Sains Malaysiana 51(2)(2022): 547-558
http://doi.org/10.17576/jsm-2022-5102-18

Physicochemical Characteristics and Microbiological Quality of Silkworm (*Bombyx mori*) Larval and Pupae Powder: Comparative Study

(Pencirian Fizikokimia dan Kualiti Mikrobiologi Larva dan Pupa Ulat Sutera (*Bombyx mori*): Kajian Perbandingan)

Loo Yuet Ying, Lim Hui Ying, Noor-Soffalina Sofian-Seng, Wan Aida Wan Mustapha & Noorul Syuhada Mohd Razali*

ABSTRACT

Silkworm (Bombyx mori) *has been reported to exhibit diverse health benefits. A comparative study was conducted to evaluate the physicochemical (colour, water activity, pH, water solubility, proximate, mineral, amino acid, and fatty acid composition) and microbiological qualities of* Bombyx mori *silkworm powder produced from two developmental stages (larvae and pupae). Silkworm pupae powder (SPP) had significantly higher ($p<0.05$) lightness (L*) and yellowness (b*) values than silkworm larval powder (SLP). SPP had significantly higher ($p<0.05$) water activity and solubility than SLP. The pH values of both silkworm powders were not statistically different. SLP had significantly higher ($p<0.05$) protein and moisture but significantly lower ($p<0.05$) fat, ash, fibre, and total calorie content as compared to SPP. The total amino acid content of SPP was significantly higher ($p<0.05$) than SLP. SLP had significantly higher ($p<0.05$) protein efficiency ratio than SPP, indicating that SLP contained higher amount of essential amino acid. Both silkworm powder contained 20 types of fatty acids with SPP exhibited significantly higher ($p<0.05$) polyunsaturated fatty acid composition than SLP. Both silkworm powders had a low n-6/n-3 ratio, which may be beneficial to human health. Both SLP and SPP could be a good source of iron and rich in magnesium and zinc. SPP had a significantly lower ($p<0.05$) yeast and mould count as compared to SLP. The finding of this study suggested that silkworm powder produced from two different developmental stages may provide beneficial health effects in humans derived from the protein, minerals, fatty acids, and amino acids content.*

Keywords: Amino acid; fatty acid; mineral; proximate composition

ABSTRAK

Ulat sutera (Bombyx mori) *telah menunjukkan banyak kebaikan kepada kesihatan manusia. Kajian perbandingan telah dijalankan untuk menilai kualiti fizikokimia (warna, aktiviti air, pH, keterlarutan dalam air, komposisi proksimat, mineral, asid amino dan asid lemak) dan mikrobiologi serbuk ulat sutera* Bombyx mori *yang diperbuat daripada dua peringkat perkembangan yang berbeza (larva dan pupa). Serbuk ulat sutera pupa (SPP) mempunyai nilai kecerahan (L*) dan kekuningan (b*) yang tinggi secara signifikan ($p<0.05$) berbanding serbuk ulat sutera larva (SLP). SPP mempunyai aktiviti air dan keterlarutan dalam air lebih tinggi secara signifikan ($p<0.05$) berbanding SLP. Nilai pH untuk kedua-dua serbuk ulat sutera tidak berbeza secara signifikan. SLP mempunyai kandungan protein dan air yang tinggi secara signifikan ($p<0.05$) tetapi kandungan lemak, abu, serat dan jumlah kalori yang lebih rendah secara signifikan ($p<0.05$) berbanding SPP. Jumlah asid amino SPP adalah lebih tinggi secara signifikan ($p<0.05$) berbanding SLP. SLP mempunyai nilai nisbah kecekapan protein yang lebih tinggi secara signifikan ($p<0.05$) berbanding SPP dan menunjukkan SLP mempunyai kandungan asid amino perlu yang lebih tinggi. SLP dan SPP mempunyai 20 jenis asid lemak. SPP mempunyai kandungan asid lemak politaktepu yang lebih tinggi secara signifikan ($p<0.05$) berbanding SLP. Kedua-dua serbuk ulat sutera mempunyai nilai nisbah n-6/n-3 yang rendah yang berpotensi untuk memberi manfaat kepada kesihatan pengguna. SLP dan SPP juga boleh menjadi sumber zat besi dan kaya dengan magnesium dan zink. SPP mempunyai kiraan yis dan kulat yang lebih rendah secara signifikan ($p<0.05$) berbanding SLP. Penemuan ini menunjukkan serbuk ulat sutera daripada dua peringkat perkembangan yang berbeza boleh memberi manfaat kepada manusia kerana mengandungi protein, mineral, asid amino dan asid lemak.*

Kata kunci: Asid amino; asid lemak; komposisi proksimat; mineral

Introduction

The use of silkworm powder as food and animal feed has a long history in many Asian countries (Sheikh et al. 2018) given that it is rich in protein content, essential amino acids, polyunsaturated fatty acids, and minerals (Anuduang et al. 2020). It has also been reported to have medicinal values, including the capability of regulating blood glucose levels in human (Ryu et al. 2013), prolong health span and increase human resistance towards Parkinson's disease (Nguyen et al. 2016), serve as an antibacterial agent in wound dressing or as an agent for hepatoprotective activity, anti-diabetes, anti-genotoxicity, anti-cancer, and anti-obesity (Kim et al. 2018). This yellowish powder is generally produced from the pupae of the silkworm (*Bombyx mori*), which is a monophagous insect that was fed exclusively on mulberry leaves. At the larval stage, the silkworms moult four times with five instars, i.e., intervals between moulting, and the larvae produce silk at the fifth instar, spinning it into a cocoon to surround the whole body, before they enter pupae phase. In the sericulture industries, the pupae are killed by boiling, drying, or soaking in sodium hydroxide before the silkworm becomes a moth (Jintasataporn 2012).

Although the nutritional value of silkworm powder has generally been established (Ademola et al. 2017; Kweon et al. 2019), most of these studies focused on examining either the larval (Ji et al. 2016b) or pupae silkworm powder (Trina et al. 2014), and little is known on the variation in the food components between the larval and pupae silkworm powders. A good understanding of these variations is, therefore, essential before the silkworm powder could be proposed as a potential future food in the pre-emptive battle against future food insecurity. In this study, a comparative approach was used to evaluate the physicochemical characteristics and microbiological quality of the larval and pupae silkworm powders. Given that the main food components of these two types of silkworm powders are similar, compositional analyses, such as amino acid, fatty acid and minerals, are deemed appropriate to determine the compositional differences between silkworm powders produced from different developmental stages.

Materials and Methods

Sample preparation

In this study, larval silkworm (*Bombyx mori*) powders were prepared from fresh biological entities following the method of Anuduang et al. (2020). The fresh fifth instar larvae of silkworm, obtained from Natural Thai Golden Silk Ltd, Payao Province, Thailand, were treated with hot water at 90 °C for 5 min. The samples were then dried in a hot-air desiccator (model: Kluynamthai, Thailand) at 80 °C for 5 h. Meanwhile, the silkworm pupae were purchased from a retail market trading edible insect-related product, known as Thailand Unique in Bangkok, Thailand. The silkworm pupae were also treated with hot water but at 100 °C for 5 min. The samples were then dried to a consistent weight in a hot-air dryer at 110-125 °C. Both dried silkworm larvae and pupae were grounded into powder form using a blender at 25,000 rpm (model: NT-1000D Nanotech, Thailand) and filtered using a 1.0-mm mesh sieve and kept in an air-tight container. Samples were kept at room temperature until further use.

Proximate analysis

This study used the protocols of the Association of the Official Analytical Chemists (AOAC 1990) to measure the moisture (method 934.01), ash (method 923.03), total fat (method 991.36), and crude fibre content (method 978.10) of SLP and SPP, and the protein content (N × 6.25) was examined using the Kjeldahl method (984.13). The carbohydrate content was calculated by subtracting the sum of moisture, protein, fat, and ash from 100 (Muanghorn et al. 2018). The caloric content of SLP and SPP was determined by multiplying the amount of fat, protein, and carbohydrate with their respective calorific value. The calorie content was calculated as follows (amount of protein × 4.0 kcal/g) + (amount of fat × 9.0 kcal/g) + (amount of carbohydrate × 4.0 kcal/g). All analyses were performed in triplicate and expressed as mean ± standard deviation.

Physicochemical analysis

The physicochemical analyses encompassed four tests, i.e., colour, water activity, pH, and water solubility index. The colour of SLP and SPP was measured using a chromameter (model: CR-400, Konica Minolta, Japan) and pre-calibrated with a white tile. The colour measurement was expressed as International Commission on Illumination (CIE) colour value, where L* determining brightness, a* determined redness or greenness, and b* determined yellowness or blueness. For each type of silkworm powders, 2 g of samples were used to measure their respective colours at room temperature. Also, the pH value for SLP and SPP was measured by dissolving 400 mg samples of each in 20 mL of distilled water.

Meanwhile, water activity was analysed to examine the stability of food products against chemical,

biochemical and microbial changes. The threshold value of water activity for food safety is 0.60, above which microorganism and mould would grow, causing chemical changes to a food item (Beuchat et al. 2013). In this study, the water activity of SLP and SPP was measured using a water activity meter (model: LabMaster-aw neo, Novasina Lab, Lachen), in which 2 g of samples for each type of silkworm powders were assayed at room temperature. For measuring water solubility index (WSI), 2.5 g of SLP and SPP samples were each dissolved in 30 mL distilled water in a 50 mL centrifuge tube, incubated in a water bath at 37 °C for 30 min, and then centrifuged at 5,100 × g for 30 min. The supernatant was collected and dried using a convection oven (model: Memmert, Schwabach, Germany) at 105 °C until a consistent weight was obtained. The dried weight over the initial powder weight was recorded as WSI, which is an indicative parameter for the solubility of biomolecules (starches, proteins, sugars, water-soluble fibre) in excessive water (Chang et al. 2018). All analyses were performed in triplicate and expressed as mean ± standard deviation.

AMINO ACID PROFILE

The amino acid profile of SLP and SPP was determined using high-performance liquid chromatography (HPLC) methods (Ng et al. 2020). The samples of SLP and SPP were each hydrolysed using three methods for the determinations of different amino acids: The acid hydrolysis method, in which each sample (0.30 g) was added with 5 mL of 6 N hydrochloric acid (HCl) and incubated at 110 °C for 24 h, and followed by derivatisation process using α-aminobutyric acid (AABA). The acid hydrolysis was used to quantify glutamic acid (Glu), proline (Pro), tyrosine (Tyr), aspartic acid (Asp), threonine (Thr), histidine (His), glycine (Gly), alanine (Ala), isoleucine (Ile), leucine (Leu), serine (Ser), phenylalanine (Phe), valine (Val), lysine (Lys), and arginine (Arg). The performic oxidation method, in which each sample (0.3 g) was added with 2 mL performic acid, incubated at 4 °C for 16 h, and followed by the addition of 0.4 mL hydrogen bromide (HBr). The mixtures were incubated at 4 °C for 30 min. Each sample was then dried at 100 °C, hydrolysed with 6 N HCl, and incubated at 110 °C for 24 h. The performic oxidation was used to quantify cysteine (Cys) and methionine (Met). The alkaline hydrolysis method, in which each sample (0.3 g) was added with 15 mL 4.3 N lithium oxide (Li_2O), incubated at 120 °C for 16 h, hydrolysed with 6 N HCl, incubated at 110 °C for 24 h, and then pH adjusted to 4.5 with HCl. The alkaline hydrolysis was used to quantify tryptophan (Trp).

For both acid hydrolysis and performic oxidation, the derivatisation process was performed using AccQ Fluor reagent with α-aminobutyric acid (AABA) as an internal standard. Samples and standards (10 µL) were analysed by HPLC (Waters 2475, Waters Co., Milford, MA, USA), equipped with a Waters 410 Scanning Fluorescence and AccQ Tag column (particle size of 4 µm; 150 × 3.9 mm). The mobile phases consisted of AccQ Tag Eluent A (200 mL of AccQ Tag in 2 L of Milli-Q water) and AccQ Tag Eluent B (60% acetonitrile acid) with a linear gradient conditioned by a fluorescence detector (λ excitation at 250 nm and λ emission at 395 nm). For alkaline hydrolysis, samples were analysed using HPLC equipped with Nova Pak C18 column (3.9 × 150 mm; Waters, Waters Corp, USA). The mobile phase was 0.0085 M sodium acetate (pH 4.0) and methanol at a ratio of 21.7: 3.3 (flow rate: 1 mL min^{-1}). Detection was through a fluorescence detector (λ excitation at 285 nm and λ emission at 345 nm). Meanwhile, the protein quality was measured with the protein efficiency ratio (PER) by considering the requirement of essential amino acid. Specifically, PER was calculated using the total essential amino acid profile from the protein sample based on (1):

$$PER = (0.06320 \times \Sigma AA) - 0.1539 \quad (1)$$

where ΣAA is the sum of essential amino acids, i.e. Thr, Val, Met, Ile, Leu, Phe, Lys, His, arginine (Arg), and Tyr.

FATTY ACID COMPOSITION

The fats of SLP and SPP samples were extracted using the Soxhlet extraction method (Ahmad Jelani et al. 2019) and the fatty acid composition was analysed using method described by Trattner et al. (2015) with little modification. The fatty acid methyl esters (FAMEs) of each sample were prepared by adding the lipid extract with 10 mL 0.01 M sodium hydroxide (NaOH) in methanol and incubated at 60-65 °C for 30 min, after which they were collected and dissolved in hexane. The fatty acid compositions were analysed by gas chromatography (GC; Agilent 6890, USA). The FAMEs were separated using GC with a polar-fused capillary column, the split injector (split ratio: 50 mL/min), and the flame ionisation detector (FID). Helium (initial pressure 80 kPa) was used as carrier gas while nitrogen was used as the makeup gas. The type of fatty acids contained in each sample was determined by comparing with an external standard (K 110 Alltech-Applied Science Labs, USA). The fatty acid composition of SLP and SPP was expressed as a percentage (%) of the total fatty acid.

MINERAL COMPOSITION

The mineral composition of SLP and SPP was examined using inductively coupled plasma mass spectroscopy (ICP-MS; PerkinElmer Sciex, Elan 9000, USA). Approximately 0.5 g samples of SLP and SPP were each digested with 1 mL nitric acid (HNO_3) for assay, and the results were expressed as mg kg^{-1} (Lenzi et al. 2019). The threshold values for the food labels 'source of' and 'high in' were calculated using the nutrient reference values of 800 mg for calcium, 14 mg for iron, 300 mg for magnesium, and 15 mg for zinc in 100 g of an edible portion (FAO & WHO 2007).

MICROBIOLOGICAL ANALYSIS

The number of microorganisms in the SLP and SPP samples was determined using the aerobic plate count method (Maturin & Peeler 2001), in which 5 g samples of SLP and SPP were each dissolved in 45 mL maximum recovery diluent (MRD) solution in a sample-to-MRD ratio of 1:9. The mixture was diluted in MRD by a 10-fold serial dilution (10^{-2}, 10^{-3}, and 10^{-4}), after which 10 µL of each dilution was spread onto the plate count agar (PCA; Merck, Darmstadt, Germany) for bacteria, and dichloran rose-bengal chloramphenicol (DRBC agar; Merck, Darmstadt, Germany) for yeast and mould. The PCA plates were incubated at 37 °C for 24 h, and the DRBC agar plates were incubated at 25 °C for 5 days. The results were expressed as log colony-forming units per gram (log CFU/g).

STATISTICAL ANALYSIS

Statistical analysis was carried out using the SPSS software package (IBM SPSS Statistics Version 23, United States). All analyses were performed in triplicates and three independent experimental trials were carried out. One-way Analysis of Variance (ANOVA) was used to examine all data. Turkey's multiple-range test (post-hoc) was used to determine the mean comparison between two samples. The differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

PROXIMATE COMPOSITION

Table 1 summarises the proximate composition of SLP and SPP, with SLP containing 61.5 ± 1.1% dry weight (dw) of protein, which was significantly ($p<0.05$) higher than that of SPP (58.7 ± 0.4% dw). These findings are parallel with that of Yang et al. (2009), who reported that the SLP contained 60% dw of protein content, while Rumpold and Schlüter (2013) found that crude protein in SPP ranged from 48.7 to 58.0% dw. High protein content is expected from both SLP and SPP since the natural diet of silkworms is mulberry leaves. Previous finding found that mulberry leaves have high protein content (Yu et al. 2018).

Meanwhile, fats are the second-highest component in silkworm powder, in which the fat content in SLP (25.2 ± 0.6% dw) was significantly ($p<0.05$) higher than that of SPP (22.3 ± 0.7% dw). These results agreed with Kim et al. (2016) finding, who reported the fat content of silkworm powder is in the range of 18.9 - 36.0% fat dw. In addition, Rodríguez-Ortega et al. (2016) in his report stated that edible insects contain great amount of unsaturated fatty acid such as oleic, linoleic, and linolenic acid which are mainly found. Also, the calorific value of SLP (481.7 ± 2.8 kcal) was significantly ($p<0.05$) lower than that of SPP (497.8 ± 1.2 kcal), and these results were within the range of 435 - 500 kcal/100g as reported by Akande et al. (2020a).

Besides, the ash content of SLP (3.2 ± 0.1% dw) was significantly ($p<0.05$) lower than that of SPP (4.2 ± 0.1% dw). In this study, SPP contained a significantly higher ($p<0.05$) amount of crude fibre (7.7 ± 0.3% dw) than SLP (4.3 ± 0.2% dw). The ash content reflects the quantity of minerals content. According to previous reports, silkworm larvae and pupae are rich in minerals including iron, zinc, manganese, calcium, and phosphorus (Akande et al. 2020b; Omotoso 2015). The total carbohydrates in SLP and SPP were 8.2 ± 0.9% and 9.1 ± 0.5% dw, respectively, with no statistical difference ($p>0.05$). The nutritional value of both silkworm powders may depend on their developmental stages, sex, feed composition, rearing technique, and climates (Adámková et al. 2017).

TABLE 1. The proximate composition of silkworm (*Bombyx mori*) larval and pupae powder

Sample	Protein (% in dry weight)	Fat (%)	Moisture content (%)	Ash (%)	Crude fibre (%)	Carbohydrate (%)	Energy (kcal/100 g)
SLP	61.5 ± 1.1[a]	25.2 ± 0.6[a]	4.3 ± 0.1[a]	3.2 ± 0.1[a]	4.3 ± 0.2[a]	8.2 ± 0.9[a]	481.7 ± 2.8[a]
SPP	58.7 ± 0.4[b]	22.3 ± 0.7[b]	2.9 ± 0.0[b]	4.2 ± 0.1[b]	7.7 ± 0.3[b]	9.1 ± 0.5[a]	497.8 ± 1.2[b]

The results are expressed as Mean ± SD ($n = 3$). [a–b] Different letters in the same column denote statistical significance at $p<0.05$

PHYSICOCHEMICAL CHARACTERISTIC

Figure 1 shows the visual observation of SLP and SPP. SLP as shown in Figure 1(a) was brown-yellowish while SPP as shown in Figure 1(b) appeared to be brown in colour but visually darker than SLP. Table 2 shows the colour parameters (L*, a*, b*) for SLP and SPP, in which the L* (lightness) value of SLP (40.3 ± 4.9) was significantly ($p<0.05$) higher than that of SPP (28.5 ± 1.1). For redness (a*), the value of SLP (0.83 ± 0.13) was significantly ($p<0.05$) lower than that of SPP (2.2 ± 0.2). The yellowness (b*) value of SLP (32.4 ± 2.2) was observed to be significantly ($p<0.05$) higher than that of SPP (21.5 ± 0.3). In general, silkworm larvae were pure white with brown spots on their abdominal segments, while pupae appeared shining yellowish-brown (Gurjar et al. 2018). High temperature during drying triggered Maillard reaction, i.e. the rearrangements of amino acid and reducing sugars in rings that reflect light, was more readily to occur in SPP (110-125 °C) than SLP (80 °C), thereby contributing toward browning in SPP (Tamanna & Mahmood 2015). In contrast, the pH values of SLP and SPP were not statistically different, with their respective pH values recorded as 6.63 ± 0.07 and 6.58 ± 0.02. The pH values in this study were in congruence with that of Kim et al. (2016), who reported that the pH value of silkworm powder was 6.43.

Meanwhile, the water activity as shown in Table 2 was significantly ($p<0.05$) lower in SLP (0.30 ± 0.01) than in SPP (0.45 ± 0.02), suggesting that both powders were stable against chemical, biochemical, and microbial changes and hence safe for human consumption. In general, dried food products with water activity less than 0.6 showed no growth of microorganisms (Chang et al. 2018) probably due to the inhibition of cell division activities (Beuchat et al. 2013).

Table 2 shows that the water solubility index (WSI) of SPP (31.7 ± 3.4%) was significantly higher ($p<0.05$) than SLP (23.0 ± 1.1%). The solubility in water for SLP and SPP in this study was considered low given that their WSI values were substantially lower than 50%. The solubility of powder appeared to be related to moisture content, where low moisture content would result in high WSI (Tchabo et al. 2018). Incidentally, the moisture content was low for SLP (4.3 ± 0.1%) and SPP (2.9 ± 0.0%) in this study as shown in Table 2. However, the solubility of silkworm powder could be enhanced via hydrolysis with protease enzyme by converting the protein into smaller peptides (Anootthato et al. 2019), in which the WSI of silkworm powder was improved from 17.9 to 93.5%.

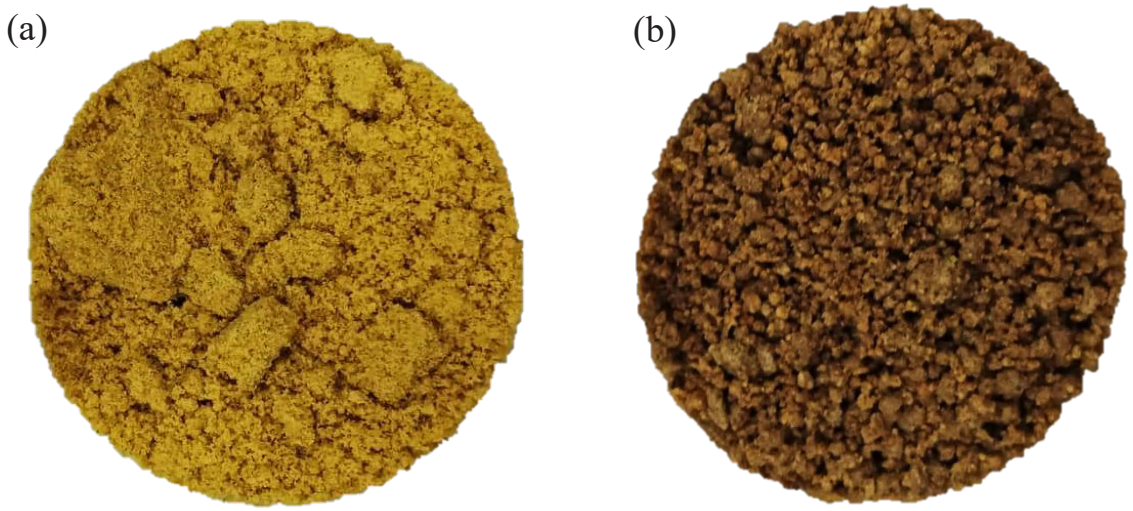

FIGURE 1. The visual appearance and colour of silkworm (*Bombyx mori*): (a) larvae powder, and (b) silkworm pupae powder

TABLE 2. Physicochemical properties of silkworm (*Bombyx mori*) larval and pupae powder

Sample	Water activity	pH	Water solubility index (%)	Colour		
				L*	a*	b*
SLP	0.3 ± 0.0[a]	6.6 ± 0.1[a]	23.0 ± 1.1[a]	40.3 ± 4.9[a]	0.83 ± 0.13[a]	32.4 ± 2.2[a]
SPP	0.5 ± 0.0[b]	6.5 ± 0.0[a]	31.7 ± 3.4[b]	28.5 ± 1.1[b]	2.2 ± 0.2[b]	21.5 ± 0.3[b]

The results are expressed as Mean ± SD (n = 3). [a–b] Different letters in the same column denote statistical significance at $p<0.05$

AMINO ACID PROFILE

Table 3 shows that both SLP and SPP exhibited nine essential amino acids and nine non-essential amino acids while Figure 2 shows the typical chromatogram for the amino acid composition in SLP. The most abundant amino acid in SLP was Glu (12.0%AA/ΣAA), while Gly (16.5%AA/ΣAA) was the most commonly detected amino

TABLE 3. Amino acid profile of silkworm (*Bombyx mori*) larval and pupae powder

Amino acid	SLP	SPP
Essential amino acid (%AA/ΣAA)		
Leu	7.2 ± 0.0[a]	4.2 ± 0.0[b]
Lys	6.9 ± 0.1[a]	4.8 ± 0.1[a]
Val	5.9 ± 0.4[ab]	4.5 ± 0.0[ab]
Ile	5.3 ± 0.9[b]	2.9 ± 0.0[c]
Thr	4.9 ± 0.0[bc]	4.0 ± 0.1[b]
Phe	4.9 ± 0.1[bc]	3.1 ± 0.0[c]
His	3.6 ± 0.1[cd]	2.3 ± 0.1[d]
Met	3.1 ± 0.0[d]	1.6 ± 0.0[e]
Trp	0.7 ± 0.1[e]	1.2 ± 0.2[e]
Non-essential Amino Acid (%AA/ΣAA)		
Glu	12.0 ± 0.3[a]	7.5 ± 0.1[d]
Asp	10.7 ± 0.2[b]	7.8 ± 0.1[d]
Ala	6.1 ± 0.7[c]	12.9 ± 0.1[b]
Tyr	5.9 ± 0.2[c]	8.2 ± 0.0[d]
Arg	5.6 ± 0.2[cd]	3.7 ± 0.1[e]
Ser	5.3 ± 0.1[cd]	9.8 ± 0.2[c]
Gly	5.2 ± 0.2[cd]	16.5 ± 0.2[a]
Pro	4.9 ± 0.3[d]	2.8 ± 0.1[ef]
Cys	1.8 ± 0.0[e]	1.8 ± 0.2[f]
Total essential amino acid	42.5[A]	28.7[B]
Total non-essential amino acid	57.5[A]	71.3[B]
Total amino acids by sample weight (g 100 g^{-1} samples)	37.3 ± 0.9[A]	47.8 ± 1.3[B]
Protein efficiency ratio (PER)	3.2[A]	2.3[B]

The results are expressed as Mean ± SD (n = 3). [a–b] Different letters in the same column denotes intragroup statistical significance at $p<0.05$. [A–B] Different letters denotes intergroup statistical significance at $p<0.05$

acid in SPP. The amount of total essential amino acid in SLP (42.5%AA/ΣAA) was significantly ($p<0.05$) higher than that of SPP (28.7%AA/ΣAA). In contrast, the amount of non-essential amino acid ($p<0.05$) in SPP (71.3%AA/ΣAA) was significantly ($p<0.05$) higher than that of SLP (57.5%AA/ΣAA). Glu was the most abundant non-essential amino acid and followed by Asp. This result is consistent with the finding of Hu et al. (2018), who reported that Glu was the main amino acid in silkworm larvae. Given that the contents of Glu and Asp in dried food generally range from 11.7 to 34.3%AA/ΣAA and from 7.8 to 13.8%AA/ΣAA, respectively (NutritionValue.Org 2020). The results of this study suggested that the silkworm powder had a high content for these two amino acids, making it a potential flavour enhancer, particularly for umami flavour. The umami flavour makes the food taste delicious or more pleasant, and they are commonly found in meat, fish, and dairy products (Dewi et al. 2016). Besides, SLP had significantly ($p<0.05$) higher protein efficiency ratio (PER = 3.2) than SPP (PER = 2.3). Given that the PER for plant proteins (i.e. soy protein and beans) usually range from 1.2 to 2.4 while animal proteins range from 3.1 to 3.7 (Mariotti 2017), the result in this study suggested that SLP could be an alternative protein source.

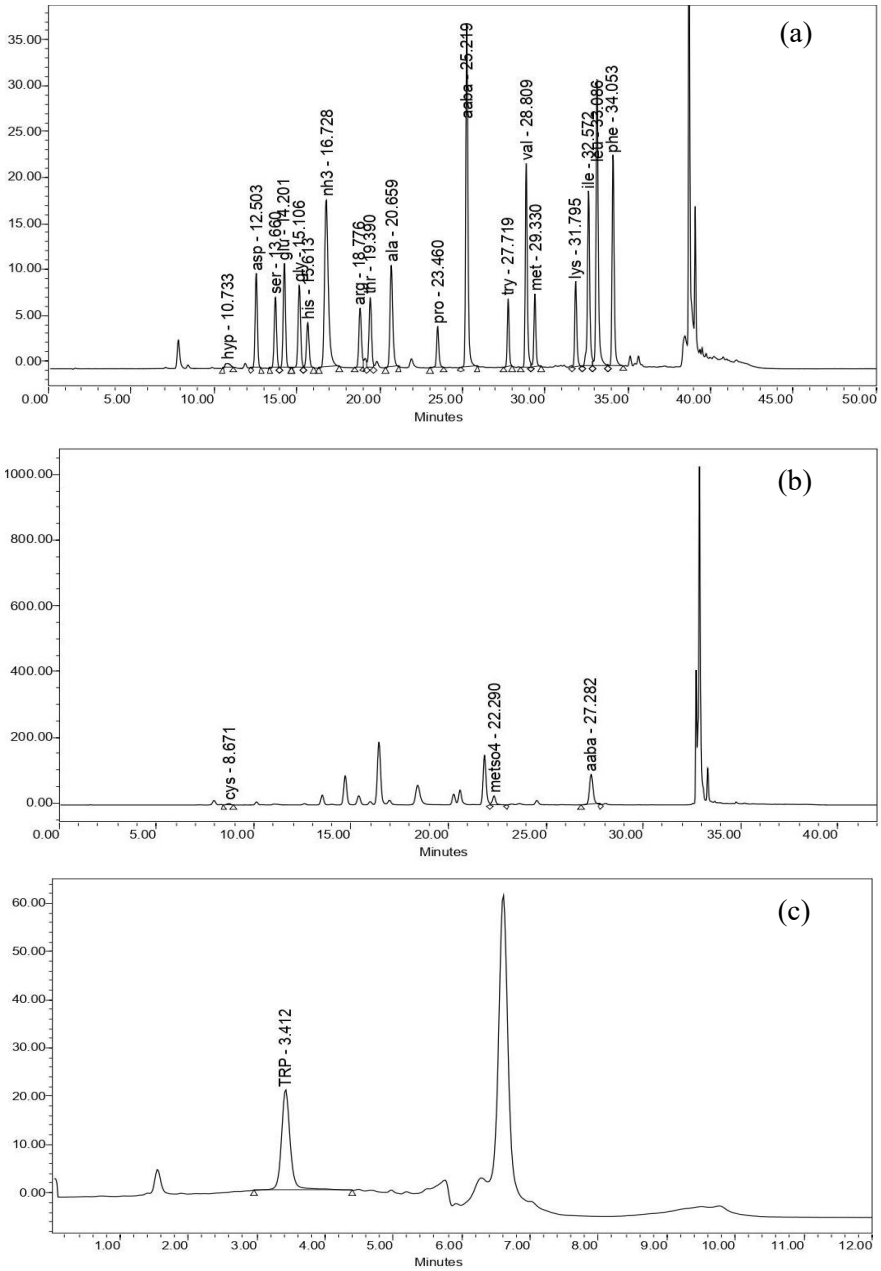

FIGURE 2. The typical chromatogram of amino acid profile for silkworm (*Bombyx mori*) larvae powder through: (a) hydrochloric acid hydrolysis, (b) performic acid hydrolysis, where *cya* denotes cysteine, and *metso*4 represents methionine, and (c) alkaline hydrolysis

FATTY ACID COMPOSITION

Table 4 summarises the fatty acid composition of SLP and SPP with SLP containing a significantly ($p<0.05$) higher amount of saturated fatty acid (SFA; 34.7 ± 0.1%) than SPP (34.5 ± 0.1%). The palmitic acid (C16:0) was the most abundant SFA in both SLP (33.2 ± 0.1%) and SPP (33.3 ± 0.1%). These results were consistent with the finding of Ji et al. (2016a) and Ekpo et al. (2009), who reported that the palmitic acid was the main component of SFA in silkworm powder. High amount of palmitic acid contributes to an interesting polymorphic property of food such as consistency and plasticity in various products including baked goods (Devi & Khatkar 2016). Meanwhile, the monounsaturated fatty acid (MUFA) was the most predominant unsaturated fatty acid in this study, with SPP (57.0 ± 0.1%) having a significantly ($p<0.05$) higher amount of MUFA than SLP (54.2 ± 0.1%). In particular, the oleic acid (C18:1n-9c) was the main component of MUFA in both SLP (43.0 ± 0.2%) and SPP (47.1 ± 0.1%), which is to the finding of Chieco et al. (2019). In comparison, α-linolenic acid (C18:3n-3) was the highest composition of polyunsaturated fatty acid (PUFA) in both silkworm powders in this study. Besides, the index for evaluating the nutritional value of dietary fat, i.e. the n-3/n-6 ratio (Zhao et al. 2010), of SPP (33.1, or an n-6/n-3 ratio of 0.03) was higher than that of SLP (13.5 or an n-6/n-3 ratio of 0.07). Given that the UK Department of Health (HMSO 1994) recommended that the value of the n-6/n-3 ratio of dried food product has to be lower than 4.0, therefore, the findings of this study suggested that the silkworm powder could serve as a potential health supplement for n-3 unsaturated fatty acids, and its relatively low n-6/n-3 ratio was probably due to its high content of α-linolenic acid.

TABLE 4. The fatty acid profile of silkworm (*Bombyx mori*) larval and pupae powder

Fatty acid methyl ester	Structure	SLP	SPP
Saturated fatty acid (SFA, %)			
Capric acid	C10:0	0.01 ± 0.00^{a}	0.02 ± 0.00^{b}
Lauric acid	C12:0	0.05 ± 0.00^{a}	0.1 ± 0.00^{b}
Myristic acid	C14:0	0.27 ± 0.01^{a}	0.32 ± 0.00^{b}
Pentadecanoic acid	C15:0	0.10 ± 0.00^{a}	0.06 ± 0.00^{b}
Palmitic acid	C16:0	33.2 ± 0.1^{a}	33.3 ± 0.1^{a}
Heptadecanoic acid	C17:0	0.49 ± 0.00^{a}	0.28 ± 0.00^{b}
Stearic acid	C18:0	0.05 ± 0.00^{b}	-
Arachidic acid	C20:0	0.4 ± 0.02^{a}	0.32 ± 0.00^{b}
Henicosanoic acid	C21:0	-	0.05 ± 0.01^{d}
Behenic acid	C22:0	0.05 ± 0.00^{d}	-
Monounsaturated fatty acid (MUFA, %)			
Myristoleic acid	C14:1	-	0.01 ± 0.00^{d}
Cis-10-Pentadecenoic acid	C15:1	0.13 ± 0.00^{a}	0.14 ± 0.00^{a}
Palmitoleic acid	C16:1	0.96 ± 0.00^{a}	1.6 ± 0.0^{b}
Cis-10-Heptadecanoic acid	C17:1	0.09 ± 0.00^{a}	0.08 ± 0.0^{a}
Elaidic acid (trans)	C18:1n-9t	9.9 ± 0.0^{ac}	8.1 ± 0.0^{b}
Oleic acid (cis)	C18:1n-9c	43.0 ± 0.2^{a}	47.1 ± 0.1^{ab}
Polyunsaturated fatty acid (PUFA, %)			
Linolelaidic acid (Trans)	C18:2n-6t	0.11 ± 0.00^{a}	0.06 ± 0.00^{b}
Linoleic acid (Cis)	C18:2n-6c	0.52 ± 0.02^{ab}	0.07 ± 0.01^{b}
α-Linolenic acid	C18:3n-3	10.4 ± 0.0^{a}	8.3 ± 0.0^{bc}
Arachidonic acid	C20:4n-6	0.14 ± 0.00^{a}	0.12 ± 0.00^{b}
ΣSFA (%)		34.7 ± 0.1^{A}	34.5 ± 0.1^{B}
ΣMUFA (%)		54.2 ± 0.1^{B}	57.0 ± 0.1^{A}
ΣPUFA (%)		11.1 ± 0.0^{A}	8.5 ± 0.0^{B}
Ratio n-3/n-6		13.5	33.1
Ratio n-6/n-3		0.07	0.03

The results are expressed as Mean ± SD (n = 3). $^{a-b}$ Different letters in the same column denotes intragroup statistical significance at $p<0.05$. $^{A-B}$ Different letters denotes intergroup statistical significance at $p<0.05$

MINERAL COMPOSITION

Table 5 shows the mineral composition of silkworm larvae and pupae powder, in which the distribution of elements in SPP was significantly ($p<0.05$) higher than SLP. In this study, potassium (K) was the highest amount of macromineral found in both SLP and SPP which were 640.1 ± 7.5 and 934.3 ± 8.6 mg/100 g, respectively. Meanwhile, the sodium-to-potassium (Na: K) ratio for SLP and SPP were 0.03 and 0.02, respectively. Given that the Na: K ratio is a key predictor for non-communicable diseases, such as blood pressure, stroke, and cardiovascular disease (Lança de Morais et al. 2018), the finding of this study suggested that both silkworm powders may have the potential of reducing the risk of non-communicable diseases in human. On the other hand, Zinc (Zn) was the most abundant micromineral found in both SLP and SPP, with their respective value of 7.1 ± 0.1 and 6.1 ± 0.2 mg/100 g. Besides, both SLP and SPP could be a good source of iron (3.5 ± 0.0 and 3.8 ± 0.0 mg/100 g for SLP and SPP, respectively), and they were rich in magnesium (229.4 ± 2.0 and 314.4 ± 3.2 mg/100 g for SLP and SPP, respectively) as well. The low amount of calcium in silkworm powder, i.e. 55.5 ± 0.4 and 37.8 ± 0.4 mg/100 g for SLP and SPP, respectively, was probably due to the absence of internal skeleton in silkworms (Köhler et al. 2019). The mineral contents in both silkworm powders differed from each other, and it may be due to differences in developmental stages of silkworm (larval and pupae stage) and the method used in processing the powder (Ji et al. 2016b).

TABLE 5. The mineral composition of silkworm larval and pupae powder

Mineral	SLP (mg/100 g)	SPP (mg/100 g)	'Source of'	'High in'
Macromineral				
Calcium (Ca)	55.5 ± 0.4^{d}	37.8 ± 0.4^{d}	120	240
Sodium (Na)	17.8 ± 2.8^{cd}	17.6 ± 3.1^{cd}		
Potassium (K)	640.1 ± 7.5^{a}	934.3 ± 8.6^{a}		
Magnesium (Mg)	229.4 ± 2.0^{e}	314.4 ± 3.2^{c}	45	90
Phosphorus (P)	420.6 ± 6.4^{b}	691.1 ± 7.1^{b}		
Micromineral				
Iron (Fe)	3.5 ± 0.1^{B}	3.8 ± 0.0^{B}	2.1	4.2
Zinc (Zn)	7.1 ± 0.1^{A}	6.1 ± 0.2^{A}	2.25	4.5
Manganese (Mn)	0.91 ± 0.01^{C}	1.4 ± 0.0^{B}		
Copper (Cu)	1.1 ± 0.0^{C}	1.2 ± 0.0^{B}		

The results expressed as Mean ± SD ($n = 3$). $^{a-b}$ Different letters denotes statistical significance of macromineral at $p<0.05$.

$^{A-B}$ Different letters denotes statistical significance of micromineral at $p<0.05$.

'Source of' and 'High in' - nutrition claims that express the nutrient level in food product (refer to Nutrient Reference Values (NRVs) which stated in the Codex Guidelines for Nutrition Labelling)

MICROBIOLOGICAL QUALITY

The total aerobic count (TAC) is an indicator for bacterial contamination in food. The aerobic count for SLP and SPP were not significantly different ($p>0.05$) i.e. 3.5 ± 0.4 and 3.3 ± 0.1 log CFU/g, respectively. However, SLP had significantly ($p < 0.05$) higher yeast and mould count (2.9 ± 0.2 log CFU yeast and mould/g) as compared to SPP (2.7 ± 0.1 log CFU/g). Although there are no microbial limits set for edible insect powder, the results for TAC and yeast and mound count (YMC) in this study

complied with the limits set by the US Food and Drug Administrations which is less 10^4 CFU/g (4 log CFU/g) for both TAC and YMC (Kenneth 2013). Meanwhile, Chitrakar et al. (2019) reported that some pathogenic microorganisms such as *Salmonella* spp. and *Cronobacter* spp. could survive in dried food. The presence of microorganisms in the silkworm powder could be due to the cross-contamination during unhygienic handling, processing, contaminated container, and improper storage method (Braide et al. 2011). Therefore, there is a need to increase awareness of proper handling practices among food handlers to minimise the risk of cross-contamination and foodborne infection.

Conclusion

Silkworm larvae powder exhibited greater amount of crude protein, total essential amino acid, and unsaturated fatty acid than silkworm pupae powder. The high protein efficiency ratio of silkworm larvae powder indicated that it contained a higher amount of essential amino acid than silkworm pupae powder. However, silkworm pupae powder contains greater amount of fat, ash, crude fibre, carbohydrate, and energy than silkworm larvae powder. Besides, silkworm larvae powder had a higher n-3/n-6 ratio and solubility in water with a lower microorganisms count. This study showed that both silkworm powders were a good source of iron, and they were rich in magnesium and zinc. The high nutrient in silkworm powder could offer immense potential for mitigating nutrient deficiency, especially protein deficiency in developing countries. Further study on functional and/or bioactive properties of silkworm powder is, therefore, essential.

Acknowledgements

This study was funded by the Dana Modal Insan (MI-2020-007) research grant provided by Universiti Kebangsaan Malaysia.

References

Adámková, A., Mlček, J., Kouřimská, L., Borkovcová, M., Bušina, T., Adámek, M., Bednářová, M. & Krajsa, J. 2017. Nutritional potential of selected insect species reared on the island of Sumatra. *International Journal of Environmental Research and Public Health* 14(5): 521.

Ademola, O.A., Omolara, A.H. & Abioye, O.R. 2017. Amino acids profile of bee brood, soldier termite, snout beetle larva, silkworm larva, and pupa: Nutritional implications. *Advances in Analytical Chemistry* 7: 31-38.

Ahmad Jelani, N., Azlan, A., Khoo, H. & Razman, M. 2019. Fatty acid profile and antioxidant properties of oils extracted from dabai pulp using supercritical carbon dioxide extraction. *International Food Research Journal* 26(5): 1587-1598.

Akande, A.O., Jolayemi, O.S., Adelugba, V.A. & Akande, S.T. 2020a. Silkworm pupae (*Bombyx mori*) and locusts as alternative protein sources for high-energy biscuits. *Journal of Asia-Pacific Entomology* 23(1): 234-241.

Akande, O.A., Falade, O.O., Badejo, A.A. & Adekoya, I. 2020b. Assessment of mulberry silkworm pupae and African palm weevil larvae as alternative protein sources in snack fillings. *Heliyon Journal* 6(4): e03754.

Anootthato, S., Therdthai, N. & Ritthiruangdej, P. 2019. Characterization of protein hydrolysate from silkworm pupae (*Bombyx mori*). *Journal of Food Processing and Preservation* 43(8): e14021.

Anuduang, A., Loo, Y.Y., Jomduang, S., Lim, S.J. & Wan Mustapha, W.A. 2020. Effect of thermal processing on physico-chemical and antioxidant properties in mulberry silkworm (*Bombyx mori* L.) powder. *Foods* 9(7): 1-11.

AOAC. 1990. *Official Methods of Analysis of AOAC International*. Association of Official Analytical Chemists (AOAC).

Beuchat, L.R., Komitopoulou, E., Beckers, H., Betts, R.P., Bourdichon, F., Fanning, S., Joosten, H.M. & Ter Kuile, B.H. 2013. Low-water activity foods: Increased concern as vehicles of foodborne pathogens. *Journal of Food Protection* 76(1): 150-172.

Braide, W., Oranusi, S., Udegbunam, L., Oguoma, O., Akobondu, C. & Nwaoguikpe, R. 2011. Microbiological quality of an edible caterpillar of an emperor moth, *Bunaea alcinoe*. *Journal of Ecology and the Natural Environment* 3(5): 176-180.

Chang, L.S., Karim, R., Abdulkarim, S.M. & Ghazali, H.M. 2018. Production and characterization of enzyme-treated spray-dried soursop (*Annona muricata* L.) powder. *Journal of Food Process Engineering* 41(5): e12688.

Chieco, C., Morrone, L., Bertazza, G., Cappellozza, S., Saviane, A., Gai, F., Di Virgilio, N. & Rossi, F. 2019. The effect of strain and rearing medium on the chemical composition, fatty acid profile and carotenoid content in Silkworm (*Bombyx mori*) pupae. *Animals* 9(3): 103.

Chitrakar, B., Zhang, M. & Adhikari, B. 2019. Dehydrated foods: Are they microbiologically safe? *Critical Reviews in Food Science and Nutrition* 59(17): 2734-2745.

Devi, A. & Khatkar, B.S. 2016. Physicochemical, rheological and functional properties of fats and oils in relation to cookie quality: A review. *Journal of Food Science and Technology* 53(10): 3633-3641.

Dewi, E.N., Amalia, U. & Mel, M. 2016. The effect of different treatments to the amino acid contents of micro algae *Spirulina* sp. *Aquatic Procedia* 7: 59-65.

Ekpo, K., Onigbinde, A. & Asia, I. 2009. Pharmaceutical potentials of the oils of some popular insects consumed in southern Nigeria. *African Journal of Pharmacy and Pharmacology* 3(2): 051-057.

FAO & WHO. 2007. *Codex Alimentarius, Food Labelling:* Fifth edition. Food and Agriculture Organization (FAO) and World Health Organization (WHO).

Gurjar, T.S., Siddhapara, M.R. & Surani, P.M. 2018. Biology of mulberry silkworm, *Bombyx mori* L. on mulberry, *Morus alba* L. *Journal of Entomology* 6(4): 276-280.

HMSO. 1994. *Nutritional Aspects of Cardiovascular Disease.* Her Majesty's Stationery Office (HMSO).

Hu, M.B., Wang, J.L., Liu, Y.J., Yuan, X., Li, J.H., Wu, C.J. & Li, L. 2018. Structure characterization and antioxidant properties of proteins extracted from the larva of *Bombyx mori* L. *Tropical Journal of Pharmaceutical Research* 17(11): 2177-2182.

Ji, S.D., Kim, N.S., Kweon, H., Choi, B.H., Kim, K.Y. & Koh, Y.H. 2016a. Nutrition composition differences among steamed and freeze-dried mature silkworm larval powders made from 3 *Bombyx mori* varieties weaving different colored cocoons. *International Journal of Industrial Entomology* 33(1): 6-14.

Ji, S.D., Kim, N.S., Kweon, H., Choi, B.H., Yoon, S.M., Kim, K.Y. & Koh, Y.H. 2016b. Nutrient compositions of *Bombyx mori* mature silkworm larval powders suggest their possible health improvement effects in humans. *Journal of Asia-Pacific Entomology* 19(4): 1027-1033.

Jintasataporn, O. 2012. Production performance of broiler chickens fed with silkworm pupa (*Bombyx mori*). *Journal of Agricultural Science and Technology B* 2: 505-510.

Kenneth, Y. 2013. *Revised Guidelines for the Assessment of Microbiological Quality of Processed Foods.* Department of Health Food and Drugs Administration, Republic of Philippines.

Kim, H.W., Setyabrata, D., Lee, Y.J., Jones, O.G. & Kim, Y.H.B. 2016. Pre-treated mealworm larvae and silkworm pupae as a novel protein ingredient in emulsion sausages. *Innovative Food Science & Emerging Technologies* 38: 116-123.

Kim, J.E., Lee, M.R., Choi, J.Y., Park, J.J., Kim, H.R., Song, B.R., Choi, Y.W., Kim, K.M. & Hwang, D.Y. 2018. Anti-obesity effect of ethanol extracts from silkworm (*Bombyx mori*) pupae powder fermented with *Cordyceps militaris* in the primary adipocytes and high fat diet-induced obesity model mice. *Journal of Life Science* 28(7): 786-794.

Köhler, R., Kariuki, L., Lambert, C. & Biesalski, H. 2019. Protein, amino acid and mineral composition of some edible insects from Thailand. *Journal of Asia-Pacific Entomology* 22(1): 372-378.

Kweon, H., Jo, Y.Y., Kim, H., Ju, W.T. & Lee, J.H. 2019. Proximate and nutritional compositions of freeze-dried silkworm powder as edible insect resources. *Journal of Sericultural and Entomological Science* 55(2): 33-39.

Lança de Morais, I., Lunet, N., Albuquerque, G., Gelormini, M., Casal, S., Damasceno, A., Pinho, O., Moreira, P., Jewell, J. & Breda, J. 2018. The sodium and potassium content of the most commonly available street foods in Tajikistan and Kyrgyzstan in the context of the FEEDCities Project. *Nutrients* 10(1): 98.

Lenzi, A., Orlandini, A., Bulgari, R., Ferrante, A. & Bruschi, P. 2019. Antioxidant and mineral composition of three wild leafy species: A comparison between microgreens and baby greens. *Foods* 8(10): 487.

Mariotti, F. 2017. *Vegetarian and Plant-Based Diets in Health and Disease Prevention*. Massachusetts: Academic Press. pp. 621-642.

Maturin, L. & Peeler, J. 2001. *BAM Aerobic Plate Count.* Food and Drug Administration (FDA).

Muanghorn, W., Konsue, N., Sham, H., Othman, Z., Mohamed, F., Noor, N.M., Othman, N., Akmal, N.S.S.M.N., Fauzi, N.A. & Solomen, M.M.P.D. 2018. Effects of gamma irradiation on tropomyosin allergen, proximate composition and mineral elements in giant freshwater prawn (*Macrobrachium rosenbergii*). *Journal of Food Science and Technology* 55(5): 1960-1965.

Ng, S.R., Noor, H.S.M., Ramachandran, R., Tan, H.Y., Ch, S.E., Chang, L.S., Babji, A.S. & Lim, S.J. 2020. Recovery of glycopeptides by enzymatic hydrolysis of edible bird's nest: The physicochemical characteristics and protein profile. *Journal of Food Measurement and Characterization* 14(5): 2635-2645.

Nguyen, P., Kim, K.Y., Kim, A.Y., Kim, N.S., Kweon, H., Ji, S.D. & Koh, Y.H. 2016. Increased healthspan and resistance to Parkinson's disease in Drosophila by boiled and freeze-dried mature silkworm larval powder. *Journal of Asia-Pacific Entomology* 19(2): 551-561.

NutritionValue.Org. 2020. Nutritional value of product (USDA national nutrient database for standard reference). https://www.nutritionvalue.org/.

Omotoso, O.T. 2015. An evaluation of the nutrients and some anti-nutrients in silkworm, *Bombyx mori* L. (*Bombycidae: Lepidoptera*). *Jordan Journal of Biological Sciences* 8(1): 45-50.

Rodríguez-Ortega, A., Pino-Moreno, J.M., Ángeles-Campos, S.C., García-Pérez, Á., Barrón-Yánez, R.M. & Callejas-Hernández, J. 2016. Nutritional value of larvae and pupae of silkworm (*Bombyx mori*) (*Lepidoptera: Bombycidae*). *Revista Colombiana de Entomología* 42(1): 69-74.

Rumpold, B.A. & Schlüter, O.K. 2013. Potential and challenges of insects as an innovative source for food and feed production. *Innovative Food Science & Emerging Technologies* 17: 1-11.

Ryu, K.S., Lee, H.S., Kim, K.Y., Kim, M.J., Sung, G.B., Ji, S.D. & Kang, P.D. 2013. 1-Deoxynojirimycin content and blood glucose-lowering effect of silkworm (*Bombyx mori*) extract powder. *International Journal of Industrial Entomology* 27(2): 237-242.

Sheikh, I., Banday, M., Baba, I., Adil, S., Nissa, S.S., Zaffer, B. & Bulbul, K. 2018. Utilization of silkworm pupae meal as an alternative source of protein in the diet of livestock and poultry: A review. *Journal of Entomology and Zoology Studies* 6(4): 1010-1016.

Tamanna, N. & Mahmood, N. 2015. Food processing and Maillard reaction products: Effect on human health and

nutrition. *International Journal of Food Science* 2015: 526762.

Tchabo, W., Ma, Y., Kaptso, G.K., Kwaw, E., Cheno, R.W., Wu, M., Osae, R., Ma, S. & Farooq, M. 2018. Carrier effects on the chemical and physical properties of freeze-dried encapsulated mulberry leaf extract powder. *Acta Chimica Slovenica* 65(4): 823-835.

Trattner, S., Becker, W., Wretling, S., Öhrvik, V. & Mattisson, I. 2015. Fatty acid composition of Swedish bakery products, with emphasis on trans-fatty acids. *Food Chemistry* 175: 423-430.

Trina, A., Kusharto Clara, M.K. & Muntikah, A.E.T. 2014. Pury, a fine powder made from silkworm pupae: Utilization and it's effects on nutritive value and organoleptic quality of traditional snack foods. *Journal of Food and Nutrition Research* 2(9): 582-586.

Yang, Y., Tang, L., Tong, L. & Liu, H. 2009. Silkworms culture as a source of protein for humans in space. *Advances in Space Research* 43(8): 1236-1242.

Yu, Y., Li, H., Zhang, B., Wang, J., Shi, X., Huang, J., Yang, J., Zhang, Y. & Deng, Z. 2018. Nutritional and functional components of mulberry leaves from different varieties: Evaluation of their potential as food materials. *International Journal of Food Properties* 21(1): 1495-1507.

Zhao, F., Zhuang, P., Song, C., Shi, Z.H. & Zhang, L.Z. 2010. Amino acid and fatty acid compositions and nutritional quality of muscle in the pomfret, *Pampus punctatissimus*. *Food Chemistry* 118(2): 224-227.

Loo Yuet Ying, Lim Hui Ying, Noor-Soffalina Sofian-Seng, Wan Aida Wan Mustapha & Noorul Syuhada Mohd Razali*
Department of Food Sciences
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor Darul Ehsan
Malaysia

Noor-Soffalina Sofian-Seng, Wan Aida Wan Mustapha & Noorul Syuhada Mohd Razali*
Innovation Centre for Confectionery (MANIS)
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor Darul Ehsan
Malaysia

*Corresponding author; email: syuhada_ns@ukm.edu.my

Received: 26 March 2021
Accepted: 29 June 2021