies had similar abilities in utilising amino acids in each diet. The first limiting amino acid in both diets was tyrosine for both colonies. It is evident from Table 12 that utilisation of amino acid was better in the SPI diet than in the AA diet, which suggests that there are much more balanced proteins in the SPI diet for the larvae to utilise. Table 5 in section 3.2 also shows that the AA diet is less balanced in terms of amino acid profile than the SPI diet; with this in mind, a balanced amino acid profile in the diet is crucial in BSF larvae development.

Spranghers et al. (2017) found that BSF larvae in their diet were mainly limited by histidine in chicken feed, digest, and vegetable waste. Larvae reared on restaurant waste appeared to be limited by glycine, as shown in Table 13. Among the feeding substrates used in their study, chicken feed provided the highest content of balanced protein, as the ratio of the amino acids to lysine was almost balanced (^{3} 1), except for histidine, with a ratio of 0.99, which was only slightly below the right amount. Restaurant waste had the second highest amount of protein that was in the right amount for BSF larvae to utilise, and vegetable waste provided the least balanced protein for BSF larvae. In addition to the balanced protein, in Spranghers et al. (2017) digestate (a substrate that resulted from fermented vegetable waste) resulted in the least body weight, providing supporting evidence to indicate the significance of having a balanced ratio to the development of BSF larvae.

Table 12. Balanced protein content in the diet utilised by BSF larvae in the current study (AA/Lys in diet divided by AA/Lys in larvae.

Amino acid	BSF[1]	AA Larvae experiment 2	SPI Larvae experiment 1	SPI Larvae experiment 2
Alanine	1.06	0.04	0.41	0.35
Arginine	0.85	0.08	0.71	0.72
Aspartic Acid	1.46	0.06	0.60	0.62
Cysteine	0.11	0.10	0.93	0.88
Glutamic Acid	1.55	0.11	1.41	1.35
Glycine	0.98	0.04	0.35	0.42
Histidine	0.54	0.05	0.34	0.35
Isoleucine	0.73	0.04	0.44	0.47
Leucine	1.12	0.07	0.58	0.61
Lysine	1.00	1.00	1.00	1.00
Methionine	0.32	0.26	0.32	0.35
Phenylalanine	0.66	0.07	0.59	0.59
Proline	0.92	0.06	0.50	0.50
Serine	0.69	0.07	0.61	0.66
Threonine	0.65	1.03	0.48	0.51
Valine	1.07	0.04	0.35	0.36
Tyrosine	1.10	0.04	0.27	0.26
Taurine	-	0.003	-	-
Tryptophan	0.15	-	-	-
Minimum	0.11	0.04	0.27	0.26
SAA[2] (g/100g)		20.80	20.39	20.39
Utilised protein content		0.77	5.59	5.37

[1] BSF balance means of four previous analyses conducted at Massey University.

[2]SAA = Sum of all amino acid in the diet.

Table 13. Balanced protein content in the diet utilised by BSF larvae in Spranghers et al. (2017) (AA/Lys in diet divided by AA/Lys in larvae).

Amino acid	BSF[1]	Chicken Feed Larvae	Digestate Larvae	Vegetable Waste Larvae	Restaurant Waste Larvae
Alanine	1.06	1.12	1.28	0.91	0.79
Arginine	0.85	1.72	1.18	1.49	1.22
Aspartic Acid	1.46	1.26	1.61	2.58	1.31
Cysteine	0.11	3.69	2.29	1.70	3.03
Glutamic Acid	1.55	2.60	1.69	1.12	2.42
Glycine	0.98	1.12	1.17	0.80	0.79
Histidine	0.54	0.99	0.67	0.67	0.87
Isoleucine	0.73	1.25	1.33	0.96	1.05
Leucine	1.12	1.59	1.31	0.98	1.21
Lysine	1.00	1.00	1.00	1.00	1.00
Methionine	0.32	1.34	1.18	0.78	1.31
Phenylalanine	0.66	1.59	1.29	1.06	1.38
Proline	0.92	1.52	0.94	0.81	1.45
Serine	0.69	1.53	1.29	1.11	1.45
Threonine	0.65	1.29	1.41	1.08	1.13
Valine	1.07	1.08	1.17	0.82	0.86
Tyrosine	1.10	-	-	-	-
Taurine	-	-	-	-	-
Tryptophan	0.15	-	-	-	-
Minimum	0.11	0.99	0.67	0.67	0.79
SAA[2]		15.23	17.41	6.41	13.74
Utilised protein content		15.13	11.58	4.30	10.87

[1] BSF balance means of four previous analyses conducted at Massey.

[2] SAA = Sum of all Amino acid in the diet

Table 14. Balanced protein content in the diet utilised by BSF larvae in Fischer et al. (2021) (AA/Lys in diet divided by AA/Lys in larvae).

Amino acid	BSF [1]	Spent Coffee Larvae	Dough Larvae	1:1 Spent Coffee: Dough Larvae
Alanine	1.06	3.10	1.03	1.31
Arginine	0.85	0.25	1.71	1.52
Aspartic Acid	1.46	4.75	1.08	1.62
Cysteine	0.11	1.31	8.07	5.82
Glutamic Acid	1.55	8.75	8.04	7.31
Glycine	0.98	4.92	1.27	1.84
Histidine	0.54	2.83	1.41	1.62
Isoleucine	0.73	4.61	1.71	2.01
Leucine	1.12	6.93	2.05	2.72
Lysine	1.00	1.00	1.00	1.00
Methionine	0.32	3.53	1.81	1.78
Phenylalanine	0.66	7.62	2.37	2.86
Proline	0.92	4.21	3.94	3.39
Serine	0.69	1.08	2.39	2.11
Threonine	0.65	2.28	1.45	1.44
Valine	1.07	4.36	1.28	1.71
Tyrosine	1.10	2.67	0.67	1.11
Taurine	-	35.29	33.91	35.15
Tryptophan	0.15	2.45	2.36	1.72
Minimum	0.11	0.25	0.67	1.00
SAA[2]		8.47	8.27	8.57
Utilised protein content		2.08	5.54	8.57

[1] BSF balance means of four previous analyses conducted at Massey.

[2] SAA = Sum of all Amino acid in the diet.

Fischer et al. (2021) provided diet that had a high concentration of taurine (Table 14). However, with the first limiting amino acid being 0.25 for arginine in spent coffee, utilisation has been significantly reduced even if the rest of the amino acids all had ratios to lysine above 1. A good amount of balanced protein was utilised in larvae reared in dough with a 0.42 increase in the first limiting amino acid ratio. Spent coffee and dough as contrasting diets had different amounts of protein provided to BSF larvae. Dough had less protein content than that in spent coffee, but with the balanced amount of amino acid readily available in dough, the

BSF larvae were able to extract more balanced protein from the dough to contribute to body weight development.

El-Dakar et al. (2021) had a relatively moderate amount of balanced protein for BSF larvae and tyrosine was the first limiting amino acid across all tested diets (Table 15). Since the first limiting factor is greater than that in the SPI diet, and the amount of total amino acid is similar, the amount of balanced protein readily available to BSF larvae is like that available in the SPI diet in the current study (Table 12). Table 15 shows that providing higher amounts of total protein does not necessarily result in higher yield of BSF larvae protein contents (see the contrast between larvae reared in goat and pig manure), where pig manure has high non-dietary protein indigestible to BSF larvae and this is shown in a deficit balancing of every amino acid. With better balanced diet such as in quail manure, the amount of balanced protein for BSF larvae is higher than that in pig manure, which resulted in a higher balanced protein content in the larvae.

Body weight, together with the total balanced protein utilisation status can provide insight into the importance of the balanced diet in the development of BSF larvae to pre-pupation. Among these three studies (El-Dakar et al., 2021; Fischer et al., 2021; Spranghers et al., 2017), El-Dakar et al. (2021) did not provide detailed information on sample weights. Chicken feed in Spranghers et al. (2017) and equal blend of spent coffee and dough in Fischer et al. (2021) had relatively balanced amino acid profiles among all analysed amino acids, and BSF larvae (pre-pupae) fed these two diets had the heaviest body weight (11.5 ±0.01g) in Fischer et al., (2021) and the second heaviest body weight (10.99±0.39g) in Spranghers et al. (2017). The lightest body weight of larvae (pre-pupae) was found in larvae fed with AA diet in the current study (0.79-1.56±0.22g across both colonies). It is evident that well balanced diet results in heavier body weight in BSF larvae.

Table 15. Balanced protein content in the diet utilised by BSF larvae in El-Dakar et al. (2021) (AA/Lys in diet divided by AA/Lys in larvae).

Amino acid	BSF [1]	Poultry manure larvae	Quail manure larvae	Goat manure larvae	Pig manure larvae
Alanine	1.06	1.44	1.63	1.27	0.85
Arginine	0.85	0.64	1.00	0.79	0.63
Aspartic Acid	1.46	1.07	1.16	1.08	0.90
Cysteine	0.11	-	-	-	-
Glutamic Acid	1.55	1.22	1.43	1.25	0.95
Glycine	0.98	1.10	1.80	1.11	0.90
Histidine	0.54	0.50	0.55	0.59	0.46
Isoleucine	0.73	1.12	1.32	1.17	0.95
Leucine	1.12	1.17	1.40	1.10	0.93
Lysine	1.00	1.00	1.00	1.00	1.00
Methionine	0.32	0.43	0.52	1.05	0.35
Phenylalanine	0.66	0.92	1.30	1.03	0.80
Proline	0.92	0.89	1.34	0.99	0.78
Serine	0.69	1.05	1.71	1.17	0.89
Threonine	0.65	1.20	1.49	1.47	1.12
Valine	1.07	1.03	1.20	0.93	0.80
Tyrosine	1.10	0.39	0.49	0.34	0.23
Taurine	-	-	-	-	-
Tryptophan	0.15	-	-	-	-
Minimum	0.11	0.39	0.49	0.34	0.23
SAA[2]		20.20	28.00	14.40	15.30
Utilised protein content		7.89	13.60	4.90	3.51

[1] BSF balance means of four previous analyses conducted at Massey.

[2] SAA = Sum of all Amino acid

Chapter 6. Conclusion

The present study has assessed the amino acid profile of black soldier fly larvae (Hermetia illucens L.) (pre-pupae) reared on different diets. Two treatment diets were prepared with distinct properties regarding their amino acid content. The study found that BSF larvae can synthesise a relatively universal amino acid profile, even with deficient protein quality in the diet. The current study also found that the development of larvae is highly related to the balance of the amino acid profile in the diet, and it is also affected by the colony, such as where the colony originated. Furthermore, current research indicates that taurine and cysteine may be essential amino acids for BSF development alongside alanine, leucine, and lysine.

The purpose of the current study was to seek answers to the following questions: 1) whether the BSF larvae require a set of essential amino acids in their diet, and 2) if BSF larvae need an ideal amino acid profile in the diet or not. Current findings successfully answered subject questions; a few amino acids have been shortlisted to be essential for BSF larvae, and a relatively balanced diet can increase the growth performance of the larvae. The current study extended the existing essential amino acid species for BSF larvae and enriched the information on the ideal balanced amino acid dietary profile for BSF larvae. Further formulations could be drafted based on the currently available information to explore additional essential amino acids to the subject insect and the ideal balance of the diet protein profile.

Together with previous studies, the current study concluded that to the best of our knowledge, BSF larvae require limited species of essential amino acids as the other production animals do, especially poultry and swine. Though the BSF offers great potential, rearing it on a relatively balanced diet can increase the efficiency of amino acid utilisation. Having said that, a balanced diet for BSF larvae is not uncommon, and peak performance can be reached by blending ingredients included in the municipal waste list.

Chapter 7. Limitations of current study and recommendation of further studies

The current study only touched on the essential amino acid profile possibly required to develop black soldier fly (Hermetia illucens L.) larvae. Further studies may this to determine the essential amino acid profile for the BSF in order to gain understanding of their protein requirements. This work is necessary not only because protein is one of the important factors that contribute to the growth efficiency of biological bodies, but also to provide a foundation to investigate integration of BSF larvae into diets for other production animals as an alternative good quality protein source.

The other limitation is that the current study did not include a great number of larvae in each replicate like the other studies did. The same experimental design can be applied in future studies to extend the sample scale for a much more accurate outcome, especially in reducing the size of the standard errors.

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Appendix

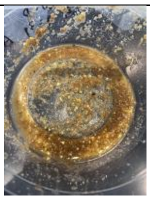
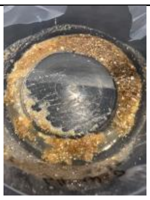
Appendix 1. Feed structure experiment description.

Purpose of the experiment: due to the water holding capacity of different feed ingredients in each diet, an optimal diet to water ratio is required for the proposed research (section 2.4).

Set up: there are 5 ratios to be tested for each diet (AA and SPI), as shown in table 16.

Result: AA diet has less water holding capacity than SPI diet, the optimal structure for AA feed is ratio 1, and the optimal structure for SPI feed is ratio 2. Apply the ratio for both feed given a constant feed weight, AA feed should contain 50% diet and 50% water, SPI feed should contain 40% diet and 60% water.

***Table 16*.** Diet to water ratio tests for each feed

Ratio ID	AA Diet	SPI diet	AA feed structure	SPI feed structure
1	**5g water: 5g diet**	5g water: 5g diet		
2	6g water: 4g diet	**6g water: 4g diet**		
3	6.5g water: 3.5g diet	6.5g water: 3.5g diet		
4	7g water: 3g diet	7g water: 3g diet		
5	7.5g water: 2.5g diet	7.5g water: 2.5g diet