Black Soldier Fly Larvae Feed with Fermented Bamboo Culms Enriched with Lactic Acid Bacteria

Nurul Azila Abdul Razak [1], Ang Chung Huap [1*], Vivien Jong Yi Mian [1], Siti Nor Ain Seri Masran [2]

Abstract

Black soldier fly (BSF) larvae have been a promising protein source use in fish and poultry meal effectively replacing plant-based protein source. Currently, there is no study on the effects of fermented bamboo culms by lactic acid bacteria to improve the nutrition of BSF larvae. The main objective of this study is to determine the protein:fat ratio and growth rate of BSF larvae fed with fermented bamboo culm fibres (*Bambusa beecheyana*) enriched with lactic acid bacteria. *Lactobacillus plantarum* and *Brevibacillus parabrevis* were successfully isolated from BSF larvae gut aged 16 days and fermentation of bamboo culms fibre were successfully carried out for 21 days. Our results showed the plot VEG217 consist of *Lactobacillus plantarum* and *Brevibacillus parabrevis* (1:1) mixed with fermented bamboo culms fibre and vegetable waste have greater average weight (111%) and length (30%) compared to negative control that only consist of BSF larvae fed with vegetable waste. Interestingly, BSF larvae fed with fermented bamboo enriched with lactic acid bacteria also puparied in much shorter time (less than 13 days) compared to negative control (18 days). All the larvae fed with fermented bamboo and lactic acid bacteria also showed improvement in the protein and fat ratio when compared to negative control (5:3). The plot VEG111 consist of Brevibacillus parabrevis mixed with fermented bamboo and vegetable wastes have the best protein:fat ratio (4:1). This result shows the potentioal of using fermented bamboo culm fibres enriched with lactic acid bacteria to improve the growth rate of BSF larvae.

Keywords: Black soldier fly larvae, Fermented bamboo culms, Lactic acid bacteria, Protein fat ratio, Growth rate

Significance | The study of plant-based protein source as the nutrition of Black soldier fly

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Introduction

Food security has been the great concern globally due to over-development, human migration, climate change and war which has led to the reduction of sustainability in food production for human and animal feeds. The demand of food supply increases in consequence of increase in population size, which someday may cause global food shortage (Jambo *et al.* 2021). Besides, climate change and over-development has been the factor to this issue leading to the shrinkage of fertile agriculture land. As the standard of living persistently increasing, cities have become more populated as a result of human migration. In Fact, this has been a serious constraint for farmers due to lack of manpower (Salami *et al.* 2021) hence leading to severe food shortage. Other than that, the current 2022 Rusia Invasion of Ukraine also a factor contributes to the food insecurity which has severely affecting the global food supply as well as animal feed. Impact, the prolonged food shortage will greatly cause inaccessibility, inadequate purchasing power, natural disaster caused by unsustainable

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agriculture practices, poverty, as well as improper utilization and distribution of food according to current studies (Anghinoni *et al.* 2021; Chen *et al.* 2021; Elbushra & Ahmed 2020; Jenderedjian & Bellows 2021; Khanna 2020).

Black soldier fly (*Hermetia illucens*) larvae has established in Australia, India, Africa and Europe (Gayatri *et al.* 2013; Martínez-Sánchez *et al.* 2011). This non-pest insect able to degrade rotten meat and decaying plant (Makkar *et al.* 2014) and has the ability to sustain in significant abiotic factors such as light, temperature, pH value and humidity (Gayatri *et al.* 2013; Martínez-Sánchez *et al.* 2011). Its high protein (40% fresh weight) and lipid (30% fresh weight) content have been a suitable replacement of fish meal, meat meal, plant protein meal in agriculture industries such as poultry, pig and aquaculture feed [10-14] (Makkar *et al.* 2014; Liu *et al.* 2018; Renna *et al.* 2017; Schiavone *et al.* 2017; Spranghers *et al.* 2018).

Probiotic bacteria play an important role in maintaining the gut health of their host, such as the case of the introduction of probiotic mixture to human's gut, *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3 and *Bifidobacterium bifidum* together with vitamins and minerals was proven to shortened the duration symptoms of upper respiratory tract infections by 21.5% on average (Walton *et al.* 2021). Similarly, probiotic bacteria also bring advantages to insects' health when introduced to the gut. For instance, Mazza *et al.* (2020) reported the supplementation of *Bacillus subtilis* to BSF larvae resulted to significant increment in average weight (0.1184 g) as compared to larvae without the probiotic (0.1167 g). Likewise, Callegari *et al.* (2020) reported the addition of *Bacillus licheniformis* HI169 and *Stenotrophomonas maltophillia* HI121 to a nutritionally deficient diet can enhance the weight and length of BSF larvae in shorter time compared to control. Both resent studies have shown the advantage of using probiotic bacteria in insect rearing process as this could benefit the agriculture industries to have sustainable and better quality in BSF larvae production in a short time.

The use of probiotic bacteria in food fermentation in food industries is very common. It promises the health benefits to the consumers as a result of improved nutrient content in the food after the fermentation period. However, there are limited information regarding the impact of nutrient content in BSF larvae after supplemented with fermented bamboo culms. Hence, this study is done to identify the protein:lipid ratio of BSF larvae after reared with agricultural wastes with fermented bamboo culm fibres enriched with lactic acid bacteria from BSF larvae gut.

Material And Methods

Ethical Approval

Ethical approval for this study was provided by Laboratory Animal Facility and Management (LAFAM) and Universiti Teknologi MARA Committee on Animal Research & Ethics (UiTM CARE) Puncak Alam Campus, Selangor, Malaysia; Amendment 5/2020; Reference number: UiTM CARE 5/2021/(349/2021).

Isolation and Identification of Bacteria

BSF larvae samples were obtained from Satoyama Farm Sdn. Bhd. organization operated in Kuching, Sarawak, Malaysia. Ten BSF larvae aged 16 days (of approximately 1000 larvae) were collected randomly. The dissection and gut extraction were done according to a method by Li *et al.* (2021). The bacteria cultures were grown on de Man, Rogosa & Sharpe (MRS) agar at 37°C in 24 hrs by using spread plate method and streak plate method in order to obtain pure bacterial cultures. The screening of cellulolytic bacteria was done on Minimal salt medium (MSM) agar – a mixture of 2.5 g $NaNO_3$, 2.0 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, 0.1 g $CaCl_2.6H_2O$, 0.2 g CMC and 15 g agar powder) which has grown in anaerobic condition at 37°C in 7 days. DNA extraction was done with Vivantis Nucleic Acid Extraction Kit GF-1. Next, the amplification of 16S rRNA genes with polymerase chain reaction (PCR) was performed using the pair of universal primers with modified annealing temperature (Table 1). Then, the products were observed using 1% agarose gel electrophoresis before outsourced for DNA sequencing at Apical Scientific Sdn. Bhd. Selangor, Malaysia. BLAST search engine at National Center for Biotechnology (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare and detect the species of bacteria.

Fermentation of Bamboo Culm Fibres Enriched with Lactic Acid Bacteria

Fermentation of *Bambusa beecheyana* culm fibre was carried out in 1L Erlenmeyer flask, using 1000 mL of sterile MSM broth and 100 g of shredded fresh bamboo fibre (1 to 4 mm length and 10 to 30 µm diameter). 10 mL of overnight bacterial culture (LAB107 and LAB111) were then inoculated into the MSM broth (at initial pH 6.40) and the fermentation process was carried out continuously for 21 days at 37°C. The fermentation mixture was carried out in triplicates.

BSF Larvae Rearing

BSF larvae aged 7 days were collected from Satoyama Farm Sdn. Bhd. BSF rearing farm operated in Kuching, Sarawak. Agricultural wastes (kale, cabbage, spinach, water spinach, citrus fruits, palm sugar) were collected from vegetable market in Kuching, Sarawak. All the agricultural wastes were prepared according to the procedures of Zhang *et al.* (2013). 3.0 g of BSF larvae aged 7 days were added into every plot container, before cover with nylon net to avoid the larvae from escaping and protect them from external pests. This rearing process were conducted in 13 days. 10 ml of fermentation broth were sprayed into respective plot containers in every two days to keep the substrate condition favorable to BSF larvae. Citrus fruit juice was used to regulate the pH value of

VEG107, VEG111 and VEG217 substrates. 3.0 g of crushed palm sugar was sprinkled in all substrates to stimulate the larvae activity since it contains a natural source of metabolizable energy (Beale *et al.* 2022). Abiotic factors such as pH value, temperature and condition of feeding substrates were monitored. Ten larvae in each plot container were taken out to be measured (in cm) and weighed (in g) at every two days.

Crude Protein and Crude Fat

After the rearing process, BSF larvae in all plots were collected and dried at 105°C in 24 hrs to a constant weight. Crude protein and fat analysis were done based on AOAC international Standardization #930.15. The nitrogen content was determined in triplicate according to Kjeldahl Method using Gerhardt KJELDATHERM Block Heating System and Gerhardt Vapodest 45s distiller. The total nitrogen content (%) obtained in samples was multiplied with conversion factor of 4.76 in order to have accurate estimation of protein content for BSF larvae (Janssen *et al.* 2017). Crude fat content was determined after n-Hexane extraction using Soxhlet for 8 hrs in triplicate.

Statistical Analysis

The statistical analysis of the data was performed using Microsoft Excel for Windows. Mean ± standard error was calculated and interpreted for all samples, 'Time' expressed in 'Days' was applied on larvae weight, larvae length and pH of substrates. Larvae weight was measured in gram (g) while larvae length was measured in centimeter (cm). Crude protein and fat content in larvae samples were expressed in percentage (%) of 100 g of DW.

Results

PCR Amplification and Identification of Bacteria

We successfully obtain a distinct PCR band for all bacterial isolates with molecular size of 1.5 kbp when amplified with 16s rRNA primers. DNA BLAST analysis has identified the the bacteria as *Lactobacillus plantarum* and *Brevibacillus parabrevis*.

Nutrient Composition of Fresh and Fermented Bamboo

Nutrient compositions of bamboo culms (*Bambusa beecheyana*) indicated fresh bamboo culm is high in carbohydrate content (61.16% of total mass), while very low in both crude fat (0.08%) and crude protein (0.4%). Meanwhile, fermented bamboo culms enriched with *Lactobacillus plantarum* and *Brevibacillus parabrevis* show significant decrease in carbohydrate content (45.20% to 47.10%) while the crude fat and protein content roses by 2.70% to 5.10% and 15.90% to 17.20% respectively after 21 days of fermentation process.

Average weight and average length of BSF larvae

BSF larvae fed in VEG217 plot gained the most significant increment in both average length ($p>0.05$) and weight ($p<0.05$) as compared to negative control and other experiment groups. The average length (Figure 3) for BSF larvae in plot VEG107 ($p>0.05$), VEG111 ($p>0.05$) and VEG217 ($p>0.05$) show continuous increment from 0.80±0.01 cm on Day-7 until their maximum length on Day-16 which are 2.28±0.12 cm, 2.18±0.21 and 2.33±0.14 cm respectively. This was about 28% to 30% increment compared to the negative control. The average weight (Figure 4) for BSF larvae in plot VEG107 ($p>0.05$), VEG111 ($p<0.05$) and VEG217 ($p<0.05$) show gradual increment from 0.01±0.01 cm on Day-7 until their maximum weight on Day-16 which are 0.19±0.02 g, 0.17±0.04 g and 0.23±0.03 g respectively. This was about 57% to 111% increment compared to the negative control.

Crude protein and fat analysis of BSF larvae

BSF larvae treated in VEG107 ($p<0.05$) has the most significant crude protein content with 11.87±0.12% compared to VEG111 ($p<0.05$) and VEG217 ($p<0.05$) with 11.82±0.12% and 11.46±0.09% respectively. All the BSF larvae in three treatments resulted to higher crude protein content than negative control (10.5±0.10%). Meanwhile, crude fat content of BSF larvae treated in VEG111 ($p<0.05$) was found to be the lowest with 3.4±0.09% as compared to VEG217 ($p<0.05$) and VEG107 ($p<0.05$) with 5.1±0.11% and 6.70±0.08% respectively. This shows that the crude fat content of BSF larvae treated in VEG 217 and VEG111 are lower than negative control (6.26±0.13%), especially the ones in VEG111 which resulted two times lower than the negative control. However, the crude fat content in VEG107 treatment is slightly higher than negative control by 0.44%. Based on the crude protein and fat of BSF larvae obtained, both values were expressed in simplest ratio (Table 2) in order to observe the enhancement in both nutrient content as compared to N/C. Our study found out that all BSF larvae in plot trials treated with probiotic bacteria shows the increment in crude protein content and drops in crude fat content. When compared to N/C (5:3 ratio), it was proven that VEG111 has the significant enhancement in crude protein:fat ratio with 4:1 followed by VEG217 and VEG107 with 23:10 and 12:7 respectively.

Discussion

Identification of bacteria

In this study, bacterial isolates from BSF larvae gut has successfully identified as *Lactobacillus plantarum* and *Brevibacillus parabrevis*. *Lactobacillus plantarum* is a facultative heterofermentative bacteria which can be found in *Apis florea* (honey bee) gastrointestinal tract (Parichehreh *et al.* 2018). Usually, this species is utilized as starter culture and preservatives in food industries (Arasu *et al.* 2013). It is also found as flexible and versatile species which able to grow at 15°C until 44°C (Lorenzo *et al.* 2018) with the optimum temperature at 37°C (Matejčeková *et al.* 2016). This lactic acid bacteria grow at the optimum pH value that is close to neutrality that is pH 5.8 - 6.0 (Krieger-Weber *et al.* 2020). As suggested in previous study, *Lactobacillus plantarum* performs

Table 1. Universal primer pairs with modified annealing temperature according to samples

Samples	Universal primer pair	Annealing temperature (°C)
Lactobacillus plantarum	27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 957R (5'-TCGAATTAAACCACATGCTCCA-3')	61
Brevibacillus parabrevis	27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')	53

Table 2. Simplified ratio of protein:fat (%) contained in BSF larvae. *The protein and fat ratio were calculated according to the percentage of crude protein and fat obtained.

Sample	*Protein to Fat Ratio
VEG107	12:7
VEG111	4:1
VEG217	23:10
N/C	5:3

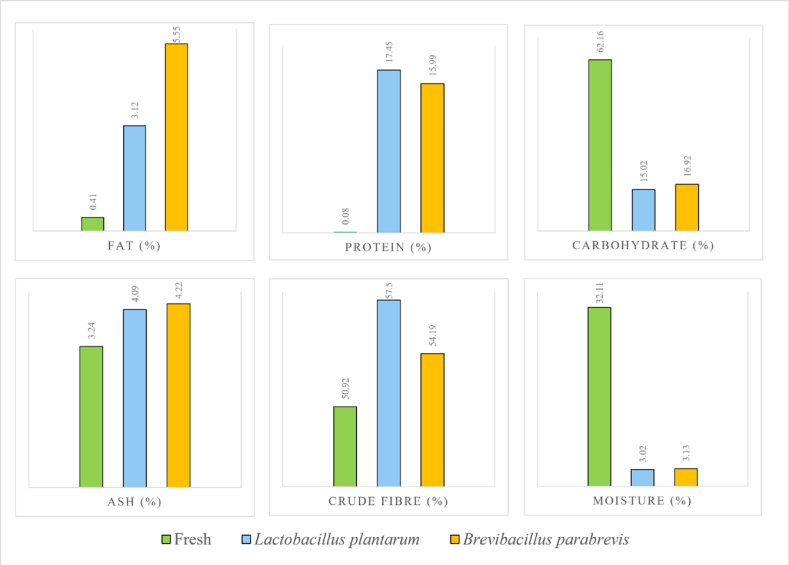

Figure 1. (Mean±SD) Nutrient composition of fresh and fermented bamboo culms enriched with *Lactobacillus plantarum* and *Brevibacillus parabrevis*.

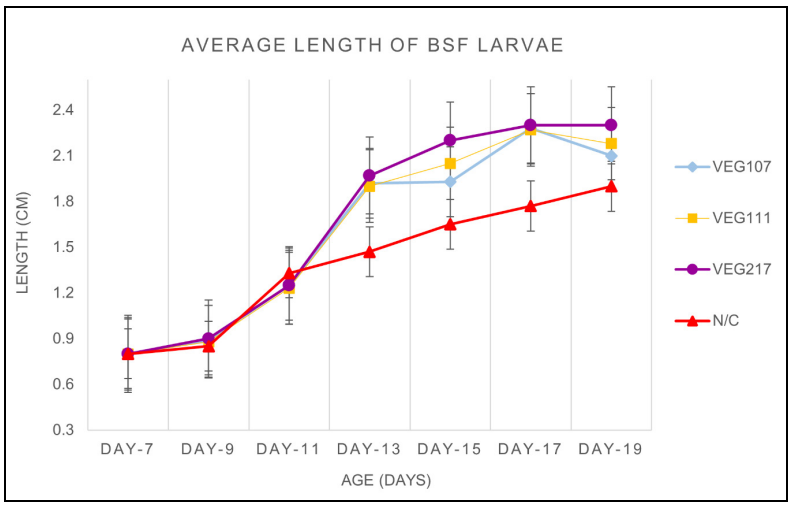

Figure 2. (Mean±SD) Average length of BSF larvae reared on seven different substrates for 13 days.

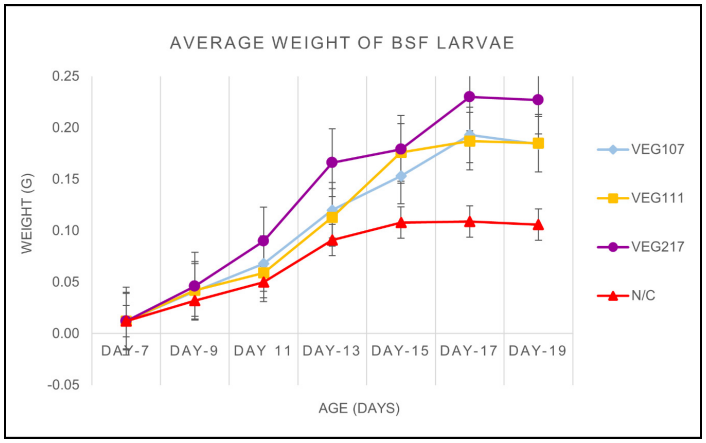

Figure 3. (Mean±SD) Average weight of BSF larvae reared on seven different substrates for 13 days.

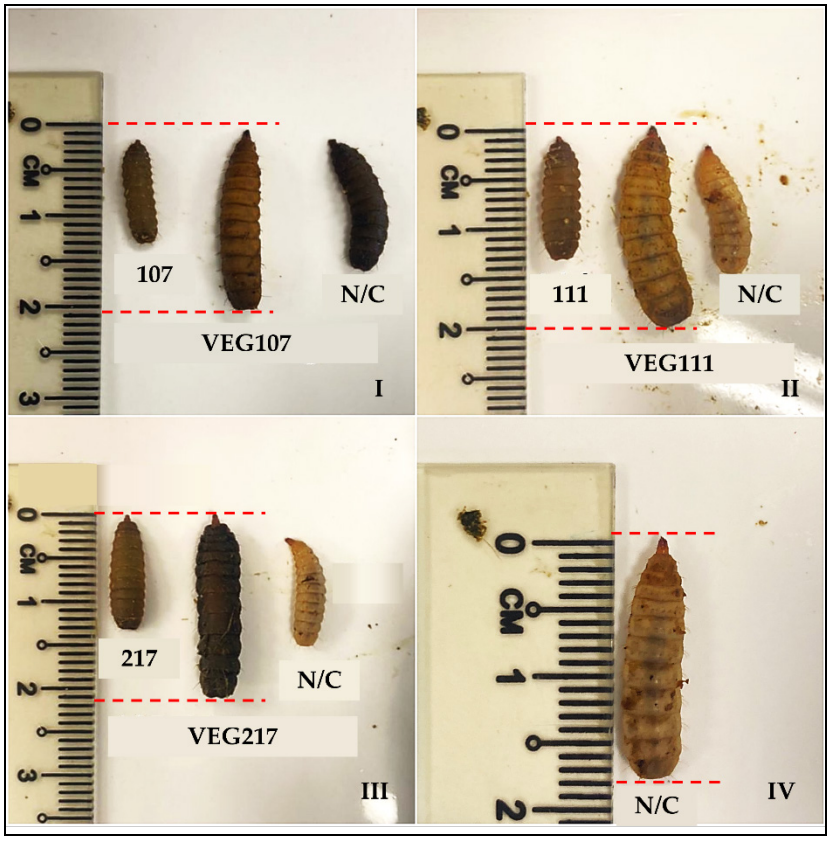

Figure 4: The measurements of BSF larvae from every plot after 13 days of rearing process. (a) The com-parison of live BSF larvae length in different plot. 107, 111 and 217 are the larvae fed only with fermented bamboo culm fibres; (b) The comparison of dried BSF larvae length – VEG107 (I), VEG111(II), VEG217 (III) and N/C (IV).

ideal fermentation process at higher pH value as compared to lower pH value (pH 3.5) – inhibited from obtaining energy from the metabolism of glucose (Henick-Kling 1988). Meanwhile, *Brevibacillus parabrevis* can be found in earthworm viscera gut (Kim *et al*. 2010) and *Rhynchophorus ferrugineus* (sago worm) (Raio *et al*. 2016). This Gram-positive bacteria is able to produce acid from cellobiose, glucose, maltose and mannitol and can withstand pH value of 5.0 - 9.0 (Hooda *et al*. 2018).

The supplementation of these bacteria has promoted gut health of BSF larvae. It was believed that our bacteria have improved the larvae gut microbiome which resulted to the better digestion and enhancement in nutrient absorption hence promoting the growth in shorter time as well as to have better nutrient composition as feedstock. Besides, these bacteria has antimicrobial and antifungal properties that fight pathogens which could prevent the larvae from diseases. As reported by Arasu *et al.* (2016), *Lactobacillus* strains exhibited antimicrobial activities against pathogens in gastrointestinal tract through secretion of extracellular metabolites such as lactic acid, acetic acid, succinic acid and bacteriocins.

Lactobacillus plantarum is safe for consumption as it has numerous promising benefits to human health (Arasu *et al*. 2016). Since this strain is commonly found in human gastrointestinal tract (Matejčeková *et al*. 2016), it promotes health benefit to the gut in terms of digestion, which results to the better absorption of nutrients. For instance, Won *et al*. (2012) discovered that supplementation of *Lactobacillus plantarum* inhibit dermatitis by intensify the type I helper T cell activation and regulatory T cell activation. Like *Lactobacillus plantarum*, *Brevibacilus parabrevis* has successfully promotes the growth of BSF larvae feedstock in this study as well as shortening the harvesting time with marketable size. According to Talib *et al*. (2017), the survival rate of *Scylla paramamosain* (mud crab) larvae has increased after supplemented with *Brevibacillus parabrevis*. However, this endospore-forming species has ability to cause food poisoning (Wang *et al*. 2018) which is probably not suitable for human consumption.

Average length and weight of BSF larvae

Lactic acid bacteria supplementation has significant impact to our BSF larvae in terms of growth rate and larval health. *Brevibacillus parabrevis* is capable of generating antimicrobial peptides (Ding & Sherman 2010; Storelli *et al*. 2011), to eliminate pathogenic microorganisms *Vibrio parahaemolyticus* in their hosts (Talib *et al*. 2017). *Lactobacillus plantarum* promote the larval growth by improving dietary uptake and preventing malnutrition in the host (Storelli *et al*. 2011; Erkosar *et al*. 2015; Schwarzer *et al*. 2016). The weight and length gain of BSF larvae observed in this study suggested the positive effect of supplementing the larval nutrients with beneficial probiotic bacteria, as the healthy guts would result to more efficient nutrient absorptions. This finding was supported by the study of Ravi *et al.* (2020), where they observed the rate of nutrient absorption in BSF larvae has improved significantly when fed with waste substrates enriched with specific strain of microorganisms. Likewise, Somroo *et al*. (2019) also reported the BSF larvae fed with soybean curd residues pre-treated with *Lactobacillus buchneri* (L3-9) has higher bioconversion rate (6.9±0.3% compared to negative control, 5.0±0.3%) which then give a rise to protein and fat content.

Consortium of bacteria also play important role in improving the feeding and growth condition of BSF larvae. As in this study, the BSF larvae feedstock enriched with *Lactobacillus* plantarum and *Brevibacillus parabrevis* in the ratio of 1:1 possessed the highest growth rate as compared to larvae supplemented with single bacterial strains. Mazza *et al.* (2020) also reported the inoculation of four different bacteria: *Kocuria marina*, *Lysinibacillus*, *Proteus mirabilis* and *Bacillus subtilis* at a ratio of 4:1:1:1 contributed in enhancing BSF larvae weight by 28.57% from control, compared to *Bacillus subtilis* alone, which only contributed 17.79% of weight gain from control.

Protein:fat ratio of BSF larvae

The treatments with probiotic bacteria has proven the enhancement of protein:fat content in BSF larvae. Interestingly, our nutrient analysis indicated the BSF larvae supplemented with bacterial isolates, *Brevibacillus Parabrevis*, have the highest crude protein content as compared to other bacterial combination. Nonetheless, when fed with both Lactobacillus plantarum and Brevibacillus parabrevis, BSF larvae also show good protein to lipid ratio (23:10). Our finding suggested that the additional bacterial strains into the feedstock of BSF larvae, which also include the use of fermented bamboo fibres is capable of reducing the lipids content while maintaining the essential protein level. Moreover, the addition of both bacteria into feed substrates have significantly increase the weight and length as well as shorten the time for BSF larvae to achieve the ideal length and weight. This finding is tally with Mazza *et al*. (2020), where the introduction of *Bacillus subtilis* and *Proteus mirabilis* in chicken manure have promoted the weight gain of BSF larvae after 14 days by 17.79% and 18.60% which are higher than control group. Similarly, Kooienga *et al*. (2020) discovered that BSF larvae treated with *Arthrobacter* AK-19 had increased in weight up to 107% from day 2 to day 3 as compared to control group which only rose by 28%.

The enhanced protein:lipid ratio of BSF larvae after enriched with *Lactobacillus plantarum* and *Brevibacillus parabrevis* could be beneficial feedstock, which may improvise the poultry in terms of health and development. As reported by Moula *et al.* (2018), the growth trend of Ardennaise chicken fed with 8% of fresh BSF larvae in 11 weeks is significantly higher with 26% of mean protein content as compared to control group (without BSF larvae) with 24% of mean protein content. Additionally, they also discovered

both male and female chicken aged 80 days old in experimental groups have higher weight in their pectoral muscle, drumsticks and thighs, wings and heart as compared to control group. Correspondingly, other study by Sumbule *et al.* (2021) has proven by adding 25% BSF larvae in 75% fishmeal diet gives heavier weight to Isa Brown layer chicks after 8 weeks, contrasted with the group fed with BSF larvae only and the diet group without BSF larvae (negative control).

Conclusions

This study indicates that the addition of fermented bamboo culms enriched with 1:1 ratio of *Lactobacillus plantarum* and *Brevibacillus parabrevis* able to enhance the growth, feeding behavior of BSF larvae as well as able to reach a harvestable size in shorter time. Besides, it was clearly pointed out that the protein:fat ratio of BSF larvae was improvised by introducing these two probiotics in bamboo culms fibre to the feeding substrates, which was able to maintain the protein:fat ratio of BSF larvae to an acceptable level. The data from this study has contributed to the understanding of the impact of nutritional value of BSF larvae with the presence of probiotic bacteria in fermented bamboo culm fibres and this has opened pathways to researchers in engaging in deeper studies utilizing these bacterial species at industrial scale in order to optimize the protein:lipid ratio for the use in live feedstock. Moreover, the data in this study can be useful information for achieving a sustainable agro-industries in a long-run. For instance, the formulation can be used for farmers to develop high protein and low lipid BSF larvae as feedstock for poultry and fish. This could bring a great outcome where they can improvise the food chain by producing the better quality of poultry and fish before supplying to the end-consumer. The utilization of BSF larvae with better protein:fat ratio as feedstock definitely able to substitute other food source for the livestock as the global warming and climate changes have greatly affect the number of crops production.

Author Contributions

A.C.H. and V.J.M. supevised the project. A.C.H. and V.J.M. conceptualized and recievd the grant. A.C.H. and N.A.A.R. conceptualized, preparied samples and performed the experiment. N.A.A.R. analyzed larvae growth. S.N.A.S.M. contributed to the supervision of animal ethics application. A.C.H. and N.A.A.R. contributed to the interpretation of the results. N.A.A.R. wrote the paper. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing financial interests

The authors have no conflict of interest.

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