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Research Article

Nutritional Properties Evaluation of Blowfly Larvae from Fish and Chicken Wastes for Asian Sea Bass Feed Formulation Application

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ABSTRACT

This present research characterized the proximate nutrient, fatty acids and amino acids compositions of the wild third instar blowfly larvae collected from fish and chicken wastes as well as its mixture, in view of aquaculture feeding for Asian sea bass (*Lates calcarifer*). Analyses of crude protein, crude lipids, crude fiber, ash, carbohydrates, amino as well as fatty acids were performed. Results revealed that the larvae that fed on the mixed substrates (chicken and fish, 50:50 ratio) had significantly the highest nutritional values (51.47 ± 0.32% of crude protein, 29.4 ± 1.47% of crude fat, 4.81 ± 0.83% of crude fiber, 4.85 ± 0.01% of ash and 12.71 ± 1.67% of carbohydrate) for formulating feeds for Asian sea bass when compared with that of chicken and fish wastes alone ($P < 0.05$). Similarly, significantly higher percentages of fatty acids (DHA and EPA) and amino acids (Arg, Lys, Met, Trp, His, Val, Ile, Leu and Phe) were observed in the larvae from mixed-substrates when compared with that of the remaining two substrates ($P < 0.05$). The findings supported the utilization of blowfly larvae harvested on mixed substrate as a possible candidate of nutrients for Asian sea bass feed formulations.

Keywords: Amino acids, Asian sea bass, Fatty acids, Proximate nutrient, Wild third instar blowfly larvae

Introduction

It has been indicated that the human population is continuously rising, demanding a sustainable supply of food resources [1]. Being the major source for protein and essential nutrients, seafood has been consumed by about 17% of the population worldwide, and its consumption is expected to further increase in tandem with the increasing human population. This condition has resulted in the booming of fisheries and aquaculture sectors, catering to such economic demands [2, 3]. Studies reported that for human consumption, approximately 75% of the total agricultural lands as well as about 8% of total water resources, have been utilized for livestock farming [2, 4]. In addition, considering that humans and animals require similar resources for food, the ever-rising population of humans has resulted in stiff competition for food resources, subsequently elevating the overall production costs for livestock activities [5].

Although the use of fishmeal has been recommended [6] for animal feed formulations, mainly due to its high composition of essential amino acids and proteins [7], the decreasing supply of natural pelagic fishes due to frequent catchment activities and the destruction of marine ecology has triggered many studies for exploring its alternative avenues [8]. In this context, the use of soybeans as protein sources for animal feeds has been suggested [9]. However, since soybean has been reported as lacking in several essential amino acids,

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namely cysteine and methionine [10], which are required for animal growth, and because it contains anti-nutrient substances such as hemagglutinin and trypsin inhibitor [11], its applicability for animal feeds may prove limited.

Considering that the consumption of insects has been gaining popularity in many countries, especially in Asia, Africa and Latin America [2, 12], utilization of insect rearing for sustaining feed security has been suggested by many previous researchers [e.g. 13, 14]. In this regard, the use of larvae and pupae of *Hermetia illucens* (black soldier flies) for animal feeds has been largely reported in the body of literature, attributable to its availability and nutritional benefits [13]. Despite these advantages for use as animal feeds, *H. illucens* requires a lengthy duration (weeks to months) to complete its life cycle [15], rendering longer for maintaining the colony before harvesting. In addition, the rearing process for *H. illucens* may attract unwanted public criticism regarding its cleanliness because they are commonly found in pig manure [13] and poultry manure/droppings [1]. These substrates can be disgusting to some quarters of the community and may not comply with the concept of "Halalan Tayyiba", a prevailing concept in Islamic teaching for choosing "good food for consumption and not to make halal criteria as the sole focus" [16]. Muslims comprise about 1.57 billion of the human population worldwide [17], so a special focus for addressing this issue may acquire scientific and economic importance.

In this context, Asian sea bass (*Lates calcarifer*) has been regarded as one of the high-value fishes, attributable to its nutritional contents [18]. It has been estimated that more than 60,000 tons of Asian sea bass were produced in the year 2012 alone [19]. Since the production cost for fishmeal has been astonishingly increasing, which in turn increases the overall costs for culturing fishery commodities, finding alternative resources, such as the use of necrophagous insect larvae, appears relevant.

Considering the need to sustain the continuous supplies of food for human consumption and since feeding for aquaculture/livestock commodities such as giant prawns and fishes can be expensive, the quest for exploring the possibility of using necrophagous larvae for substituting and/or supplementing feed requirements may prove to be economically pertinent. Secondly, it is pertinent to indicate that many studies do not provide clear indications on the type of rearing media used [e.g. 1, 5], instar stages of larvae analyzed [e.g. 1], and the ambient temperature at which the insects grew [e.g. 1, 5, 14]. Taking into account that those factors may directly affect the growth of the larvae as well as its composition of nutrients, and the fact that such information remains lacking in many papers, proper comparisons with those data may be difficult to accomplish. Therefore, this present study that characterized the proximate nutrient, fatty acids and amino acids compositions of the wild third instar blowfly larvae collected from fish and chicken wastes as well as its mixture at known ambient field conditions, in view of aquaculture feeding for Asian sea bass (*L. calcarifer*), merits consideration.

Material and Methods

Substrates and location of study

This research was designed in view of utilizing the wild fully grown third instar Calliphorid larvae collected on three types of wastes *viz.* mixed-fish waste substrate, chicken waste substrate and the mixed-substrates (fish and chicken wastes at 50:50) during November-December 2016 for formulating feeds for the economically important Asian Seabass. The wastes (fish offal as well as discarded chicken internal organs, head, legs and bones) were collected freshly from the nearby grocery markets at Taman Pulai Indah and Taman Universiti morning market and transported to the decomposition site in separate sealed containers.

The location used for the decomposition process was an abandoned building that was fully protected from rain with missing windows located within Universiti Teknologi Malaysia, Johor Bahru campus. The harvester was devised for facilitating the collection of Calliphorid larvae, as shown in Figure 1 below. Six units of mixed-substrates (weighing 2 kg each) were placed in a customized rectangular wooden tray (length: 100 cm; width: 65 cm; height: 80 cm) that contained a layer of wheat flour (about 1.5 cm thick). Both ends of the harvester were attached to polyvinyl chloride (PVC) funnels, channeled towards the collecting plastic containers containing about 2 cm thick of wheat flour. Prior to the analysis, the larvae were dried and transformed into a powdery form [20]. Because the majority of the wild blowfly larvae were observed to reach their fully grown size on the 5th and 6th day of decomposition, they were harvested during these days for analyses.

Figure 1. Photographs of the (a) location of the decomposition site, (b) placement of the harvester in the building and (c) its close-up view

Considering that insects like blowflies are poikilotherms [21, 22], their developmental patterns are largely dependent on ambient temperatures. Therefore, recording the ambient temperature and relative hu-
midity data, as well as total daily rainfall, acquires significance for enabling suitable comparisons to be made by other researchers.

Taxonomic identification

The fresh blowfly larvae samples were mounted following the procedure described by previous researchers [23]. For assigning the instar, observation on the posterior spiracles (number of spiracular slits) was made [21]. The taxonomic keys provided by Omar [24] and Sukontason et al. [25] were used to identify the species.

Characterization of blowfly larvae for Asian sea-bass feed formulation

Proximate analysis

Following the methods suggested by the AOAC [26], analyses of crude protein, crude fat, crude fibre, moisture and ash in the third instar larvae of blowfly larvae were performed.

Amino Acids analysis

This present research evaluated the concentrations of 17 different amino acids (aspartic acid (Asp), serine (Ser), glutamic acid (Glu), glycine (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), tyrosine (Tyr), valine (Val), lysine (Lys), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), methionine sulfone ($MetSO_2$) and tryptophan (Trp)) in the third instar blowfly larvae. With exceptions to $MetSO_2$ and Trp, the standard solutions for the other 15 amino acids (1.250, 0.625, 0.313 and 0.078 mM) were prepared by serial diluting the stock solution of amino acids (2.5 mM) with suitable amounts of HCl (0.1 N) followed by the addition of 400 µL of the internal standard each. DL-2-aminobutyric (AABA) (≥ 99% purity) at 2.5 mM was used as the internal standard. The different concentrations of $MetSO_2$ (1.250, 0.625, 0.313 and 0.078 mM) and Trp (1, 0.7, 0.5, 0.3 and 0.1 M) for constructing the calibration curves were prepared *via* serial dilutions from the stock solution of 10 mM of $MetSO_2$ and 1 M of Trp.

The analysis of 16 amino acids as well as Trp were conducted using HPLC. The ranges for calibration curves for the 16 amino acids (including MetSO2) and Trp were 0-1.250 mM and 0-1 mM, respectively. The obtained calibration curve was accepted when the coefficient of determination (R^2) was ≥ 0.995 and the % relative standard deviation (%RSD) was less than 20% [27]. While the limit of detection (LOD) is the lowest concentration of an analyte determined with a noise ratio of at least 3 : 1, the limit of quantitation (LOQ) refers to the lowest concentration of an analyte in a calibration curve with signal to noise of at least 10 : 1 [27].

Fatty Acid analysis

Analysis of fatty acid of the harvested larvae from the three different substrates (mixed-fish waste substrate, chicken waste substrate and mixed-substrate (50:50)) was conducted by a certified MS ISO/IEC 17025 private laboratory using Gas chromatography-flame-ionization detection (FID) (in-house method, No. G3).

Formulation for Asian sea bass feed and its costing

To enable feeding trials to be attempted by future researchers on Asian sea bass, three theoretical formulations using wild blowfly larvae harvested in this present research were calculated following the FAO's suggested feed ingredients [19]. The ingredients included crude protein, crude fat, wheat meal and rice bran. For exploring the economics of using blowfly larvae as feed for Asian sea bass, a comparison of direct pricing, as suggested by Nor *et al.* [28], with that of fishmeal in Malaysia was also made.

Statistical analysis

Data analyses were conducted using the IBM SPSS version 20 software. One way ANOVA with Tukey-Kramer post hoc test was used for comparing the differences in the proximate composition of moisture, crude protein and crude fibre among the larvae that fed on the three kinds of waste substrates *i.e.* fish and chicken for rearing the species [29].

Results and Discussion

Species of wild blowfly larvae and their taxonomic identifications

It was observed that three species of wild necrophagous blowflies (*viz. Chrysomya megacephala*, *Chrysomya rufifacies* and *Hemipyrellia tagaliana*) infested all three different substrates, with *C. megacephala* and *C. rufifacies* being the more dominant species. This finding was consistent with that reported by Zulkifli [30], who investigated the composition and life cycle of necrophagous flies in Johor.

In this research, the larvae were reared in an abandoned concrete building that was fully protected from rain with open windows. It was observed that the mean ambient temperature, mean relative humidity and total daily rainfall during 12^{th}- 17^{th} November 2016 ranged between 24.6-29.5°C, 80.1-91.0% and 2.1-13.1 mm, respectively. As for the period of 15^{th}-20^{th} December 2016, the same were 27.5-29.1°C, 76.6-93.2% and 1.3-23.1 mm, correspondingly. The small variations observed in the mean ambient temperature, mean relative humidity and total daily rainfall during this research were consistent with the prevailing environmental conditions for the low land of Peninsular Malaysia [31]. Therefore, it can construed that the infesting wild necrophagous blowfly larvae had developed at similar ambient conditions throughout the six days of the rearing process. Necrophagous insects are poikilotherms (unable to generate body heat), and they grow faster at higher temperatures. Recording the ambient temperature, relative humidity, and total daily rainfall data would provide the underlying condition at which the larvae grew. Such an aspect is important for standardizing the proximate amino acid and fatty acid contents discussion.

Nutritional characterization of the wild blowfly larvae

Proximate analyses of the third instar larvae

While the optimum crude protein and fat contents vary with levels and sizes of the fish, the percentages have been reported to range between 40-55% [32, 33] and 18-23% [32, 33], respectively. Similar to other fishes, the specific requirement for dietary carbohydrates for Asian sea bass remains unreported [34, 35]. Being a carnivorous species, Asian sea bass has been shown to have limited capacity in utilizing dietary carbohydrates for energy, demonstrating limited glycaemic control [34]. Nonetheless, better weight gain was observed in the fish fed with 20% than that of 15% carbohydrates [35]. Therefore, it has been proposed that feeds meant for carnivorous fishes should contain about 20% soluble carbohydrates instead of 25-45% for omnivorous species [34].

While specific requirement for crude fibre in feeds for Asian sea bass has not been reported, the general amount suggested for fishes is less than 7% of the diet to minimize the amount of undigested material entering the culture system [34, 36]. Similarly, specific requirement for ash in feeds for Asian sea bass remains lacking; however, the general percentage of ash in fishmeal is about 10% [37]. Data on proximate analyses in the fully grown wild third instar larvae of blowfly are presented in Table 1. It was observed that the larvae harvested from the mixed-substrates (that contained mixed-fish waste substrate and chicken waste substrate (ratio of 50:50)) had the highest proportion of crude protein (51.47 ± 0.32%, $P < 0.05$) when compared with that of mixed-fish waste substrate (36.84 ± 0.37%) and chicken waste substrate (45.14 ± 0.11%) alone. The highest proportion of crude protein in the mixed-substrates can be attributable to complementary contributions of protein from both types of wastes. Moreover, significantly higher proportion of crude protein was observed in the larvae that infested chicken waste substrates ($P < 0.05$) than that of mixed-fish waste substrates.

Interestingly, the proportions of crude protein observed in the wild blowfly larvae analyzed here were observably higher than that of *H. illucens* larvae reported by previous researchers [5] , signifying their high potential to be used as protein sources. The proportions of crude protein reported here were lower than that of commercially available fishmeal products (58.7-73%) reported in the

Table 1. Proximate composition of the fully grown wild third instar blowfly larvae harvested

Proximate Compositions (%)	Mixed- fish waste Substrate	Chicken waste Substrate	Mixed-fish and chicken wastes Substrate (50:50)	Comparisons of Proximate Compositions among the three groups of Substrates		
	(A)	(B)	(C)	(A) vs (B)	(A) vs (C)	(B) vs (C)
Crude Protein	36.84 ± 0.37	45.14 ± 0.11	51.47 ± 0.32	P < 0.05	P < 0.05	P < 0.05
Crude Fat	27.5 ± 1.38	24.7 ± 1.24	29.4 ± 1.47	P > 0.05	P > 0.05	P < 0.05
Carbohydrate	29.56 ± 1.02	25.09 ± 1.23	12.71 ± 1.67	P < 0.05	P < 0.05	P < 0.05
Crude Fibre	1.65 ± 0.32	0.51 ± 0.15	4.81 ± 0.83	P > 0.05	P < 0.05	P < 0.05
Ash (%)	4.98 ± 0.05	4.00 ± 0.03	4.85 ± 0.01	P < 0.05	P < 0.05	P < 0.05

Comparisons of the proximate compositions of the larvae harvested were performed using ANOVA with Tukey-Kramer post hoc test. Level of significance of 0.05 was used for assigning the significant differences among the different groups of rearing substrates (*viz* mixed-fish waste substrate, chicken waste substrate, and mixed-substrates). The data are presented as mean ± standard deviation.

literature [2, 5]. However, the crude protein proportions in the wild larvae (36.84-51.47%) appear to be adequate not only for the Asian sea bass (40-55%) [38], but also broiler chickens (18-23%), layer chickens (12.5-18.8%), turkeys (14-28%) and ducks (15-22%) [39]. Similar to crude protein, the highest proportion of crude fat was also observed for larvae from the mixed-substrates (29.40 ± 1.47%, P < 0.05) when compared with the remaining two others. As for the larvae from mixed-fish waste substrate and chicken waste substrates, the proportions of crude fat were 27.50 ± 1.38% and 24.7 ± 1.24%, respectively. Considering the proportions of crude protein and crude fat required for the Asian sea bass feed that ranged between 40-55% [38] and 18-23% [35], respectively, the larvae on the mixed as well as chicken wastes substrates had sufficient amounts of these nutrients. Therefore, such larvae harvested from these two substrates can be considered as potential candidates for replacing fishmeal for the Asian sea bass. Although the proportion of crude protein in larvae from the mixed-fish waste substrate was slightly lower than the required proportions for the Asian sea bass, partial replacement of fishmeal may still be possible.

It was evident that the larvae from the mixed-fish waste substrate (29.56 ± 1.02%, P < 0.05) had the highest proportions of carbohydrates, followed by the chicken wastes (25.09 ± 1.23%) and mixed-substrates (12.71 ± 1.67%). Importantly, while the Asian sea bass may be able to absorb soluble carbohydrates like glucose quickly, the same may not be the case for the complex ones [33, 35]. Since the amount of soluble carbohydrates in the form of glycogen in animal tissues (like blowfly larvae) is considered small [34] and because the Asian sea bass has limited capacity at digesting complex carbohydrates [33, 35], the amount of these nutrients in feeds meant for this economically important fish must be restricted. Since the proportion of carbohydrates in feeds for fishes like the Asian sea bass should not exceed 20% [34], and because the wild blowfly larvae obtained from the mixed-substrates had only 12.71% while having adequate amount of crude protein and crude fat, their usefulness for formulating feeds for this important seafood commodity could not be ruled out. The fact that higher proportions of carbohydrates were observed for wild blowfly larvae in the remaining two substrates, their practical values for formulating feeds for omnivorous animals can therefore be suggested.

As for crude fibre, the lowest proportion was observed in wild blowfly larvae from the chicken waste substrates (0.51 ± 0.15%, P < 0.05) when compared with that of mixed-fish waste substrate (1.65 ± 0.32%) and mixed-fish and chicken waste substrate (4.81 ± 0.83%). Considering that the proportions of crude fibre observed in wild blowfly larvae from the three different substrates did not exceed the recommended 7% [34, 36], and since the ash content also fell well within the acceptable limit of about 10% [37], suitability of the larvae to be used for aquaculture feeds is supported. While higher crude fibre (> 7%) may lead to more undigested material in the culture system [34], ash content has been associated with the presence of minerals like phosphorus and calcium [40]. Therefore, further studies focusing on the compositions of minerals in wild blowfly larvae, in view of its practical values in animal feeds formulation, may prove interesting.

Table 2. Analytical figures of merit (Calibration range, R2, regression equations, LODs and LOQs) for the 17 amino acids in the fully grown wild third instar blowfly larvae

Amino acids	Range of CalibrationCurves (mM)	Calibration Parameters		LOD (mM)	LOQ (mM)	Relative Standard Deviation (%)
		Coefficient of Determinants (R^2)	Equation			
Aspartic acid	0.078-1.25	0.999	y = 76086x + 362.05	0.004	0.015	12.99
Serine	0.078-1.25	0.996	y = 64584x + 1995.9	0.014	0.045	11.30
Glutamic acid	0.078-1.25	0.999	y = 78195x - 535.19	0.004	0.014	14.67
Glycine	0.078-1.25	0.997	y = 62468x + 2679.5	0.002	0.008	7.15
Histidine	0.078-1.25	0.997	y = 68212x + 3942.9	0.013	0.043	10.42
Arginine	0.078-1.25	0.995	y = 58575x + 3795.8	0.009	0.029	15.98
Threonine	0.078-1.25	0.996	y = 78481x + 343.71	0.008	0.027	14.27
Alanine	0.078-1.25	0.998	y = 77813x + 1373	0.009	0.029	7.99
Proline	0.078-1.25	0.995	y = 88453x + 3386	0.026	0.087	16.88
Tyrosine	0.078-1.25	0.997	y = 65486x + 1008.7	0.010	0.035	8.94
Valine	0.078-1.25	0.997	y = 86846x + 320.87	0.003	0.010	9.73
Lysine	0.078-1.25	0.998	y = 61324x + 1725.3	0.002	0.006	6.30
Isoleucine	0.078-1.25	0.995	y = 85630x + 3673.4	0.015	0.049	10.94
Leucine	0.078-1.25	0.999	y = 83928x + 3206.4	0.013	0.043	10.90
Phenylalanine	0.078-1.25	0.998	y = 81291x + 2153.4	0.013	0.045	12.82
Methionine Sulfone	0.078-1.25	0.998	y = 43968x + 2288.5	0.005	0.018	12.68
Tryptophan	0.100-1.00	0.995	y = 48262x - 522.96	0.007	0.022	14.16

Amino acid compositions of the third instar larvae

Since the analysis of amino acids in the wild third instar blowfly larvae was performed at the Johor Fishery Research Institute, Ministry of Agriculture and Agro-Based Industry Malaysia following the validated analytical method and instrumentation, performing the complete validation analytical procedure, once again, appears irrelevant. Therefore partial validation alone was performed and the analytical figures of merit as well as the LODs and LOQs for all the 17 amino acids (Asp, Ser, Glu, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Lys, Ile, Leu, Phe, MetSO2 and Trp) in the wild third instar blowfly larvae analyzed here are presented in Table 2. Following the criteria suggested by previous researchers [27, 41], each calibration curve was only accepted when the R2 ≥ 0.995 with RSD < 20%. Considering that all the calibration curves for amino acids obtained in this present research adhered fully to the prescribed criteria, linear responses can be inferred, advocating the suitability of analytical method used for determining amino acids in the larvae. Using the data from

Table 3. Amino acids Compositions of the fully grown wild third instar blowfly larvae harvested

Amino acids Compositions (%)	Mixed- fish waste Sub-strate	Chicken wasteSub-strate	Mixed-fish and chicken wastes (50:50) Sub-strate	Comparisons of Amino acids Compositionsamong the three groups of Substrates		
	(A)	(B)	(C)	(A) vs (B)	(A) vs (C)	(B) vs (C
Aspartic acid	3.79 ± 0.17	3.57 ± 0.20	4.96 ± 0.18	P > 0.05	P < 0.05	P < 0.05
Serine	3.58 + 0.16	3.85 ± 0.29	5.26 ± 0.36	P > 0.05	P < 0.05	P < 0.05
Glutamic acid	6.76 ± 0.52	6.73 ± 0.49	8.00 ± 0.61	P > 0.05	P > 0.05	P > 0.05
Glycine	2.55 ± 0.13	2.79 ± 0.25	4.07 ± 0.23	P > 0.05	P < 0.05	P < 0.05
Histidine*	1.91 ± 0.18	2.47 ± 0.20	3.65 ± 0.22	P < 0.05	P < 0.05	P < 0.05
Arginine*	6.51 ± 0.33	5.77 ± 0.33	8.55 ± 0.66	P > 0.05	P < 0.05	P < 0.05
Threonine	2.56 ± 0.12	2.32 ± 0.20	3.94 ± 0.31	P > 0.05	P < 0.05	P < 0.05
Alanine	5.11 ± 0.36	4.69 ± 0.28	6.03 ± 0.37	P > 0.05	P < 0.05	P < 0.05
Proline	1.91 ± 0.20	1.69 ± 0.11	2.47 ± 0.15	P > 0.05	P < 0.05	P < 0.05
Tyrosine	3.74 ± 0.34	4.18 ± 0.53	5.69 ± 2.09	P > 0.05	P > 0.05	P > 0.05
Valine*	5.48 ± 0.26	5.39 ± 0.44	6.21 ± 0.64	P > 0.05	P > 0.05	P > 0.05
Lysine*	5.37 ± 0.38	6.46 ± 0.54	8.05 ± 0.44	P > 0.05	P < 0.05	P < 0.05
Isoleucine*	4.79 ± 0.44	4.12 ± 0.29	6.59 ± 0.24	P > 0.05	P < 0.05	P < 0.05
Leucine*	7.94 ± 0.48	6.71 ± 0. 79	9.34 ± 1.16	P > 0.05	P > 0.05	P < 0.05
Phenylalanine*	6.63 ± 0.46	6.00 ± 0.35	8.30 ± 0.96	P > 0.05	P < 0.05	P < 0.05
Methionine sul-fone*	1.87 ± 0.12	1.41 ± 0.15	2.61 ± 0.12	P < 0.05	P < 0.05	P < 0.05
Tryptophan*	0.64 ± 0.03	0.51 ± 0.01	0.89 ± 0.15	P < 0.05	P < 0.05	P < 0.05

ANOVA with Tukey Kramer post hoc test was used for comparing the concentrations of amino acids in wild third instar Calliphorid larvae harvested from the three groups of substrates. Level of significance of 0.05 was used for assigning the statistical significance. The data are presented as mean ± standard deviation.

calibration curves, the LODs (signal to noise ratio, 3:1) and LOQs (signal to noise ratio, 10:1) for Asp, Ser, Glu, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Lys, Ile, Leu, Phe, MetSO2 and Trp were calculated to range between 0.002 – 0.026 mM and 0.006 – 0.087 mM, respectively (Table 2).

Analysis on amino acid compositions in the fully grown wild third instar blowfly larvae using RP-HPLC with fluorescence detector revealed the presence of 17 different amino acids, with 9 of which being essential ones. The essential amino acids were Met (in the form of Methionine sulfone (MeSO2)), Lys, Arg, Trp, His, Val, Ile, Leu and Phe. The remaining amino acids included Asp, Ser, Glu, Gly, Thr, Ala, Pro and Tyr (Table 3).

It was observed that the larvae harvested from the mixed-substrates had significantly the highest amount of Trp (P < 0.05; mean: 0.89 ± 0.15%) when compared with those from mixed-fish waste (mean: 0.64 ± 0.03%) and chicken waste (mean: 0.51 ± 0.01%) substrates, individually. Although no statistical significant difference was observed in the larvae harvested from mixed-fish waste with that of chicken waste, significantly higher amount of Lys (P < 0.05) was observed in the larvae harvested from mixed-substrates (mean: 8.05 ± 0.44%) when compared with that of mixed-fish waste (mean: 5.37 ± 0.38%) and chicken waste (6.46 ± 0.54%) substrate individually. Similarly with Arg, larvae harvested from mixed substrate (mean: 8.55 ± 0.66%) were significantly higher (P < 0.05) when compared with the remaining 2 substrates (mixed-fish waste: mean: 6.51 ± 0.33%; chicken waste: 5.77 ± 0.33%).

Following the same pattern, statistical significance was observed in the amounts of MetSO2 in the wild third instar blowfly larvae harvested from all the different substrates (P< 0.05). While 2.61 ± 0.12 % of Met was found in the wild blowfly larvae from mixed- substrates, the same was found at 1.87 ± 0.12% and 1.41 ± 0.15% for mixed-fish waste and chicken waste substrate, correspondingly. With an exception to the findings reported by Makkar et al. [2] that utilized cattle manure as substrate for BSFL, the results on the four essential amino acids in the wild third instar blowfly larvae obtained here were found higher than that of BSFL [13]. Interestingly, review of literature reveals that the amounts of Trp, Lys, Arg and Met in BSFL harvested from manures [2, 13] were considerably higher when compared with those from chicken feed, vegetable waste and restaurant waste

Table 4. Fatty Acids Compositions of the fully grown wild third instar blowfly larvae harvested

Fatty acids Compositions (%)	Mixed- fish waste Substrate	Chicken waste Substrate	Mixed-fish and chicken wastes (50:50) Substrate	Comparisons of Fatty acids Compositions among the three groups of Substrates		
	(A)	(B)	(C)	(A) VS (B)	(A) VS (C)	(B) VS (C)
Oleic Acid C18:1n-9	6.54 ± 0.33	11.50 ± 0.58	8.68 ± 0.43	P < 0.05	P < 0.05	P < 0.05
Palmitic acid C16:0	10.00 ± 0.50	8.88 ± 0.44	6.97 ± 0.35	P < 0.05	P < 0.05	P < 0.05
Linoleic acid C18:2n-6[b]	5.33 ± 0.27	10.81 ± 0.54	13.11 ± 0.66	P < 0.05	P < 0.05	P < 0.05
Stearic acid C18:0	1.97 ± 0.10	1.14 ± 0.06	1.60 ± 0.08	P < 0.05	P < 0.05	P < 0.05
Myristic acid C14:0	1.73 ± 0.09	0.18 ± 0.01	0.38 ± 0.16	P < 0.05	P < 0.05	P > 0.05
Eicosenoic acid C20:2	0.08*	0.06	0.14 ± 0.01	P < 0.05	P < 0.05	P < 0.05
Erucic Acid C22:1n-9	0.10 ± 0.01	ND	0.30 ± 0.02	NA	P < 0.05	NA
Docasahexaenoic acid (DHA)[a] C22:6n-3	0.44 ± 0.02	ND	1.08 ± 0.05	NA	P < 0.05	NA
Pentadecanoic acid C15:0	0.22 ± 0.01	ND	0.07	NA	P < 0.05	NA
Palmitoleic acid C16:1	0.26 ± 0.01	ND	0.13 ± 0.01	NA	P < 0.05	NA
Cis-heptadecoic acid C17:1	2.88 ± 0.14	ND	1.83 ± 0.09	NA	P < 0.05	NA
Heneicosanoic acid C21:0	0.12 ± 0.01	ND	0.14 ± 0.01	NA	P < 0.05	NA
Behenic acid C22:0	0.07*	ND	0.11	NA	P < 0.05	NA
Caproic acid C6:0	0.12 ± 0.01	ND	0.03	NA	P < 0.05	NA
Eicosapentaenoic acid (EPA)[a] C20:5n-3	0.74 ± 0.04	ND	1.28 ± 0.06	NA	P < 0.05	NA
Gamma Linolenic acid C18:3n-6	0.08*	0.10 ± .01	NA	P < 0.05	NA	NA
Lauric Acid C12:0	0.87 ± 0.04	ND	ND	NA	NA	NA
Caprylic acid C8:0	0.18 ± 0.01	ND	ND	NA	NA	NA
Capric acid C10:0	0.09*	ND	ND	NA	NA	NA
Heptadecanoic acid C17:0	0.32 ± 0.02	ND	0.35 ± 0.05	P < 0.05	NA	NA
Elaidic acid C18:2n-6	0.38 ± 0.01	ND	0.39 ± 0.02	NA		
Arachidic acid (ARA)[b] C20:0	0.017*	ND	0.18 ± 0.01	NA		
Alpha Linolenic acid (LNA)[b] C18:3n-3	0.22 ± 0.01	ND	0.23 ± 0.01	NA		

[a] Represents essential fatty acids for Asian sea bass [32 ,45]

[b] Represents fatty acids suggested to be classified as essential for Asian sea bass [45]

* Data presented as sole value since variations in values were not observed in the different replicates of larvae analyzed.

ANOVA with Tukey Kramer post hoc test was used for comparing the concentrations of fatty acids in wild third instar Calliphorid larvae harvested from the three groups of substrates. Independent samples t-test was used for certain fatty acids that were only detected in two of the groups. Level of significance of 0.05 was used for assigning the statistical significance. The data are presented as mean ± standard deviation.

[42]. Notwithstanding, the amounts of Trp, Lys and Arg reported here were also comparable with those in fishmeal [4], except for Met. It is pertinent to indicate here that while the amounts of Trp, Lys and Arg observed here had exceeded the amounts prescribed for Asian Seabass (Trp: 0.4-0.5 %; Lys: 4.9 %; Arg: 3.8%), the same was slightly lower for Met (2.2 %) harvested from mixed-fish waste and chicken waste substrate individually [35].

While significant differences in the amounts of Val in the wild third instar blowfly larvae harvested from the three different substrates were not observed ($P > 0.05$), significantly higher amount of His (2.17 ± 0.16%), was observed in larvae from mixed-fish waste substrate ($P < 0.05$) when compared with the remaining two others. Significantly higher Phe (8.30 ± 0.96%) were observed in larvae from mixed-fish waste substrate ($P < 0.05$) when compared with that of larvae from chicken waste substrate alone. On the other hand, significantly lower amount of Leu (6.71 ± 0.79%) was observed in larvae from chicken waste substrate ($P < 0.05$) when compared with that of larvae harvested from mixed-substrates. The mean amounts of Val, Lys, Ile, Leu and Phe observed in wild third instar blowfly larvae from all the three different substrates ranged between 5.39-6.21%, 5.37-8.05%, 4.12-6.59%, 6.71-9.34%, 6.00-8.30%, respectively (Table 3). Considering that the specific requirements for these essential amino acids for fishes remain lacking, suitable comparisons and discussions with the findings reported here could not be attempted. Since all the wild third instar blowfly larvae analyzed here had sufficient amounts of Trp, Lys and Arg, with slightly lower amount of Met (harvested from mixed-fish waste and chicken waste substrate individually), its usefulness for formulating feeds for Asian sea

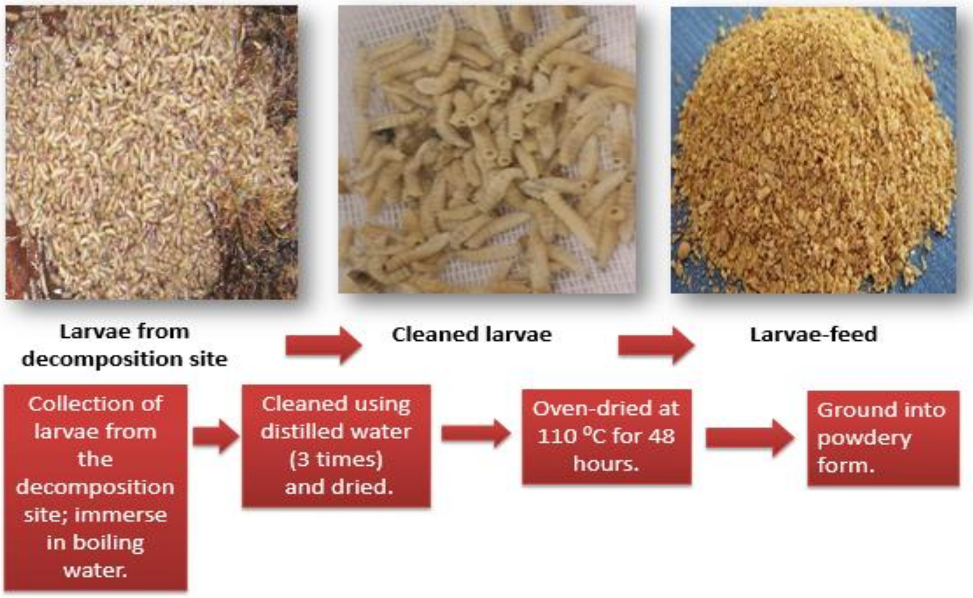

Figure 2. Stages in the production of Calliphorid larvae meal

bass or at least partially replacing the use of fishmeal, cannot be neglected.

Fatty acid compositions of the third instar larvae

Interestingly, although essential fatty acids namely EPA and DHA (both known as lcPUFA) as well as LNA are important to promote positive feeding behavior that leads to better growth in fishes [35], specific studies at examining their requirements for diadromous fishes like Asian sea bass remain limited. In this present research a total of 23 different fatty acids were detected in the wild third instar blowfly larvae, three of which being essential fatty acids *viz.* LNA, EPA and DHA (Table 4). The remaining fatty acids found included Oleic acid, Palmitic acid, Linoleic acid, Stearic acid, myristic acid, Eicosenaoic acid, Erucic acid, Pentadecanoic acid, Palmitoleic acid, Cis- heptadecoic acid, Heneicosanoic acid, Behenic acid, Caproic acid, Gamma Linolenoc acid, Lauric acid, Caprylic acid, Capric acid, Heptadecanoic acid, Elaidic acid and Arachidic acid (ARA) (Table 4).

Results revealed that all the 23 fatty acids were recovered in wild third instar blowfly larvae harvested from the mixed-fish waste substrates. As for the wild third instar blowfly larvae reared on chicken waste substrates alone, 16 of the fatty acids (*viz.* Erucic acid, Docasahexaenoic acid, pentadecanoic acid, Palmitoleic acid, Cis-heptadecoic acid, Heneicosanoic acid, Behenic acid, Caproic acid, Eicosaoentaenoic acid, Lauric acid, Caprylic acid, Capric acid, Heptadecanoic acid, Elaidic acid, Arachidic acid and Alpha Linolenic acid) remained undetected. This finding concurs with the indications made by St-Hilare *et al.* [43] that 'black soldier fly prepupae incorporate a-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) when fish offal is included in their diet' and the fact that such fatty acids were found lower in the larvae when reared on cow manure. Significantly higher amounts of DHA (1.08 ± 0.05%) and EPA (1.28 ± 0.06%) were observed in wild third instar blowfly larvae reared on mixed-substrate ($P < 0.05$) when compared with that of mixed-fish waste substrates alone (DHA: 0.44 ±0.02%; EPA: 0.74 ± 0.04%). The amount of DHA in wild third instar blowfly larvae reared on mixed-substrate was found closely marginal to the 1.15% amount prescribed by previous researchers [35].

Comparison made with the previously reported studies on BSF on varying substrates [42, 43] reveals lower amounts of EPA (0.01 – 0.35%) and DHA (0.01 – 0.17%) than that of the same for wild third instar blowfly larvae reported in this present research. Since the amount of EPA in wild third instar blowfly larvae from the mixed-substrate (1.28 ± 0.06%) had exceeded the prescribed amount of 0.75% [43], and can potentially become toxic to Asian sea bass, extracting this excessive amount of EPA from such larvae may prove to be a prudent approach. The extracted excessive EPA can later be used for other applications such as in nutraceutical and food additives industries [44]. Considering that the addition of 1% of ARA has led to many undesired effects on juvenile Asian sea bass [45], and since the means of ARA found in the wild third instar blowfly larvae for both the mixed-fish waste and mixed-substrate reported here ranged between 0.17- 0.18%, the negative impact of consuming wild third instar blowfly larvae by the Asian sea bass appears limited. Because the means of LNA in such larvae harvested from both the mixed-fish waste (0.22 ± 0.01%) and mixed-fish and chicken waste (0.23 ± 0.01%) substrates were lower than the prescribed amount of 0.45% for Asian sea bass [35], further studies for optimizing the quality of wild third instar blowfly larvae harvested maybe required. The fact that insects like the wild third instar blowfly larvae are poikilotherms [46] optimizing the rearing conditions particularly the ambient temperature by which the larvae are growing can potentially address this issue.

Formulation of potential feeds for Asian sea bass using the wild third instar blowfly larvae meal

Whereas 40-55% of protein and 18-23% of fat have been recommended for formulating feeds for rearing Asian sea bass [32, 33, 35, 38] the mid-ranges of these two nutrients were chosen for constructing the three theoretical formulations (Diets 2, 3 and 4) based on the three different substrates utilized in this present research (Table 5). Figure 2 showed the stages in the production of Calliphorid larvae meal. While formulating the diets, specific indications made by the National Research Council (NRC) (2011) that the inclusion of fishmeal, wheat meal and rice bran must not be more than 60%, 25% and 15%, accordingly, were considered [47]. Moreover, the diets must also contain fixed values of dicalcium phosphate, choline chloride, ascorbic acid, vitamin premix and carrageenan at 0.01%, 0.1%, 0.05%, 0.5% and 2.5%, correspondingly.

Diet 2 formulated from dried wild third instar blowfly larvae collected from the mixed-substrates that constituted 57.0% of the total diet had provided the estimated of 46.6% of crude protein, 20.1% of crude fat and 19.3% of carbohydrate (in the form of nitrogen free extract, NFE). In addition to low estimated ash (9.3%) and fibre (3.8%), the diet only required 19.0% and 0.5% of fishmeal and fish oil, respectively, with the protein contribution from the dried larvae being estimated at 75% of the total. As for the Diet 3 formulated from the chicken waste substrate, dried blowfly meal and fishmeal constituted 56.0% and 27.0% of the total Diet 3, correspondingly. While Diet 3 also required low percentage of fish oil (2.0%), the protein contribution from the dried wild third instar blowfly larvae to the diet was estimated at 67.5%. It was estimated that the Diet 3 would provide 47.0%, 19.5%, 19.2%, 9.9% and 1.3% of crude protein, crude fat, carbohydrates (NFE), ash and fibre, correspondingly. In addition, dried blowfly meal constituted 39.0% of the total ingredient for Diet 4 (mixed-fish waste substrate); 43.0% and 3.0% of the diet being the fishmeal and fish oil, respectively. The protein contribution from the dried wild third instar blowfly larvae for Diet 4 was estimated at 47.6%.

To put all these three different diets into perspective, the control diet (Diet 1) that was formulated by totally factoring out the dried blowfly meal would require 59.0% of fishmeal and 12.3% of fish oil. This has resulted in 48.1% of crude protein, 19.9% of crude fat, 19.6% of carbohydrates (NFE), 12.7% of ash and 1.4% of fibre. Because the percentages of crude protein, crude fat and carbohydrates (NFE) as well as fibre calculated for Diets 2, 3 and 4 fell well within the suggested percentages for formulating feeds for the Asian sea bass [32, 33, 35, 38], they can be construed as potential candidates for partial replacements of fishmeal. Therefore, further studies involving the feeding trial utilizing these three different diets for culturing Asian sea bass prove necessary to elucidate their real potential in the field.

Table 6 represents the costing for each ingredient used in the experimental diet formulations. At the moment of writing, the overall cost for fishmeal in Malaysia was MYR 17.00 per kg [4]; the same was only estimated at MYR 1.84 for 1 Kg of dried blowfly meal. It was estimated that the total cost for producing Diets 1 (control), 2 (mixed-substrates, 57.0% replacement of diet), 3 (chicken waste substrate, 56% replacement of diet) and 4 (mixed-fish waste substrate, 39% replacement of diet) were MYR 18.38, MYR 9.98, MYR 11.33 and MYR 13.34, respectively. The total costs for producing Diets 2, 3 and 4 were 45.7%, 38.4% and 27.4% cheaper, correspondingly, when compared with that of Diet 1 that devoid of any inclusion of dried blowfly meal. Considering the favorable nutritional compositions and cost-effectiveness, the usefulness of Diet 2 formulation for Asian sea bass culturing appears empirically supported.

Table 5. Formulations for Asian Sea bass feed

Diets	Diet 1	Diet 2	Diet 3	Diet 4
Ingredients (%)				
Dried Calliphorid meal	-	57	56	39
Danish Fishmeal	59	19	27	43
Wheat Meal	17	11.8	3.3	3.3
Rice Bran	5	5	5	5
Fish oil	12.3	0.5	2.0	3.0
Dicalcium phosphate	1.0	1.0	1.0	1.0
Choline Chloride	0.1	0.1	0.1	0.1
Vitamin C	0.1	0.1	0.1	0.1
Vitamin Mix	1.5	1.5	1.5	1.5
Mineral Mix	1.5	1.5	1.5	1.5
Carrageenan	2.5	2.5	2.5	2.5
Protein Contribution from Calliphoridlar-vae (%)	0.0	75.0	67.5	47.6
Estimated Proximate Composition of each diet (%)				
Crude Protein	48.1	46.6	47.0	46.5
Crude Fat	19.9	20.1	19.5	19.9
Nitrogen Free Extract	19.6	19.3	19.2	18.8
Ash	12.7	9.3	9.9	11.6
Fibre	1.4	3.8	1.3	1.8

Nutritional requirements for Asian seabass feed: Crude protein: 40-55% [32, 33, 35]; Crude fat: 18-23% [32, 35]; Carbohydrates: 15-20% [35]. Diet 1 (Fishmeal,Control), Diet 2 (Calliphorid larvae reared on mixed-substrates), Diet 3 (Calliphorid larvae rearedon chicken waste substrate), and Diet 4 (Calliphorid larvae reared onmixed-fish wastesubstrate).

Table 6. Costing for each ingredient used in the experimental diet formulations

Ingredients/Diet	Cost/Kg (MYR)	Diet 1 (cost in MYR/Kg)	Diet 2 (cost in MYR/Kg)	Diet 3 (cost in MYR/Kg)	Diet 4 (cost in MYR/Kg)
Danish fishmeal	17.00	590 g (10.03)	190 g (3.23)	270 g (4.59)	390 g (6.63)
Dried Calliphoridmeal	1.84	-	570 g (1.05)	560 g (1.03)	430 g (0.79)
Wheat meal	3.60	170 g (0.61)	118 g (0.43)	33 g (0.12)	33 g (0.12)
Rice bran	1.00	50 g (0.05)	50 g (0.05)	50 g (0.05)	50 g (0.05)
Fish oil	20.80	123 g (2.56)	5 g (0.10)	20 g (0.42)	30 g (0.624)
Dicalciumphosphate	1.55	10 g (0.02)	10 g (0.02)	10 g (0.02)	10 g (0.02)
Choline chloride	1.55	1 g (0.002)	1 g (0.002)	1 g (0.002)	1 g (0.002)
Vitamin C	160.00	1 g (0.16)	1 g (0.16)	1 g (0.16)	1 g (0.16)
Vitamin mix	110.00	15 g (1.65)	15 g (1.65)	15 g (1.65)	15 g (1.65)
Mineral mix	20.00	15 g (0.30)	15 g (0.30)	15 g (0.30)	15 g (0.30)
Carrageenan	120.00	25 g (3.00)	25 g (3.00)	25 g (3.00)	25 g (3.00)
Total (MYR/kg)	**-**	**18.38**	**9.98**	**11.33**	**13.34**

Assumptions:

a. Cost for each ingredient used in the experimental diet formulation in 2018.
b. About 12 kg of wastes were required to produce 2.58 kg of wet larvae (equivalent to approximately 650 g of dried larvae). Therefore, 18.4 kg of waste was required to produce 1 Kg of dried larvae.
c. Since the price of 1 kg of wastes purchased from local groceries was MYR 0.10, the total cost of MYR 1.84 was required for producing 1 kg of dried larvae.

Conclusion

Considering that the blowfly larvae harvested from the mixed- substrates (50:50) on the 5th and 6th day of decomposition had the most appropriate amounts of crude protein, crude fat, carbohydrates, crude fiber, essential amino acids and fatty acids, its utilization for formulation of feeds for Asian sea bass appears empirically supported. The blowfly larvae meal investigated here were cost efficient for Asian sea bass feeding and environmentally friendly. This would be of applied values in aquaculture, paving the way for reducing dependency towards the endangered pelagic fishes as the main source for nutrients.

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