Killing process on the nutrient content, product stability and in vitro digestibility of black soldier fly (Hermetia illucens) larvae meals

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Research

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Abstract

BACKGROUND

Black soldier fly (BSF; *Hermetia illucens*) is considered as a potential sustainable insect alternative source of protein for animal feed; however, the quality of BSF meal is greatly influenced by the killing method. Therefore, the purpose of this article is to compare the quality of BSF obtained by different killing methods.

RESULTS

BSF at the 18-day-old prepupae stage were separated into six different killing methods ($n = 3$): 1. blending, 2. freezing, 3. CO_2 treatment, 4. vacuum, 5. Blanching and 6. humane method. After killing, were obtained by hot air oven drying and grinding. The chemical composition and *in vitro* digestibility calculated from sediment was not affected by the killing method, except that blending provided the worst BSF quality for all measured parameters ($P < 0.05$). The highest quality of BSF was obtained from the heat treatment procedures (blanching and the humane method), as they produced lower acidity after killing, total viable counts, browning reaction (enzymatic and non-enzymatic), darkness, moisture, fat acidity, protein and lipid oxidation during storage compared with other killing procedures ($P < 0.05$). Interestingly, the highest of free amino acids in the supernatant after *in vitro* digestibility of BSF samples was observed with the humane killing method ($P < 0.05$), whereas other parameters were similar to those obtained with blanching.

CONCLIUSION

Blanching and humane method produce BSF with the nutritional quality, safety and stability. humane method did not produce clearly different outcomes to blanching; therefore, the selection of one of these techniques over the other should depend on the regulations in each country.

Background

Nutrition is a major factor which influences the yield, health and profits of the animal production industry. Raw protein materials are the most expensive component in feed formulation when compared with other groups of nutrients. Therefore, protein sources of high quality with a reasonable cost should be an important consideration. Fishmeal and soybean meal have been used as general protein sources for the feed industry; however, these raw materials contain several disadvantages. Quality fluctuation and pathogen contamination are observed as disadvantages for fishmeal, whereas an imbalance in amino acids, low digestibility and anti-nutritional factors are presented in soybean meal. Moreover, these ingredients affect environmental deterioration [1, 2]. Therefore, alternative protein raw materials should be sought as a solution to these problems. In recent years, there has been increasing interest in insects as an alternative sustainable protein source for animals, especially in fish, poultry and swine [3]. Their suitable chemical composition, low-cost production procedures, high feed conversion efficiency and rapid, environmentally friendly productive cycle are advantages of insect production [1, 2]. Moreover, most recent experimental studies reported several benefits on growth performance, health and product quality using insects in feed formulation at the appropriate ratio [1, 2]. However, the use of insects as an alternative protein source faces many challenges, including large scale production with stability of product quality, price competitiveness with general protein sources and country-specific regulatory barriers [3]. Fortunately, consumers accept animal products produced from insect-fed animals; however, safety and quality control should be verified and noticed by professional auditors along the production process [3]. Insects are considered as a novel protein source; therefore, attention must be paid to the above challenges. In addition, the number of publications on the use of insects as a protein source for animals has only increased in the last decade [1, 2]. Therefore, these challenges and barriers should be addressed in the near future.

Several insect species are discovered and used for several benefits, whereas black soldier fly (*Hermetia illucens*) has received the most attention as an alternative protein source for animals in tropical and temperate countries [4]. The large diversity of diets (organic waste: manure, food waste and industrial by-products), higher feed conversion ratio, absence of disease transmission to humans and inability to concentrate pesticides or mycotoxins from their diets in their body mass are some of the reasons driving interest in this insect species from researchers, insect farmers, animal producers and feed manufactures [4]. Not only have waste management systems based on black soldier flies been established in several industries, but high-value products can also be obtained after the processing of products such as biodiesel, antimicrobial peptides and chitosan [4].

Consistent quality and safety are the most important considerations for black soldier fly farming and processing units. As we know, the diet is the most influential factor on the quality of black soldier fly pupae meal (BSF) [5]. However, the killing method is also a highly influential factor on BSF quality [1, 6–8]. Heat treatment, freezing, asphyxiation and mechanical disruption are the general procedures to euthanize black soldier fly prepupae [7]. The fast killing method with heat treatment (blanching in boiling water for a short time) has been suggested and can decrease the amount of microbial contamination, conserve nutrient quality, prevent degeneration of the end product and provide a bright colour which is acceptable to consumers, whereas slow killing methods without heat treatment (freezing, asphyxiation and blending) provided adverse consequences [6–8]. In contrast, Montevecchi *et al* [9]. reported that blending was the best procedure for preserving fat integrity. Freeze drying or blending without any heat treatment was performed after killing procedures to obtain a sample for further laboratory analysis [6–9], which was different from the general processing procedure of baking killed black soldier fly prepupae in an oven until it reached less than 10% moisture, grinding and storing in a vacuum pack and/or frozen at − 20 °C. Moreover, animals must be unconscious during the killing procedure according to regulations regarding animal welfare in livestock and to promote a good-quality end product. The sensory response to pain is presented in insects; therefore, ethical aspects should also be considered [10]. Therefore, the effects of different killing procedures (heating, freezing, asphyxiation or humane method) on the quality of oven-dried BSF were evaluated in the current study.

Materials And Methods

Animals, killing procedures and processing

BSF prepupae at 18 days of age were obtained from a commercial black soldier fly farm (Orgafeed Co. Ltd., Bangkok, Thailand) on which fattening broiler chicken diet and palm kernel meal were fed as the main diets. After an adaptive period in a temperature-controlled room (27 °C) for 4 h, black soldier flies were separated randomly into six groups with three replicates (1 kg of black soldier fly prepupae in fresh matter per replicate). The killing methods were adapted from other research studies [6–9]. Six different killing procedures were performed as follows: 1. blending (insects were blended for 2 min by homogenizer), 2. freezing (placing the insects at -20 °C for 6 h), 3. carbon dioxide treatment (CO_2, a plastic container containing the insects was filled with 100% CO_2 and left at room temperature for 120 h), 4. vacuum (insects was placed under vacuum for 120 h), 5. blanching (insects were immerged in boiling water for 40 sec) and 6. humane method (insects were treated with CO_2 for 10 min after which the blanching procedure was performed). This study was conducted following the standard guidelines approved by the Institutional Animal Care and Use Committee of Kasetsart University, Bangkok, Thailand (ACKU63-VET-008). Then, insect samples were dried at 60 °C for 48 h, ground to a size of 1 mm size and kept at -20 °C (Except, the sample for microbial analysis).

Microbial analysis

BSF after killing in different methods (before dried) were collected into sterile tubes. The samples were immediately transported to Laboratory in Kasetsart University veterinary teaching hospital for bacterial culture and analysis.

Chemical compositions and physico-chemical properties

Chemical compositions

The proximate composition; moisture, crude protein, ether extract, crude fibre, crude ash and nitrogen-free extract (NFE) of the BSF dried samples were determined using the standard methods as described by AOAC [11].

pH and colour

The pH was measured in duplicate by homogenizing one g of BSF powder in nine mL of 15-ohm pure distilled water. The pH of the homogenate was monitored by using a digital pH-meter (Seven compact S220, Mettler Toledo, Ayer Raja Crescent, Singapore). The colour (Lightness -L*, redness -a*, yellowness -b* values; CIE 1976) was measured in duplicate with a portable colorimeter Chroma Meter CR-400 Minolta (Minolta Sensing Inc., Osaka, Japan)

Enzymatic and non-enzyme browning reaction

Enzymatic browning reaction

Five grams of sample were mixed with 10 ml of 50 mM phosphate buffer pH 6.0 and homogenized in an ice bath. The homogenate was centrifuged at 5,000 rpm for 10 min at 4^{o}C and the supernatant kept for analysis of tyrosinase activity. Next, 1.2 ml of 50 mM phosphate buffer pH 6.0 with 800 µl of 10 mM L-DOPA (substrate for tyrosinase) was added to 50 µl of the supernatant, 250 µl of enzyme and substrate and the reaction incubated at 30^{o}C for 5 min in a microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, Winooski, VT). Absorbance was measured at 475 nm. One unit of enzyme corresponds to the amount that catalyses the transformation of 10 mM of L-DOPA to dopachrome from tyrosinase per minute under standard conditions of pH, temperature and substrate concentration [12].

Non-enzymatic browning reaction

A modified version of the method described by Hwang *et al* [13]. was performed. Briefly, 1 g of sample was mixed with 7 ml of 50 mM CaCl and 50 mM Tris-HCl (pH 7.0). The mixture was vortexed and centrifuged at 10,000 g for 15 min at 4 °C. To observe the progress of the Maillard reaction, the absorbance of 250 µl of supernatant at 440 nm and 550 was recorded for the early and late Maillard reactions, respectively. The formula for calculating the browning index is Browning Index (BI) = (Absorbance at 420 nm – Absorbance at 550 nm).

Protein oxidation

Protein oxidation, as measured by the total carbonyl content, was evaluated at weeks 1, 4 and 12 by derivatization using the DNPH method with slight modifications (Armenteros *et al.* [14]). The ground BSF samples (2 g/sample) were homogenized in 20 ml pyrophosphate buffer (pH 7.4) consisting of 2.0 mM $Na_4P_2O_7$, 10 mM Tris–maleate, 100 mM KCl, 2.0 mM $MgCl_2$ and 2.0 mM EGTA using an ultra-turrax homogenizer for 30 s. The homogenates were divided into two equal aliquots of 0.1 ml. Then, proteins were precipitated in both aliquots by adding 1 ml of 10% TCA and centrifuged for 5 min at 5000 g (4 °C). The supernatants were removed, and one pellet was treated with 1 ml 2 N HCl (for quantifying protein concentration) and the second pellet with an equal volume of 0.2% (w/v) DNPH in 2 N HCl (for carbonyl concentration measurement). Both samples were incubated for 1 h at room temperature (vortexed every 15 min). After that, samples containing 0.2% DNPH were precipitated with 1 ml of 10% TCA and washed twice with 1 ml of 1:1 ethanol/ethyl acetate (v/v), vortexed, and centrifuged for 5 min at 10,000 g (4 °C). The pellets were then dissolved in 750 µl of 20 mM sodium phosphate buffer pH 6.5 containing 6 M guanidine hydrochloride, stirred and centrifuged for 2 min at 5000 rpm (4 °C) to remove insoluble fragments. Protein concentration was calculated from absorption at 750 nm using BSA (bovine serum albumin) as the standard (Lowry *et al.* [15]). A number of carbonyls were measured at 370 nm in

250 µl sample using a microplate reader and expressed as nmol of carbonyl per mg of protein using the adsorption coefficient for the protein hydrazones (21.0 mM^{-1} cm^{-1}).

Fat acidity

AACC [16] procedures were followed with slight modification to determine fat acidity. Briefly, 2 g of sample powder was dried at 110 °C for 2 h and the weight was recorded. *The* Sample was transferred to a thimble, which was plugged with cotton. For fat extraction, 50 mL of petroleum ether was added. Soxtech method was used for extraction, and the solvent was evaporated. *The* extract was dissolved in 50 mL of toluene-alcohol-phenolphthalein solution (TAP) and placed in a 250 mL flask. *The e*xtract solution was titrated with 0.0178 N KOH to the endpoint matching the standard colour.

Storage trail

Lipid oxidation

Lipid oxidation was measured at weeks 1 and 4 according to the method of Larouche *et al*. [7] Thiobarbituric acid-reactive substances (TBARs) were measured in BSF powder as follows: 0.5 g of each ground BSF powder (TBARs) homogenised in 10 ml of cold 1.15% phosphoric acid was centrifuged at 3,000 rpm for 10 min at 4 °C. A 400 µl aliquot of supernatant was mixed with 1% TBARs and 0.1 mM of butylated hydroxytoluene in 0.05% NaOH. All tubes were then treated with 200 µl of 7% phosphoric acid and boiled for 15 min. The reaction mixtures were cooled in an ice bath for *10* min and placed in the dark, then 1.5 ml of n-butanol was added and the mixture centrifuged (Hermile Z233 MK-2, Wehingen, Germany) at 2,000 rpm for 5 min at 4 °C. Activity was evaluated with a microplate reader at 532 nm (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, Winooski, VT), that measured the absorbance of TBARs and a 1,1,3,3-tetraethoxypropane calibration curve (Botsoglou *et al.* [17]). Oxidation products were quantified as malondialdehyde (MDA) equivalents (µg MDA/kg BSF powder).

In vitro digestibility

In vitro digestibility procedures for swine were modified from Kong *et al* [18]. which consists of a two-step method. In the first step, 0.5 g of ground sample was mixed with 17.5 ml of stimulate gastric fluid (SGF containing; 25 ml of 0.1 M sodium phosphate buffer pH 6.0 and 10 ml of 0.2 M HCl pH 7.0, adjust to pH 2.0 with 1 M HCl or NaOH) to simulate digestion conditions in the stomach. Then, 0.5 ml of 5 chloramphenicol was added and 0.5 ml of freshly made pepsin solution (10 mg/ml, pepsin from porcine gastric mucosa) was also added. The reaction mixture was incubated in a shaking incubator at 39^{o}C for 6 h. In the second step, to stop the reaction of SGF and simulate digestion in the small intestine, 7.5 ml of simulated intestinal fluid solution (SIF containing 10 ml of 0.2 M sodium phosphate buffer solution pH 6.8 and 5 ml of 0.6 M NaOH were adjusted to pH 7.0) was mixed with 0.5 ml of pancreatin solution (50 mg/ml of pancreatin from porcine pancreas) and incubated for 18 h at 39^{o}C in a shaking incubator. After incubation, 2.5 ml of 20% trichloroacetic acid was added, and the sample was left for 30 min at room temperature to stop the enzyme reaction and precipitate the indigestible protein. One millilitre aliquots of supernatants were kept at -20 ^{o}C for analysis of free amino acids (*in vitro* protein digestibility; IVPD) and maltose (*in vitro* carbohydrate digestibility; IVCD), performed according to the trininobenzene sulfonic acid (TNBS) and Dinitrosalicylic acid (DNS) method, respectively. The IVPD and IVCD study in the supernatant were described in Sansuwan *et al* [19]. Residues were then filtered through a filter glass crucible in a Fibertec System cold extraction unit. The samples were rinsed twice or more with distilled water. Next, two additional rinses with 30 ml of 95% ethanol and 30 ml of 99% acetone were performed in the filter crucible. Glass filter crucibles with undigested residues were dried at 60–70^{o}C for 24 h for determination of crude protein and organic matter digestibility in the sediment as described in Kovitvadhi *et al* [20].

Statistical analysis

All statistical analyses in this study were performed by using R-statistic with the Rcmdr Package (R Development Core Team [21]). Statistical significance was accepted at $P < 0.05$. One-way analysis of variance was performed for data with a normal distribution and homogeneity of variance, with killing methods as fixed factors, to determine the consequences on BSF qualities. Duncan's new multiple range test served as a post hoc test.

Results

Microbial analysis in BSF larvae after killing

An approximately 10-fold lower microbial load was observed in the blanching and humane group compared with other groups. Several specific bacterial species were discovered in the BSF, except when killing methods with heating treatment (blanching and the humane method). No *Salmonella* sp. were observed in any group in this study (Table 1).

Table 1 Microbial load after killing BSF larvae from different killing methods

Microorganism	Killing methods					
	Mechanical		Asphyxiation		Heat	
	Blending	Freezing	CO_2	Vacuum	Blanching	Humane
TVC (CFU/g sample)	$4.1x10^{10}$	$1.6x10^{10}$	$4.8x10^{10}$	$3.5x10^{10}$	$7.7x10^{9}$	$7.2x10^{9}$
Acinetobacter sp.	l	l	l	l	l	l
Bacillus cereus	l	l	l	l	l	l
Bacillus spp.	l	l	l	l	l	l
Enterobacter aerogenes	l	l	l	l	l	l
Enterobacter cloacae	l	l	l	l	l	l
Escherichia coli	l	l	l	l	l	l
Klebsiella pneumoniae	l	l	l	l	l	l
Proteus mirabilis	l	l	l	l	l	l
Salmonella sp.	l	l	l	l	l	l

TVC; Total viable aerobic count, CFU; Colony forming Unit, l; No growth, l; Growth

Chemical composition and physicochemical parameters of BSF larvae meal

The results for the effects of different killing methods on BSF qualities (proximate composition and their parameters) are summarized in Table 2. Statistically significant differences were presented in the chemical composition of BSF between the groups ($P < 0.001$) except crude ash and fibre ($P > 0.05$). The proportion of ether extract and crude protein in the blending group were lower than in the others ($P < 0.001$). On the other hand, the blending group was higher in nitrogen-free extract followed by the freezing, humane, blanching, CO_2 and vacuum groups ($P < 0.001$) as shown in Table 2.

Table 2
Proximate compositions and physicochemical properties of BSF larvae after killing with different processing

Parameters	Killing methods						SEM	*P*
	Mechanical		Asphyxiation		Heat			
	Blending	Freezing	CO_2	Vacuum	Blanching	Humane		
Proximate composition								
Dry matter (%FM)	95.7^{ab}	96.6^{c}	96.1^{bc}	95.5^{a}	97.4^{d}	97.2^{d}	0.181	**0.001**
Crude ash (%DM)	7.29	7.30	7.50	7.44	7.04	6.90	0.068	0.47
Ether extract (%DM)	25.7^{a}	27.3^{ab}	28.1^{b}	29.1^{b}	28.1^{b}	28.4^{b}	0.331	**0.001**
Crude protein (%DM)	39.3^{a}	44.9^{b}	46.8^{b}	52.5^{c}	46.3^{b}	44.6^{b}	0.990	**0.001**
Crude fibre (%DM)	10.2	9.22	9.29	9.51	9.66	9.77	0.116	0.12
NFE (%DM)	17.5^{d}	11.4^{c}	8.28^{b}	2.69^{a}	8.95^{bc}	10.3^{bc}	1.074	**0.001**
Physicochemical properties								
pH value	6.14^{a}	6.48^{c}	6.43^{bc}	6.36^{b}	7.31^{d}	7.47^{e}	0.128	**0.001**
Browning reaction								
Enzymatic (U)	2.65^{d}	0.83^{b}	1.53^{c}	1.52^{c}	ND^{a}	ND^{a}	0.246	**0.001**
Non-enzymatic (BI)	1.27^{d}	0.65^{b}	0.59^{b}	0.79^{c}	0.17^{a}	0.15^{a}	0.094	**0.001**
Colour								
L* (Lightness)	40.2^{a}	41.4^{b}	41.3^{b}	41.4^{b}	41.7^{c}	41.7^{c}	0.043	**0.001**
a* (Redness)	0.23^{d}	0.08^{b}	0.04^{a}	0.09^{b}	0.23^{d}	0.14^{c}	0.018	**0.001**
b* (Yellowness)	0.80^{c}	0.47^{b}	0.43^{a}	0.48^{b}	0.85^{d}	0.80^{c}	0.043	**0.001**
Hue	1.30^{a}	1.41^{b}	1.49^{c}	1.40^{b}	1.31^{a}	1.41^{b}	0.016	**0.001**
Chroma	0.83^{c}	0.48^{b}	0.43^{a}	0.49^{b}	0.88^{d}	0.81^{c}	0.046	**0.001**
Fat acidity	84.9^{c}	13.9^{b}	10.4^{b}	12.2^{b}	0.77^{a}	0.55^{a}	7.527	**0.001**

NFE; Nitrogen free extract

Enzymatic browning reaction was expressed as U, BI; Browning index

Fat acidity was expressed as mg of KOH per 100 mg of dry sample

Protein oxidation was expressed in nM carbonyl

Parameters	Killing methods						SEM	*P*
	Mechanical		Asphyxiation		Heat			
	Blending	Freezing	CO_2	Vacuum	Blanching	Humane		
Protein oxidation	0.81^{bc}	1.03^{c}	0.42^{a}	0.51^{ab}	0.60^{ab}	0.71^{ab}	0.060	**0.01**
NFE; Nitrogen free extract								
Enzymatic browning reaction was expressed as U, BI; Browning index								
Fat acidity was expressed as mg of KOH per 100 mg of dry sample								
Protein oxidation was expressed in nM carbonyl								

pH value after killing declined greatly in the blending group, whereas the killing methods involving heating (blanching and the humane method) and other techniques affected the pH only slightly or caused a moderate decrease, respectively ($P < 0.001$).

The highest rate of non-enzymatic browning was observed in the blending group, whereas vacuum, freezing, CO_2, blanching and humane exhibited lower rates of browning ($P < 0.001$). On the one hand, the highest rate of enzymatic browning was found in the blending group, followed by CO_2, vacuum and freezing, while this chemical reaction was not present in the blanching and humane groups ($P < 0.001$).

BSF killed by the blanching and humane methods were lighter in colour than those killed by other methods ($P < 0.001$). Most of BSF had low redness and yellowness values; however, the blending, blanching and humane groups were higher in these values than the others ($P < 0.001$). Three colour groups were formed on the basis of the hue angle value, which were 1. blending and blanching 2. freezing, vacuum and humane and 3. CO_2 ($P < 0.001$). The colourfulness (Chroma) in descending order was blanching, humane, blending, vacuum, freezing and CO_2 ($P < 0.001$). A low value of protein oxidation was presented in the CO_2, vacuum, blanching and humane groups, whereas a low value of fat acidity was revealed only in the blanching and humane groups compared with the others ($P < 0.01$) as show in Table 2

[Table 2 **should be here find this table at the end of the document text file]**

Storage trial of BSF after killing

The ascending trend for lipid peroxidation was presented during the storage period (Fig. 1), for which high values were observed in blending, vacuum, freezing, vacuum and CO_2, respectively ($P < 0.001$).

In vitro **digestibility of BSF larvae meal**

Table 3 present the *in vitro* digestibility performances based on supernatant or end-product

evaluation of carbohydrates was the highest in blending following by freezing, vacuum and CO_2, whereas the blanching and humane methods had low values for this parameter ($P < 0.05$). In contrast, the highest value of *in vitro* crude protein digestibility was illustrated in the humane group ($P < 0.001$). The lowest *in vitro* digestibility of dry matter and crude protein based on the sediment calculation method was found in the blending group ($P < 0.05$).

Table 3
In vitro digestibility of BSF larvae meal as a feed material for livestock

***In vitro* digestibility**	**Killing methods**						**SEM**	***P***
	Mechanical		**Asphyxiation**		**Heat**			
	Blending	**Freezing**	**CO_2**	**Vacuum**	**Blanching**	**Humane**		
Supernatant								
IVCD	19.5^{d}	13.2^{c}	8.42^{b}	11.2^{bc}	2.54^{a}	1.87^{a}	1.521	**0.001**
IVPD	122^{a}	112^{a}	124^{a}	129^{a}	133^{a}	166^{b}	5.374	**0.04**
Sediment **(%)**								
Dry matter	53.3^{a}	59.4^{b}	57.1^{ab}	61.0^{b}	56.1^{ab}	59.8^{b}	0.868	**0.04**
Crude protein	31.3^{a}	48.9^{b}	53.1^{b}	54.8^{b}	52.9^{b}	51.9^{b}	2.094	**0.001**
IVCD; *in vitro* carbohydrate digestibility (mM of *D*-maltose equivalence per g feed)								
IVPD; *in vitro* protein digestibility (mM of *DL*-alanine equivalence per g feed)								
^{a,b,c} Different superscripts indicate statistically significant differences ($P < 0.05$)								

Discussions

Impact of killing method on microbial load

Heating at the appropriate temperature and for the appropriate duration has a major influence on the reduction of microbial loads in BSF after killing [7], which is able to reduce the total aerobic viable count by around 10 times compared with other methods. As we know, the growth rate of bacteria is reduced in a cold temperature environment, which can result in a low microbial load in BSF after killing by freezing methods [7]. The total bacterial count in the blending method was similar to that of asphyxiation methods in this trial; however, the study of Larouche *et al.* [7] reported a lower value of this parameter in the blending group than with the asphyxiation method. The blending method provided a homogenized sample which destroyed the cell structure and increased the contact surface between bacteria and substrates which could promote bacterial growth. Black soldier fly prepupae can survive under asphyxiation for long periods, in contrast to house crickets (*Acheta domestica* [22]). Therefore, this technique promoted anaerobic conditions and prolonged the killing process at room temperature, which led to selective growth of a specific group of microbes. For these reasons, higher microbial loads of facultative anaerobic bacteria and anaerobic bacteria were observed in the asphyxiation treatment groups [7]. The rearing method and biosecurity system are important factors which correlate with the microbial load and community in BSF [23]. The absence of contamination by *Acinetobactor* sp., *Bacillus cereus* and *Salmonella* spp. during cultivation and processing contributed to the absence of growth of these bacteria in BSF. The absence of *Salmonella* spp. in raw materials is an important quality and safety indicator according to the regulations in several countries; therefore, good agricultural and biosafety practices must be strictly performed Ministry of Agriculture and Cooperatives, 2016 [24] to prevent contamination along the production process. However, contamination with coliforms and other pathogenic bacteria was discovered in this study, which came from the BSF substrates. Heat treatment was suggested to reduce these microorganisms. The overall number of bacteria in this study seemed higher than in the study of Larouche *et al.* [7]. The different outcome may have been a consequence of the bacterial culture being immediately performed after the end of killing procedures in this study, whereas Larouche *et al.* [7] cultured microbes from a freeze-thawed sample, which would have decreased the growth rate of microbes. The acidic conditions of end products promote a prolonged shelf life

and reduces the microbial growth rate [25], which relates to the activity of acid-producing bacteria such as *Lactobacillus* sp. [7]. As described above, heat treatment reduced the microbial load, whereas asphyxiation promoted bacterial growth. High pH was observed in heat-treatment groups, and low pH was presented in asphyxiation groups, which was comparable to other research studies [7]. The total viable count after killing by heat treatment was still higher than the limitation based on the regulation on animal feed of Thailand [24], therefore a further hot drying process with appropriate storage conditions should be performed to reduce microbial loads. From our suggestion, the pathogenic bacteria were observed in the black soldier fly prepupae after killing; therefore, further heat treatments and/or drying processes must be performed to prevent infection in animals and also cross contamination of humans, which is a public health concern.

Impacts of killing methods on chemical composition and physicochemical properties

Proximate compositions

The blanching procedure decreased the moisture levels of the end product compared with other methods, which has the benefit of reducing the cost of the drying process [7]. However, oven drying and grinding was performed before moisture evaluation. The same results were observed in this study, in which the reduction of water holding capacity was caused by protein denaturation after heat treatment [7, 26]. The lowest ash or mineral components were presented in the blanching group compared to other groups in this and another study [7], which could be a consequence of heat treatment conserving organic matter in a higher proportion compared with other techniques. The effects of different killing methods on ether extract composition in this study was similar to the study of Montevecchi *et al.* [9] in which blanching and freezing produced products higher in lipid than blending. On the other hand, Larouche *et al.* [7] reported that blanching produced the lowest lipid levels compared to asphyxiation, freezing and blending. However, no difference in fat composition between freezing and blanching was also published [6]. Differences in insect preparation, extraction, storage and lipid determination are the likely causes of these diverse outcomes. A lower amount of crude protein was observed in blanching compared with freezing in this and another study [8]. However, blending contained the lowest crude protein content. Freezing and blanching did not affect total amino acids of TM [27]. In terms of overall results and information, blending was the worst method for its inability to preserve the nutrients in BSF compared with other methods.

Physicochemical properties

The colour of BSF is an absolutely important factor in terms of customer acceptance because insect meal in light brown shades of beige, tan and khaki is preferred by customers, whereas dark brown or black is not accepted. The browning reaction and melanization are the chemical reactions which influence colour alterations after killing and processing [7, 8]. The enzymatic browning reaction or polyphenol oxidation in an aerobic condition promoted the darkish effects on BSF [28, 29]. Moreover, the complex between amino acids and reducing sugars was present after the drying process, leading to a darker colour, which is called the Maillard reaction or non-enzymatic browning reaction [30, 31]. Melanin was produced as the end product of melanization, which was the innate immune response of insects to harmful environments and promoted a darker colour of BSF [8]. The low level of browning reaction in BSF with heat treatment was caused by enzyme destruction by heat [7, 8]. Heat treatment can only terminate the enzymatic browning reaction [30, 31]; however, the decline of the non-enzymatic browning reaction was observed in this study, which may have resulted from a short time available for metabolism, as the killing process was rapid by heating [7]. The slow killing methods without an enzyme destruction protocol, such as freezing and asphyxiation, promoted the activation of several metabolic process, mainly lipolysis, melanization and browning, which led to a darker colour [8]. The darker colour developed immediately after blending the prepupae of BSF because the enzymatic browning reaction between substrates and enzymes released from cell destruction was increased in the homogenized samples. Therefore, the darkest colour was found in the blending procedure [7]. For these reasons, the BSF colour is greatly influenced by the killing procedures. The lightest BSF was observed in the hot treatment group, which agreed with other research studies [7, 8]. Therefore, limitation on the initiation

of browning reaction and melanisation was a major factor to prevent colour alteration of BSF. Blanching was considered as the best killing method to preserve the integrity and quality of BSF, which agreed with the report of Larouche *et al.* [7]. In contrast, Montevecchi *et al.* [9] reported blending or grinding of live black soldier fly prepupae as the best killing method due to homogenization of BSF prepupae in a specific solvent mixture that was water free and composed by chloroform-methanol 2:1 (v/v) which water was subtracted by methanol slowing down enzymatic activity (hydrolytic) especially lipase for generated reaction of fat acidity and lipid oxidation. The mechanisms by which different killing procedures affect BSF quality were described in detail. Fat acidity was used to represent the amount of free fatty acids in samples which correlated to lipase activity respectively [9], the fat acidity in the asphyxiation and freezing group was higher than that in the heat treatment group by approximately three and ten times, which agreed with this study [6, 7]. In this study, the extremely high value of fat acidity was observed in the blending group, which represented the high lipase activity in this sample. The freezing technique only decreased lipase activity, which remains in a low temperature environment (5 °C), even at − 30 °C [32]. On the other hand, heat treatment can destroy lipase, which minimizes fat acidity and prevents the pH reduction after killing compared with other methods. Another parameter representing the integrity of BSF is lipid oxidation; the high activity of this chemical reaction promotes several adverse outcomes for consumers such as nutrient loss, lower digestibility of products, rancidity, carcinogen substances and toxicity [33]. The rate of lipid oxidation from killing procedures with heat treatments were reduced around 1–2 times compared with other methods in this and another study [7], whereas the highest lipid oxidation value was presented in the blending group. Based on the standard, the BSF subjected to heat and vacuum treatment was considered not rancid (< 1.5 mg MDA/kg) after an oven drying process [34]. Only BSF subjected to the heat treatment procedure was still slightly rancid (1.6–3.6 mgMDA/kg) after storage at 4 °C for 28 days, whereas asphyxiation, freezing and blending was recognized as rancid products (> 3.6 mgMDA/kg) [34]. The higher carbonyl content (protein oxidation) obtained in the freezing method than in the heating method in the current study may be due to the process being initiated by hydrogen abstraction from the alpha-carbon in a peptide chain. If two protein radicals are in closed proximity, they may crosslink with one another via a radical formed under high-temperature conditions, which generates free radicals (carbonyl production). The terminated of these free radicals were conjugated (i.e. radical-radical interaction). Moreover, lipid radicals (lipid oxidation) can also abstract hydrogen from protein functional groups, which can result in lipid protein cross-link [35], suggesting a possible reduced capacity for protein and lipid oxidation via the indirect effect of radicals from the heating method.

Impacts of killing methods on in vitro **digestibility**

The digestibility of BSF was an important factor in its use as a feed ingredient, which could promote nutrient utilization efficiency and decrease amount of manure to promote the idea of environmentally friendly. The initiation of browning reaction and melanization consequence on the binding between amino acids (mainly lysine) and reducing sugar which this complex was inability to digest by digestive enzymes from animals [8]. Moreover, protein aggregation was found after melanization, which decreased protein solubility and digestibility [8]. However, the occurrence of some chemical reactions, mainly melanization from the stress response during freezing, was activated, which could lead to lower digestibility of BSF as described above [8]. However, lower crude protein digestibly was observed in blanching compared with freezing in this study and others [8]. Although blanching can destroy several enzymes, heat also can denature the protein, causing aggregation and resistance to digestion by animal enzymes, which could be the cause of this consequence. Interestingly, the end products of protein digestibility in the supernatant of *in vitro* digestibility were found in the highest value in the humane method compared with other methods. Treatment with CO_2 for short periods before submersion in boiling water may influence on this outcome. However, the fact that insects were unconscious and/or had a lower response to stress during CO_2 treatment should be investigated further as the cause.

Impacts of killing methods on storage trail (lipid oxidation content)

The sample characteristics and environmental conditions during storage were the major factors which influenced the level of lipid oxidation [33]. The occurrence of free radicals and tissue damage led to lipid oxidation from the reaction between

lipids and enzymes [36, 37]. Medium-chain saturated fatty acids, called lauric acids, were a major component of the fatty acid profiles of BSF, which have a low ratio of polyunsaturated fatty acids compared with vegetable oils [4, 5]. However, saturated fatty acids were the main component of BSF. The high proportion of lipid was observed in BSF depending on their diets [4, 5]. Therefore, the characteristics of BSF carry the risk of presenting lipid oxidation. Moreover, inappropriate storage conditions of high oxygen, temperature, water activity and optimal pH for metabolism can accelerate lipid oxidation [33]. The freezing method was only able to decrease the metabolic enzyme activity but did not terminate it; therefore, lipid oxidation was still occurring [8, 9]. Moreover, the metabolomics approach confirmed that several enzymatic methods were activated during killing by freezing because the adaptation to survive in a cold environment was established as the insect is still alive during this slow killing procedure [8]. In addition, cell membranes were damaged by ice crystals obtained from the slow freezing procedures, which led the risk of higher lipid oxidation [7, 9]. On the one hand, the freezing method deteriorated the lipid quality of yellow mealworm (*Tenebrio molitor*. TM) [38]. The reduction of lipid oxidation at the first day after oven drying and grinding was observed with vacuum procedures because lipid oxidation was delayed by the lack of oxygen [33]. Blanching was considered as the best procedure to conserve the fat integrity of BSF based on the results in this study and others [6, 7] because heat treatment can destroy the enzymes which reduce the lipid oxidation process. On the other hand, blending was suggested as a way to conserve fat quality, whereas blanching caused severe deterioration of tissues, leading to high lipid oxidation [9]. According to this information and the results of this study, the lower proportion of lipids in blending methods compared with others [9] could be the cause of these different outcomes. However, further analysis could confirm this hypothesis.

Conclusions

The worst killing method was blending based on the results in this study. The chemical composition and *in vitro* digestibility of dry matter and crude protein from the evaluation from sediments were similar between freezing, CO_2, vacuum, blanching and humane methods. Blanching and the humane method with heat treatment exhibited higher pH after killing, lower total viable counts, lower browning reaction rates for both enzymatic and non-enzymatic browning reactions, brighter colours, higher moisture contents and higher product stability (fat acidity, protein oxidation and TBARs during storage) than other killing procedures. Based on the research results, blanching and humane methods were suggested as the best procedures to kill black soldier fly prepupae to preserve product quality. Only the higher total free amino acids of the supernatant after *in vitro* digestibility was found in humane methods compared to blanching. Therefore, the blanching technique was suggested in terms of cost and benefits on product quality. However, the humane method can be selected depending on the regulations based on ethics and animal welfare of each country. Moreover, cost effectiveness should be another consideration point in the real-life situation.

Abbreviations

BSF: Black solider fly; TVC:Total viable aerobic count; CFU:Colony forming Unit; NFE:Nitrogen free extract; BI:Browning index; TBARs:Thiobarbituric acid-reactive substance; MDA:Malondialdehyde; DNS:Dinitrosalicylic acid; TNBS:trininobenzene sulfonic acid; IVCD:*in vitro* carbohydrate digestibility; IVPD:*in vitro* protein digestibility

Declarations

Competing interest

There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper. The authors declare that they have no competing interest.

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Authors' contributions

All authors read and approved the final manuscript.

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Figures

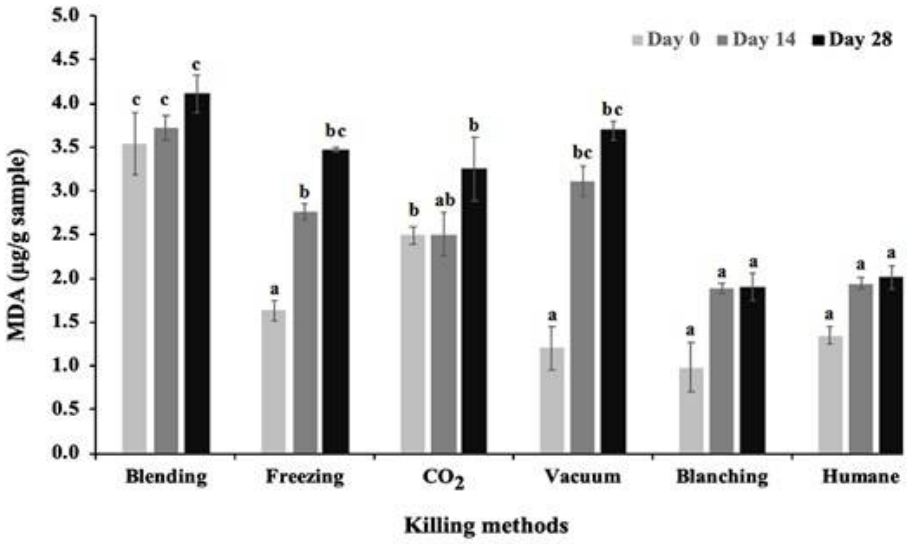

Figure 1

Storage time on TBARs contents of BSF after storage at 4 °C during 0 (), 14 () and 28 days ()