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RESEARCH ARTICLE

Does the processing of black soldier fly larvae meal affect the amino acid solubility in Atlantic salmon (*Salmo salar*)?

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Abstract

The present work aimed to evaluate protein and amino acid (AA) digestibility/solubility of different black soldier fly larvae (BSFL) based meals for Atlantic salmon *in vivo* and *in vitro*. Three types of insect meals that had been through different processing techniques were included: microwave full fat BSFL meal (BSFM), defatted BSFL meal with an enzymatic pre-treatment (BSFE) and a defatted BSFL meal without enzymatic pre-treatment (BSFH). For the *in vivo* digestibility studies only two ingredients (BSFE and BSFH) were used. The experimental diets for the different ingredients were prepared by mixing a control diet with BSFL meals at an 80:20 ratio. The *in vitro* method implied a two-stage hydrolysis involving both gastric simulation (acid hydrolysis) and gastrointestinal simulation (acid hydrolysis followed by alkaline hydrolysis), using enzymes extracted from salmon. The results showed that the AA solubility was higher in the gastrointestinal phases than the gastric phase alone, showing the importance of having both phases *in vitro* solubility for an effective protein breakdown. The AA solubility of different insect-based meals showed that neither partial defatting nor the addition of enzymatic treatment impacted the protein and AA solubility. The *in vivo* trial (56 days) recorded no differences between fish fed diets containing BSFE and BSFH for growth or body indices. The protein and AA apparent digestibility were similar for both BSFE and BSFH ingredients. Thus, in the current study no differences in nutrient digestibility were observed due to different processing methods employed to BSFL meal both *in vivo* and *in vitro*.

Keywords

availability – feed ingredient – insect meal – *in vitro* – *in vivo*

1 Introduction

The world's population is expected to grow to over 9 billion by 2050, which will intensify the demand for food by 25-70% above today's level and challenge natural resources (European Environment Agency, 2016). The aquaculture sector has been a valuable provider for the growing food demand (Golden *et al.*, 2021). Production from aquaculture has been steadily increasing, reaching 63 million tonnes in 2020 and it is projected to reach 140 M tonnes by 2050 (FAO, 2022). One of the major challenges in the aquaculture sector is the efficient use of feed and ingredients relative to production (IFF0, 2023; FAO, 2022). To overcome this challenge, several

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efforts were made on developing and investigating new protein and lipid sources for animal feeds, such as algae, blue mussel, yeast, and insect meal (Albrektsen *et al.*, 2022). Insect-based ingredients have gained significant attention for their use in fish feeds due to their high protein content and beneficial amino acids (AA), vitamins, and minerals profile (Mousavi *et al.*, 2020). They are also a natural part of the diet for many carnivorous and omnivorous fish species (Henry *et al.*, 2015). Numerous studies have explored replacing traditional protein sources with insect-based proteins in various fish species including salmonids (Liland *et al.*, 2021). Among the approved insect species for its inclusion in aquafeed, black soldier fly larvae (BSFL) meal has gained widespread attention (Liland *et al.*, 2021).

The BSFL can be included in fish diets in various processed forms. These include whole or chopped larvae, dried using methods like oven or microwave drying, freeze-ground, partially defatted (using low drying temperatures), highly defatted (using standard drying temperatures), or full fat larvae (Maulu *et al.*, 2022). The most used BSFL processing method is the partial mechanical defatting using oil press or centrifuge and full fat BSFL meal (Dortmans *et al.*, 2017). These different insect biomass processing methods can affect the nutritional composition, and digestibility in different fish species as reported in meta-analysis by Weththasinghe *et al.* (2021) and a review by English *et al.* (2021). For instance, Roques *et al.* (2020) showed that processing BSFL into hydrolysate protein resulted in higher nutrient digestibility compared to defatted insect protein meal. Different methods to process BSFL meals have also been shown to strongly affect growth performances of different fish species (Cardinaletti *et al.*, 2019; Lock *et al.*, 2016; Nogales-Mérida *et al.*, 2019; Terova *et al.*, 2019). Several strategies have been employed to improve growth, and nutrient digestibility of insect meals in animal feed. Pre-treatment of ingredients such as fermentation (Yamamoto *et al.*, 2010; Seong *et al.*, 2018), or enzyme treatment (Lin *et al.*, 2007; Yao *et al.*, 2019) are known to improve the digestibility of feed ingredients in fish species. The supplementation of exogenous protease has been studied to improve the quality of alternative protein sources in poultry (Angel *et al.*, 2011; Mahmood *et al.*, 2017; Walk *et al.*, 2018). Gasco *et al.* (2018) reported that insect meal extraction process or dietary enzyme inclusion can improve insect meal digestibility of aquafeed, however appropriate technologies have not yet been fully applied.

In vivo feeding trials can be used to determine the apparent digestibility of differently processed raw materials and are usually considered as the ideal method. However, this demands a lot of time, labor and most importantly a large number of fish for experiments. *In vitro* digestion methods have been proved to be useful and less time-consuming ways to evaluate the nutritional value of ingredients such as poultry byproducts meal, cotton seed meal, algae and insect (Lewis *et al.*, 2019; Tibbetts *et al.*, 2011; Toledo-Solís *et al.*, 2020). These tools can provide knowledge on the influence of different diet components, which is important in the nutritional evaluation of ingredients and diets for aquaculture. The *in vitro* digestion is a technique used to simulate the process of digestion that occurs in the gastrointestinal tract of animals, and can be conducted using different methods and techniques, depending on the objectives of the study and the type of material being tested (Wang *et al.*, 2021).

The main objective of this study was to investigate if processing BSFL with different techniques can affect the solubility/digestibility of insect meals. An *in vitro* method was first used to measure and compare the protein and AA solubility of BSFL meals by applying gastric phase and/or gastrointestinal phase. Secondly, an *in vivo* study was conducted to assess the impact of insect processing on apparent digestibility coefficients (ADC) of various nutrients in BSFL meals.

2 Materials and methods

Chemicals and reagents

Analytical reagent grade chemicals and Milli-Q® water (18.2 MΩ cm) (EMD Millipore Corporation, Billerica, MA, USA) were used unless otherwise stated. Tris-HCl (GE Health care), trichloroacetic acid ($C_2HCl_3O_2$; Merck Life Science AS), haemoglobin from bovine blood (95% crude protein), and casein from bovine milk (90% crude protein) from Sigma Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH, Emsure® ACS, ISO), hydrochloric acid (HCl, Emsure® ACS, ISO, 37% w/w) and hydrogen peroxide (H_2O_2, Emsure® ACS, ISO, 30% w/w) were purchased from Merck (Darmstadt, Germany).

Experimental ingredients

The three experimental ingredients were procured by Bioflytech (Alicante, Spain) and were processed differently as given in Figure 1: BSFM – microwave-dried BSF meal obtained by grinding the whole larvae (killing by blanching); BSFH meal – BSFL meal obtained by heating followed by tricanter centrifugation at BioflyTech (con-

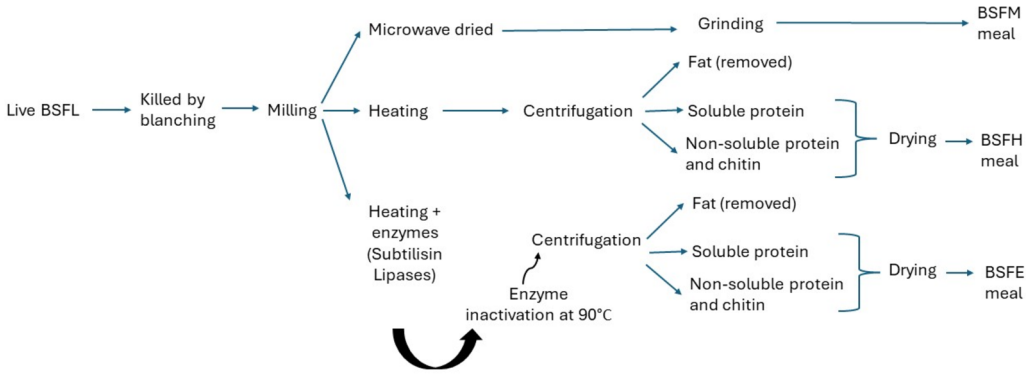

FIGURE 1 Different processing techniques included in this study leading to the production of three types of black soldier fly larvae (BSFL) meals: a microwave full fat BSFL (BSFM), defatted BSFL meal with an enzymatic pre-treatment (BSFE) and a defatted BSFL meal without enzymatic pre-treatment (BSFH).

ventional process, without enzymes); BSFE meal – BSFL meal treated enzymatically (i.e. commercial proteases; subtilisin and lipases) followed by enzyme inactivation at 90 °C and tricanter centrifugation at BioflyTech. The main purpose of pre-treating with enzyme prior to centrifugation is to increase the protein yield from insects. Resulting protein products were spray-dried to obtain protein-enriched meals. Fish meal (FM) and soy protein concentrate (SPC) were tested by the *in vitro* method and were obtained from the Norwegian fish feed monitoring programme (Sele *et al*., 2023). The proximate and amino acids composition of insect meals, FM and SPC are given in Table 1.

The method of insect meals production is detailed as follows (SUSINCHAIN, 2023):

Blanching: BSF larvae were killed by steam blanching them for 5 min in a steam oven (Rational CombiMaster Plus 61; Rational Belgium NV, Zwijndrecht, Belgium), at 100 °C and stored overnight in open trays at 4 °C.

Milling: The larvae were uploaded to the milling unit and milled to a particle size below 1 mm. This is to ensure uniform processing, accessibility for enzymes and equal temperature in the biomass.

BSFM: The larvae were dried in an industrial microwave oven (MEAM Dry 32; input power 22 kW) at MEAM (Houthalen-Helchteren, Belgium). In detail, 15 kg BSF larvae were placed in plastic trays on a conveyor belt, forming a layer of 1.5 cm. After two runs of 7.5 min at 85 °C drying temperature, the larvae dried from ±65% moisture content to a moisture content below 5%.

BSFE: The milled larvae are weighed inside the reactor and mixed with water (1:1) at acidic pH with citric acid and a cocktail of enzymes (0.2% protease Prot1 and lipases 0.75% Lip1 and 0.75% Lip2), at mid temperature under agitation for 4 h and followed by a thermal treatment at 90 °C for 2 h, which also deactivates the enzymes, and followed by centrifugation. The treatment time (2 h) might be unnecessary when producing insect meals at industrial level as the mixture is heated for 15 min at 90 °C by steam injection, resulting in faster heat transfer and more efficient energy.

BSFH: The milled larvae were proceeded to tricanter centrifugation.

Tricanter centrifugation: The larvae were passed through a tricanter centrifuge, and three fractions are obtained: oil, a liquid phase containing water and soluble proteins, and solids, containing non-soluble proteins and chitin. The separation of the three fractions is based on their different specific gravities. The amount of the liquid phase varies with the nature and quality of the raw material. Under average conditions one may estimate the volume of liquid phase about 60% while the remaining 40% makes up the solid phase.

Production of insect meals: The liquid phase is concentrated using an evaporator at 60 °C, mixed with the solid phase, loaded into the disc dryer, and finely milled to ensure the uniformity of the final insect meal.

Proximate composition and amino acid analysis analyses

The ingredients were analysed for dry matter, ash, energy, thiobarbituric acid reactive substances (TBARS), crude lipid, and crude protein content following standard procedures. Briefly, dry matter content was measured gravimetrically after drying at 104 °C for 24 h, ash content was determined by combustion in a muffle furnace flame combustion at 550 °C for 16-18 h, and total

TABLE 1 Analysed proximate and amino acid composition of the ingredients expressed as g/100 g DM or as g/100 g crude protein (in brackets)

	FM	SPC	BSFM	BSFE	BFSH
Chemical composition (%dw)					
Dry matter (DM)	93.3	93.5	94.3	93.4	93.1
Crude protein (CP)	74.5	67.4	42.4	51.4	51.6
True protein	51.0	52.5	28.8	34.3	34.2
Crude lipid	10.6	<1.0	26.5	14.0	14.3
Energy (kJ/g)	19.8	18.6	24.3	20.7	20.4
Ash	14.3*	9.0*	9.98	8.83	10.8
TBARS (nmol/g)	41.0	<4.3	61.0	80.0	80.0
Amino acid composition expressed as g/100 g DM or as g/100 g CP (in brackets)					
Hyp	0.57 (0.77)	0.08 (0.12)	<LOQ	<LOQ	<LOQ
His	1.43 (1.93)	1.77 (2.62)	0.90 (2.11)	1.08 (2.10)	1.06 (2.06)
Tau	0.67 (0.90)	ND	<LOQ	<LOQ	<LOQ
Ser	2.90 (3.92)	3.45 (5.13)	1.59 (3.75)	2.00 (3.89)	2.01 (3.89)
Arg	3.91 (5.29)	4.71 (7.00)	1.70 (4.02)	2.14 (4.16)	2.16 (4.18)
Gly	4.14 (5.60)	2.87 (4.26)	1.85 (4.37)	2.41 (4.69)	2.41 (4.66)
Asp	6.00 (8.11)	7.02 (10.41)	3.60 (8.49)	4.05 (7.88)	4.01 (7.78)
Glu	8.46 (11.44)	10.92 (16.21)	4.00 (9.41)	5.12 (10.05)	5.13 (9.96)
Thr	2.86 (3.87)	2.58 (3.83)	1.51 (3.56)	1.86 (3.62)	1.87 (3.63)
Ala	4.06 (5.49)	2.77 (4.11)	3.00 (7.19)	3.40 (6.62)	3.37 (6.54)
Pro	2.68 (3.62)	3.45 (5.11)	2.17 (5.12)	2.64 (5.13)	2.63 (5.11)
Lys	4.97 (6.72)	3.78 (5.61)	2.02 (4.77)	2.54 (4.93)	2.49 (4.82)
Tyr	2.28 (3.08)	2.41 (3.57)	2.20 (5.10)	2.50 (4.81)	2.51 (4.87)
Met	2.08 (2.82)	0.83 (1.23)	0.66 (1.56)	0.74 (1.43)	0.74 (1.43)
Val	3.27 (4.43)	2.84 (4.21)	2.14 (5.04)	2.64 (5.14)	2.63 (5.11)
Ile	2.65 (3.58)	2.77 (4.11)	1.58 (3.73)	1.89 (3.69)	1.88 (3.65)
Leu	4.91 (6.64)	5.02 (7.45)	2.65 (6.25)	3.10 (6.11)	3.14 (6.09)
Phe	2.89 (3.91)	3.78 (5.62)	1.59 (3.76)	1.93 (3.75)	1.92 (3.73)

FM = fish meal; NRC, 2011: FM (5-01-985); SPC = soy protein concentrate; Leeper *et al.*, 2022; BSFM = microwave full fat BSFL; BSFE = defatted BSFL meal with enzymatic pre-treatment; BSFH = defatted BSFL meal without enzymatic pre-treatment; TBARS = thiobarbituric acid reactive substances; ND = not determined; LOQ = limit of quantification (0.6 mg amino acid/g sample). True protein: Sum of anhydrous amino acids (except cysteine and tryptophan).

lipid was determined gravimetrically after acid hydrolysis and extraction with diethyl ether (Lie, 1991). Energy content was determined by bomb calorimetry using an IKA calorimeter C7000. TBARS were determined by a method modified from Scmedes and Hølmer (1989) with absorption measured at 532 nm and TBARS was quantified by reference to an external standard (Malondialdehyde, MDA). Total nitrogen (N) was measured with a nitrogen analyser (Vario Macro Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany) according to AOAC official methods of analysis (AOAC, 2010). Protein is presented both as calculated by multiplying the total nitrogen by 6.25 (crude protein) as well as based on true protein calculation as described by Belghit *et al.* (2019). The amino acid analysis of BSFL meals, feces and residue after two-stage hydrolysis were carried out by an ultra-performance liquid chromatography, coupled with UV detector (UPLC, Waters Acquity UPLC system, Milford, MA, USA). The quantitative determination was based on an accredited method by the Nordic Committee of Food Analysis (NMKL) and described in detail elsewhere (Belghit *et al.*, 2019b). The results were integrated by Empower 3 (Waters, Milford, MA, USA). Amino acids were quantified using standards from Thermo Fisher Scientific (product number: 20088; Rockford, IL, USA).

In vitro *digestion method*

Extraction of crude salmon enzymes

A detailed protocol describing the *in vitro* digestion methodology used is available for open access at: 10.17504/protocols.io.5jyl8j3b7g2w/v1.

The extraction of crude salmon gut enzyme method was developed based on principles described elsewhere (Alarcón *et al.*, 2002; Rahmah *et al.*, 2016; Yasumaru and Lemos, 2014). Briefly, Atlantic salmon (n = 6), weighing around 145.8 ± 4.6 g were taken from the laboratory facility at the Institute of Marine Research, Norway. The fish were fed 40 g of commercial feed at 8:00 in the morning (Supreme Plus15, Skretting, crude protein 51%). After 4 h, the fish were sacrificed using overdose (100 mg/L) of MS222, followed by a quick cephalic concussion. The fish were dissected to remove the stomach, pyloric ceca, and intestine which were thoroughly washed with cold distilled water to remove the blood stains and fat. The pH of the stomach (4.9-6) and intestines (7-9) were noted before the excision. The stomach and the intestine along with pyloric caeca were thoroughly washed with cold distilled water to remove the blood stains and fat. These tissues were chopped into smaller pieces and homogenised with cold distilled water in 1:10 ratio using a tissue homogeniser (Polytron PT 2100). The homogenisation was performed in several pulses of approximately 30 sec to avoid overheating and the entire process of homogenisation was done by keeping a glass beaker on ice to avoid damage to the tissue protein and enzymes. The homogenised samples were then centrifuged at 3,220 × *g* for 30 min at 4 °C (Fisher Scientific, Eppendorf™ 5810R Centrifuges with A-4-81 Model Rotor). The collected supernatant which constituted the crude enzyme extract were stored at –80 °C until further use. Before determination of enzyme activity or before performing the *in vitro* experiments, the crude enzyme extracts were further dialysed using 10 MWCO (molecular weight cut off) dialysing tubes to concentrate the enzyme solution.

Determination of pepsin and protease activities

Total pepsin activity of crude extract was assayed according to the method described by Anson and Mirsky (1932), using 2% haemoglobin solution as substrate. The assay was initiated by adding 5 mL of the substrate into the glass tubes named blank and test. All the tubes were placed at 37 °C for approximately 10 min to equilibrate. This was followed by addition of 1 mL of enzyme solution into the test tubes and were placed at 37 °C for 10 min to incubate. Later, the reaction was terminated by adding 10 mL of 5% trichloroacetic acid (TCA) to all tubes. One mL of pepsin was added into blank tube after adding TCA. All the tubes were mixed properly and were kept at 37 °C for 5 min. The blank and test tubes were centrifuged at 3,200 × *g* for 10 min at 4 °C and the absorbance were read at 280 nm (UV-VIS Spectrophotometer, Shimadzu, Model: UV-1800, USA). One unit of pepsin activity was defined as the change in absorbance of 0.001 per min at pH 2 at 37 °C measured as TCA soluble products.

The total protease activity of crude extract was measured according to Walter (1984). In this assay, the protease activity of the stock solution was measured using casein as the standard substrate. To begin with, 20 µL of enzyme solution was mixed with 0.5 mL of 0.1 M Tris-HCl buffer (pH 8) at room temperature. The reaction was initiated by the addition of 0.5 mL of 1% casein and kept for 30 min. Later, the reaction was terminated by the addition of 0.5 mL of 20% TCA. The solution mix were allowed to stand for 10 min at room temperature, followed by centrifugation at 16,500 × *g* for 5 min at 4 °C. The absorbance of the reaction mixture was measured at 280 nm (UV-VIS Spectrophotometer, Shimadzu, Model: UV-1800). One unit of enzyme activity is defined as the 1 µg tyrosine released per min (Walter, 1984). All the measurements were carried out in duplicates.

In vitro solubility

In this study, the *in vitro* digestion method included two steps: acidic and alkaline hydrolysis which is meant to correspond to the conditions in the stomach and the intestine, respectively. This method was described elsewhere (Radhakrishnan *et al.*, 2022) and applied to study different processed BSFL meals. The BSFL meals were crushed and freeze dried for 48 h (FreeZone 18 Liter Console, Labconco, Kansas City, MO, USA) prior to *in vitro* experiments. An appropriate amount of sample, equivalent to approximately 80 mg of protein, was weighted in a round bottom tube (13 mL). An acidic (0.01 N HCl, pH 2) and an alkaline solution (0.01 N NaOH, pH 8) were prepared by diluting HCl and a weighted amount of NaOH in Milli-Q® water. Initially, the samples were incubated with 200 µL of gastric enzyme extract and 4.8 mL of acidic solution to a volume of 5 mL. The mixture was allowed to stand for 1 h at room temperature under continuous rotation (20 rpm). After 1 h, a set of samples were stopped after the first step of digestion (gastric simulation, acidic hydrolysis) and kept for further analysis, while another set of samples were processed for the second step of digestion (gastrointestinal simulation, acid hydrolysis followed by alkaline hydrolysis). The second step of digestion was

started by incubating the samples with the 3.3 mL of intestinal crude enzyme and alkaline solution to make up to a volume of 10 mL. This mixture was again allowed to stand for 1 h at room temperature under continuous rotation (20 rpm) by keeping the ratio of 5U of pepsin or protease per mg of protein. After removing the samples from the rotator, they were submitted to centrifugation (3,000 *g*, 10 min) and the soluble fractions were transferred to new tubes. All tubes were immediately placed on ice to stop the enzyme activity. In this experiment, a set of tubes without sample was included (blanks). The purpose of these tubes is to evaluate background inputs from enzymes and working solutions. It is also recommended to use a background control with insect-based diets in suspension for assessing the autohydrolysis of the samples. However, in the current study this background control was not performed.

Soluble fractions were stored at –20 °C for AAs analysis. For the ingredients, the soluble fractions were collected from the gastric simulation phase (acid hydrolysis, GS) and from the gastrointestinal simulation phases (acid hydrolysis followed by alkaline hydrolysis, GIS). All samples were studied in duplicates (both technical and biological duplicates).

In vivo digestibility

Three experimental diets (3-mm pellet) were produced by Skretting AS (Stavanger, Norway). The three diets contained FM and SPC as protein source and fish oil and rapeseed oil as lipid source (Ctl, BSFE diet and BSFH diet). Fish meal and SPC (Ctl diet) were replaced with BSFE and BSFH at 20% and were formulated by combining 80% of the Ctl diet with 20% BSFE and BSFH (BSFE diet and BSFH diet, respectively; Table 2). However, due to the high fat content in the BSFM, it was not possible to extrude the diet and thus it was not included in the *in vivo* study.

The *in vivo* feeding trial was conducted at Matre Research Station (Institute of Marine Research, Norway). Post-smolt Atlantic salmon (~400-450 g) were randomly distributed into 9 indoor tanks with continuous light (24 h) (35 fish per tank) in triplicate. One-meter tanks contained 1,500 L filtered running seawater (25-30 L/min) with a temperature of 8.4-8.9 °C, salinity 34-35‰, and oxygen saturation was maintained above 80% during the whole experimental period. The fish were fed one of the three diets during 54 days at *ad libitum*, two times per day using automatic feeders. At the end of the trial, all fish (n = 35) were individually weighed, and the growth performance indexes were calculated. To estimate the apparent nutrient digestibility of the diets, feces were collected by manual stripping and were stored in polypropylene containers for freeze-drying. During freeze-drying samples were placed without lids in a freeze dryer at –20 °C and 0.2 mbar (FreeZone® 18 Liter; Labconco, Kansas City, MO, USA).

Formulae and calculations

True protein content was determined as sum of amino acid residues. The amino acid residues correspond to proteinogenic amino acids, i.e. the actual molecular fraction of the amino acids after the loss of one molecule of H_2O:

$$\text{True protein} = \text{AAi} * \left(\frac{\text{AAi(MW)} - \text{H}_2\text{O(MW)}}{\text{AA(MW)}}\right)$$

AAi represents the proportion of single amino acid (g amino acid per 100 g of dry weight); MW = molecular weight of a single amino acids.

Amino acid solubility of gastric simulation phase (acid hydrolysis, GS) and from the gastrointestinal simulation phases (acid hydrolysis followed by alkaline hydrolysis, GIS) during the *in vitro* tests were calculated as follows:

$$\begin{aligned}\text{AA solubility (\%)} = \big[&(\text{AA present in the soluble}\\ &\text{fraction after } \textit{in vitro} \text{ digestion})\\ &/(\text{AA present in the feed before}\\ &\textit{in vitro} \text{ digestion})\big] \times 100\end{aligned}$$

Formulae used to determine apparent digestibility coefficient (ADC) of nutrients in the diets and feed ingredients was previously described (Furukawa and Tsukahara, 1966). The ADC of nutrients in the diets and BSFL meal was calculated as follows:

$$\text{ADC diets(\%)} = 100 - 100\left(\frac{\text{Y diet}}{\text{Y feces}} * \frac{\text{N feces}}{\text{N diet}}\right)$$

where Y is concentration of the inert marker (i.e. yttrium oxide) and N is the concentration of the nutrient.

$$\begin{aligned}\text{ADC of ingredients (\%)} = &(\text{Nut}_{\text{TD}} * \text{AD}_{\text{TD}} - 0.8\\ &* \text{Nut}_{\text{RD}} * \text{AD}_{\text{RD}})\\ &/(0.2 * \text{Nut}_{\text{Ing}})\end{aligned}$$

Nut_{TD} is nutrient concentration in test diet, AD_{TD} is the apparent digestibility of nutrients in test diet, Nut_{RD} is nutrient concentration in the reference diet, AD_{RD} is

TABLE 2 Formulation and proximate composition of the experimental diets fed to Atlantic salmon

	Ctl diet	BSFE diet	BFSH diet
Ingredients (%)			
BSFE	–	20.0	–
BSFH	–	–	20.0
Fish meal	30.0	24.0	24.0
Soy protein concentrate	17.0	13.6	13.6
Wheat gluten	15.0	12.0	12.0
Wheat	10.15	8.12	8.12
Fish oil	15.0	12.0	12.0
Rapeseed oil	5.50	4.40	4.40
Lecithin, rapeseed	1.00	0.80	0.80
Vitamin premix	0.70	0.56	0.56
Mineral premix	0.70	0.56	0.56
Monosodium phosphate	2.50	2.00	2.00
L-Lysine	0.70	0.56	0.56
L-Threonine	0.10	0.08	0.08
DL-Methionine	0.30	0.24	0.24
L-Histidine	0.30	0.24	0.24
Choline chloride	0.50	0.40	0.40
Carop. Pink (10% Astax)	0.05	0.04	0.04
Yttrium oxide	0.05	0.04	0.04
Chemical composition (%dw, analysed)			
Dry matter (DM)	95.9	94.1	94.0
Crude protein (CP)	45.8	46.8	45.7
True protein	34.3	32.3	34.2
Crude lipid	27.1	25.5	26.6
Energy (kJ/g)	23.9	23.6	23.6
TBARS (nmol/g)	19.0	21.0	23.0
Yttrium oxide	0.076	0.073	0.071

apparent digestibility of nutrients in the basal diet and Nut_{Ing} is the nutrient concentration in test ingredient.

Statistical analysis

The software Statistica 13.4 (Statsoft Inc.) and GraphPad Prism (version 9.0, for Windows, GraphPad Software, La Jolla, CA, USA) were used for statistical analysis. Data were tested for normality and homogeneity of variance using a Kolomogorov-Smirnov test and Shapiro-wilk test, respectively. The *in vivo* digestibility data were analysed using one-way ANOVA to compare between test diets and reference diet. The *in vitro* solubility between different phases of digestion and ingredients were analysed using one-way ANOVA followed by Tukey's multiple comparisons. For all statistical tests, $P < 0.05$ were considered significant, and all the results are expressed as mean ± standard error. Figures and graphs were obtained by using GraphPad Prism (version 9.0, for Windows, GraphPad Software, La Jolla, CA, USA).

3 Results and discussion

Nutrient composition of ingredients

The proximate and AA composition of the different ingredients is reported in Table 1. The crude protein (74 and 67% on dry matter, DM) and crude lipid (10.6% and <1.0% DM) content of FM and SPC, respectively, were within the values found in these typical ingredients (NRC, 2011). The BSFM meal used in this study had a crude protein and fat content of 42% and 27% DM, while BSFE and BSFH had a similar crude protein (51% DM), true protein (34% DM) and crude lipid (14%) (Table 1). These differences in the proximate composition of the BSFL meals reflected the processing method

TABLE 2 (Continued)

	Ctl diet	BSFE diet	BFSH diet
Amino acid composition expressed as g/100 g DM or as g/100 g CP (in brackets)			
Hyp	0.18 (0.40)	0.17 (0.36)	0.16 (0.34)
His	0.88 (1.91)	0.89 (1.91)	0.93 (2.04)
Tau	0.15 (0.33)	0.12 (0.25)	0.12 (0.26)
Ser	2.02 (4.40)	1.89 (4.05)	2.00 (4.38)
Arg	2.33 (5.08)	2.16 (4.62)	2.27 (4.97)
Gly	2.06 (4.49)	2.05 (4.38)	2.11 (4.62)
Asp	3.40 (7.42)	3.30 (7.02)	3.60 (7.82)
Glu	8.70 (18.95)	7.30 (15.66)	8.00 (17.50)
Thr	1.54 (3.35)	1.52 (3.24)	1.61 (3.51)
Ala	1.95 (4.24)	2.13 (4.56)	2.25 (4.92)
Pro	2.88 (6.27)	2.68 (5.73)	2.81 (6.13)
Lys	3.10 (6.75)	2.84 (6.08)	3.00 (6.62)
Tyr	1.40 (3.04)	1.51 (3.23)	1.59 (3.49)
Met	0.98 (2.14)	0.90 (1.92)	0.94 (2.06)
Val	1.84 (4.01)	1.90 (4.06)	1.97 (4.31)
Ile	1.64 (3.58)	1.60 (3.42)	1.65 (3.61)
Leu	3.10 (6.76)	2.93 (6.27)	3.10 (6.73)
Phe	2.07 (4.50)	1.94 (4.15)	1.99 (4.35)

Ctl = control diet; BSFE = defatted BSFL meal with enzymatic pre-treatment; BSFH = defatted BSFL meal without enzymatic pre-treatment. True protein = Sum of anhydrous amino acids (except cysteine and tryptophan).

applied in the current study. For instance, the defatting (partial) increased the protein content by ~20% compared to full fat (Table 1). The process of defatting BSFL (complete or partial) into protein rich meal has become a frequent practice to minimise the risk of lipid oxidation (Hurtado-Ribeira *et al.*, 2023). Furthermore, no significant effect was observed in the analysed proximate composition due to enzyme pre-treatment. The AA content varied also among the different ingredients, being generally higher in FM and lower in SPC, as an example, the content of methionine was 2.8 and 1.2 g/100 g crude protein in FM, and SPC, respectively (Table 1). Similarly, to the protein content, the AA composition in the partially defatted BSFL meals (BSFE and BSFH) were higher compared to the full fat BSFL (BSFM; Table 1). In general, processing insect biomass, such as through defatting, can result in a higher protein and AA content and lower lipid levels compared to full-fat BSFL meals. Additionally, no significant differences in the proximate composition have been observed between BSFL meals treated with enzymes and those without enzyme treatment.

In vitro *solubility of ingredients*

As shown in Tables 3A and B, *in vitro* true protein and AA solubility was higher in FM followed by BSFL meals, with SPC being the least soluble (FM > BSFM = BSFE = BSFH > SPC). These results reflect the higher solubility of animal-based protein sources like FM, compared to plant-based protein source such as SPC (Hudon and de la Noüe, 1985; McClements and Grossmann, 2021). Among the differently processed BSFL meals, the true protein and most of the AAs showed similar solubility values (BSFM = BSFE = BSFH) for both phases (GS and GIS; Table 3A,B). As previously reported, insects are not eviscerated before its use and their gut has serine and cysteine-like proteases (Thie and Houseman, 1990). These endogenous proteases remain active after processing insects and can alter the protein functionality (Janssen *et al.*, 2019). Therefore, comparable results for the insect meals (i.e. BSFM, BSFE, BSFH) during the GS or the GIS phases could mean that the solubility in these samples is additionally affected by the presence of endogenous enzymes naturally occurring in insect.

The values are expressed as mean ± SE (n = 2). Statistical significance analysed through one-way ANOVA test. Different superscript letters within an individual row denote statistically significant differences in solu-

TABLE 3 (A) *In vitro* solubility (%) between differently processed BSFL meals, FM and SPC in gastric phase (GS;acidic hydrolysis); (B) *In vitro* solubility (%) between differently processed BSFL meals, FM and SPC in gastrointestinal phase (GIS;acidic hydrolysis followed by alkaline hydrolysis)

(A)	BSFM	BSFE	BSFH	FM	SPC
True protein	17.5 ± 0.21^{b}	19.6 ± 0.09^{b}	17.7 ± 0.82^{b}	31.6 ± 1.00^{a}	4.1 ± 0.41^{c}
Amino acids					
His	11.7 ± 0.57^{b}	11.6 ± 0.34^{b}	NC	15.8 ± 0.41^{a}	NC
Ser	14.7 ± 0.71^{c}	18.4 ± 0.40^{b}	14.8 ± 0.66^{c}	30.9 ± 0.61^{a}	7.9 ± 0.18^{d}
Arg	10.4 ± 0.42^{b}	13.0 ± 0.03^{b}	11.9 ± 0.24^{b}	19.2 ± 1.38^{a}	NC
Gly	24.5 ± 0.82^{cd}	28.9 ± 0.15^{b}	27.4 ± 0.26^{bc}	53.5 ± 1.25^{a}	21.4 ± 0.06^{d}
Asp	8.4 ± 1.22^{c}	14.1 ± 0.07^{b}	13.2 ± 0.30^{b}	19.9 ± 0.68^{a}	2.8 ± 0.09^{d}
Glu	24.3 ± 0.04^{c}	32.3 ± 0.04^{a}	30.2 ± 0.51^{ab}	27.4 ± 1.07^{b}	3.6 ± 0.02^{d}
Thr	9.1 ± 0.17^{b}	11.2 ± 0.12^{b}	10.4 ± 0.15^{b}	19.4 ± 0.83^{a}	1.8 ± 0.07^{c}
Ala	35.4 ± 0.51^{a}	24.4 ± 0.06^{c}	22.8 ± 0.41^{c}	31.6 ± 1.14^{b}	1.8 ± 0.09^{d}
Pro	20.4 ± 0.19^{b}	16.2 ± 0.14^{c}	14.9 ± 0.25^{c}	33.5 ± 0.34^{a}	1.7 ± 0.18^{d}
Lys	9.9 ± 0.51^{c}	13.7 ± 0.07^{b}	12.7 ± 0.33^{bc}	22.8 ± 1.06^{a}	3.7 ± 0.25^{d}
Tyr	6.9 ± 0.17^{b}	7.9 ± 0.01^{ab}	7.6 ± 0.10^{ab}	8.9 ± 0.41^{a}	NC
Met	NC	6.1 ± 0.08^{b}	6.2 ± 0.01^{b}	17.4 ± 0.79^{a}	NC
Val	10.8 ± 0.24^{b}	11.7 ± 0.08^{b}	10.9 ± 0.20^{b}	26.0 ± 0.81^{a}	1.3 ± 0.08^{c}
Ile	7.6 ± 0.30^{b}	8.6 ± 0.19^{b}	8.0 ± 0.02^{b}	16.7 ± 0.31^{a}	NC
Leu	5.4 ± 0.13^{b}	6.5 ± 0.10^{b}	6.0 ± 0.15^{b}	18.2 ± 0.59^{a}	0.6 ± 0.02^{c}
Phe	6.1 ± 0.08^{b}	7.2 ± 0.16^{b}	6.6 ± 0.24^{b}	12.9 ± 0.08^{a}	NC

(B)	BSFM	BSFE	BSFH	FM	SPC
True protein	30.4 ± 0.47^{b}	31.5 ± 0.22^{b}	30.8 ± 0.76^{b}	36.0 ± 0.21^{a}	22.3 ± 0.17^{c}
Amino acids					
His	18.7 ± 0.20^{b}	18.7 ± 0.38^{b}	18.3 ± 0.83^{b}	21.9 ± 0.55^{a}	9.9 ± 0.21^{c}
Ser	24.4 ± 0.18^{b}	24.6 ± 0.22^{b}	23.8 ± 0.22^{b}	29.8 ± 0.53^{a}	15.7 ± 0.05^{c}
Arg	23.9 ± 0.19^{a}	25.3 ± 0.01^{a}	24.1 ± 0.52^{a}	24.7 ± 0.22^{a}	12.6 ± 0.07^{b}
Gly	25.2 ± 0.19^{b}	26.1 ± 0.40^{b}	24.9 ± 0.34^{b}	35.6 ± 0.40^{a}	21.6 ± 0.22^{b}
Asp	20.8 ± 0.61^{c}	24.0 ± 0.04^{b}	24.4 ± 0.79^{b}	27.3 ± 0.04^{a}	15.1 ± 0.13^{d}
Glu	29.9 ± 1.38^{a}	33.0 ± 0.18^{a}	32.7 ± 1.08^{a}	30.1 ± 0.09^{a}	13.4 ± 0.11^{b}
Thr	23.2 ± 0.09^{b}	24.4 ± 0.07^{b}	23.6 ± 0.60^{b}	26.3 ± 0.15^{a}	18.2 ± 0.17^{c}
Ala	28.2 ± 0.27^{a}	23.2 ± 0.19^{b}	23.0 ± 0.92^{b}	29.2 ± 0.08^{a}	18.4 ± 0.20^{c}
Pro	22.3 ± 0.25^{b}	19.9 ± 0.26^{c}	19.4 ± 0.41^{c}	31.6 ± 0.18^{a}	12.3 ± 0.11^{d}
Lys	25.9 ± 0.83^{a}	27.1 ± 0.26^{a}	27.9 ± 1.30^{a}	27.8 ± 0.16^{a}	21.0 ± 0.25^{b}
Tyr	14.1 ± 0.16^{c}	15.4 ± 0.13^{b}	14.3 ± 0.22^{c}	19.3 ± 0.06^{a}	14.4 ± 0.06^{c}
Met	22.7 ± 0.13^{c}	26.3 ± 0.12^{b}	25.3 ± 0.55^{b}	21.8 ± 0.08^{c}	30.2 ± 0.31^{a}
Val	20.2 ± 0.21^{b}	20.2 ± 0.16^{b}	19.9 ± 0.43^{b}	28.0 ± 0.17^{a}	17.7 ± 0.23^{c}
Ile	19.9 ± 0.29^{b}	21.3 ± 0.14^{c}	20.9 ± 0.27^{bc}	23.8 ± 0.22^{a}	15.3 ± 0.14^{c}
Leu	18.9 ± 0.29^{b}	19.9 ± 0.12^{b}	19.6 ± 0.36^{b}	24.6 ± 0.15^{a}	14.4 ± 0.14^{c}
Phe	17.1 ± 0.15^{c}	18.3 ± 0.06^{b}	17.5 ± 0.19^{bc}	20.1 ± 0.19^{a}	10.5 ± 0.05^{d}

bility values ($P < 0.05$). BSFM = Microwave full fat; BSFE = partially defatted BSFL meal with enzymatic pre-treatment; BSFH = partially defatted BSFL meal without enzymatic pre-treatment; FM = fish meal; SPC = soy protein concentrate. True protein: Sum of anhydrous amino acids (except cysteine and tryptophan).

Further, the solubility values for serine (GS phase; Table 3A) and tyrosine (GIS phase; Table 3B) had significantly higher solubility in BSFE than BSFM and BSFH. It is generally expected as the enzymatic treatment performed on BSFE is expected to accelerate protein hydrolysis to soluble peptides. Toledo *et al.*

(2022) reported an increase in bioavailability of AA after enzyme pre-treatment of narbonne vetch (*Vicia narbonensis* NVM/ZV-156) and soybean (*Glycine max*) meal by reducing the undigestible protein-phytate complexes and increasing the total soluble protein (Toledo-Solís *et al*., 2022). In other studies, addition of protease such as flavourzyme can reduce the molecular weight of peptides, generating higher amounts of low molecular weight AAs, thus increasing their solubility (Vieira *et al*., 2016; Wen *et al*., 2019; Yang *et al*., 2020). Furthermore, higher solubility values for aspartic acid (GS phase; Table 3A) and methionine (GIS phase; Table 3B) were reported for partially defatted meals (BSFE and BSFH) compared to full fat meals (BSFM). Similar results were obtained for hemp seeds used for salmonid feeds, which upon defatting improved the protein digestibility by releasing higher amount of nitrogen content (Banskota *et al*., 2022). Similarly, Traksele *et al*. (2021), found that a low fat BSFL resulted in higher solubility (75%) compared with full fat BSFL (48%). This is because defatting process can minimise the lipid-protein interaction, resulting in increasing access of the proteases to the proteins and thereby increasing the protein solubility (Shanthakumar *et al*., 2022). This implies that BSFL meals where mechanical defatting was used, can help in improving the solubility of AAs such as methionine and aspartic acid.

In the current study, irrespective of the protein sources, the solubility of true protein increased by 12%, 41% and 82% in the GIS compared to the GS phase for FM, insect meals and SPC, respectively) (Table 3A,B). Similarly, the AAs had an overall higher ($P < 0.05$) solubility (except for glutamic acid) in the GIS phase compared to GS phase. For example, the solubility of aspartic acid increased by 60% in the GIS phase compared to in the GS phase for BSFM meal (Table 3A,B). Interestingly, for SPC in the GS phase of digestion, the AA such as histidine, tyrosine, methionine, isoleucine and phenylalanine were below the limit of quantification, thus having a very limited solubility. However, during the GIS phase, most of the AA in SPC had higher solubility compared to gastric phase, and methionine in particular had higher solubility than for the other tested ingredients ($P < 0.05$) (Table 3B). Study by Grabner and Hofer (1985), reported an increase in the relative content of total AAs in the soluble fraction in the GIS phase, indicating better digestion compared to the GS phase. It has been speculated that for proteins, change in pH from acidic to alkaline can increase the solubilization of peptides, liberating more AAs (Mohan *et al*., 2007). The acidic environment leads to enzymatic breakdown of the polypeptide chain, making it further available for alkaline proteases. These results reinforce the importance of having both the gastric and post-gastric phases *in vitro* digestibility setups for effective breakdown, as shown in earlier studies (de Jonge *et al*., 2009; Silva *et al*., 2020).

In general, the results obtained in the present study on *in vitro* solubility of different protein meals showed that animal protein sources had higher solubility when compared to plant sources and that insect meal AA solubility was higher than SPC, but lower than FM. This work showed that processing did not affect the overall solubility of the true protein of insect meals but have effects on the solubility of some individual AA, where the defatting and enzymatic treatments of BSFL increased the solubility of serine, tyrosine, methionine and aspartic acid.

In vivo *apparent digestibility of ingredients*

Atlantic salmon were fed the three diets shown in Table 2 for 56 days. There was no dietary effect observed for the weight gain, specific growth rate or for body indices between the different dietary groups (Supplementary Table S1). No mortality was recorded during the trial. Results obtained in this work on Atlantic salmon agreed with *in vivo* digestibility feeding studies performed under the same project (SUSINCHAIN), in broiler and fish (i.e. rainbow trout and sea bass, where BSFE and BSFH had similar digestibility values (unpublished data). The ADC of dry matter were not affected by enzymatic treatment, neither the ADC of protein. Both BSFE and BSFH showed a higher DM digestibility, more than 90%, where ADC of dry matter gives the measure of overall digestibility of diet (Basto *et al*., 2020; Che *et al*., 2017; Lee *et al*., 2020). The ADC of protein was ~72% in BSFE and BSFH in the current study (Table 4). These values were lower than the reported ADC in partially defatted BSFL meals in seabass and salmonids (Basto *et al*., 2020; Dumas *et al*., 2018; Radhakrishnan *et al*., 2021). The differences in the digestibility values between the current trial and earlier studies could be due to the genetic and environmental differences between studies, or the technological process applied to the insect meal production. The authors also speculate that this reduction could also be due to a variation in yttrium proportions in the diet during its formulation.

The values are expressed as mean ± SE (n = 3). Statistical significance analysed through one-way ANOVA test. BSFE = partially defatted BSFL meal with enzymatic pre-treatment; BSFH = partially defatted BSFL meal without enzymatic pre-treatment

TABLE 4 Apparent digestibility coefficient (ADC%) of dry matter, crude protein, and amino acid of the ingredients fed to Atlantic salmon

ADC	BSFE	BSFH
Dry matter	93.6 ± 0.67	93.7 ± 1.14
Crude protein	71.9 ± 2.26	72.6 ± 4.78
Amino acid (%)		
His	80.1 ± 2.10	85.1 ± 3.26
Ser	76.4 ± 1.00	84.7 ± 4.04
Arg	86.1 ± 1.34	89.8 ± 2.23
Gly	74.4 ± 2.51	81.7 ± 7.72
Asp	77.1 ± 1.89	94.7 ± 6.20
Glu	84.9 ± 0.98	90.4 ± 2.74
Thr	74.1 ± 2.93	83.5 ± 5.31
Ala	82.5 ± 2.19	87.9 ± 3.93
Pro	80.2 ± 1.77	84.7 ± 3.44
Lys	80.7 ± 1.24	86.5 ± 3.18
Tyr	83.7 ± 2.45	88.5 ± 3.56
Met	83.6 ± 1.61	88.3 ± 3.16
Val	81.3 ± 1.92	85.9 ± 3.76
Ile	82.0 ± 1.67	86.2 ± 3.68
Leu	82.7 ± 1.63	87.3 ± 3.24
Phe	84.9 ± 1.59	87.9 ± 2.63

Protein digestibility is associated with fish ability to hydrolyse proteins into small peptides and AAs (NRC, 2011). It was assumed that treating with enzymes might have a possible effect on increasing the protein digestibility. However, no such differences were observed in the ADC of protein in insect meal treated with or without enzymes. Similarly, the digestibility of the individual AA in tested BSFL meals were not significantly different. Similar observations were reported in ADC of nutrients, dry matter, or energy digestibility in rainbow trout where protease supplementation had no effect on cottonseed meal, canola meal, sunflower meal, meat bone meal, and feather meal (Lee *et al.*, 2022). It could be also seen that though there exist no statistical significance among the different insect meals, the BSFE showed a relatively lower values compared to BSFH. Supplementation of exogenous enzymes are studied to improve the digestibility of nutrients in the fish diet (Dalsgaard *et al.*, 2012; Shi *et al.*, 2016). However current study did not witness any difference with or without enzyme treatment.

Comparing results between *in vivo* and *in vitro* solubility is a crucial aspect of the present research. The AA digestibility data indicated that the recorded values for the *in vivo* system overshadow consistently the equivalent values in the *in vitro* system by factors of ~3-5. Lewis *et al.* (2018) also reported higher ADC values compared to *in vitro* solubility of poultry by product meals in barramundi (Lewis *et al.*, 2019). The lack of numerical agreement between *in vivo* and *in vitro* digestibility values is probably related to the selected operating conditions of the *in vitro* methods. However, the results obtained from both *in vivo* and *in vitro* showed similar pattern on ingredient digestibility.

4 Conclusions

In general, the current study showed that the processing techniques applied to black soldier fly larvae meal did not affect the solubility of protein and amino acids both *in vitro* and *in vivo*. The *in vitro* solubility of protein and AA was found to be higher in animal protein sources compared to plant-based sources. Specifically, the solubility of AA in insect meal was greater than in SPC, yet lower than in FM. Although no improvements in true protein solubility were observed, supplementation with the enzyme complex has resulted in higher solubility in some specific amino acids *in vitro*. Whereas no such differences were observed *in vivo*. Overall, this research demonstrates that processing of insect meal via different methods can change the proximate composition of ingredients. Further, in the current study, the addition of enzymes into the processing techniques did not have an impact on increasing the digestibility of nutrients.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.26021350

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Author contributions

Gopika Radhakrishnan conducted the experiments, analysed, and interpreted the data, drafted, and revised the paper; Marta Silva supervised the study, interpreted the data, edited and revised the paper; Nina S Liland supervised the study, interpreted the data, edited and revised the paper: Rosita Secci analysed, and interpreted; Pedro Araujo analysed, and interpreted the data, drafted, and revised the paper; Antony Jesu Prabhu Philip designed the study, supervised the study, interpreted the data, edited and revised the paper; Ikram Belghit designed the study, supervised the study, interpreted the data, edited and revised the paper.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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