Journal of Insects as Food and Feed, 2022; 8(9): 953-966
SPECIAL ISSUE: *Insects on the monogastric menu*

In vitro assessment of protein digestibility and mineral solubility of black soldier fly larvae meals for monogastric animals

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Received: 5 November 2021 / Accepted: 23 May 2022

RESEARCH ARTICLE

Abstract

Animal farming is a fast-growing sector which demands a large supply of feed materials and the search for novel feed ingredients with a smaller impact on the global environment has been intensified. However, before the inclusion of these feed ingredients in animal feed, a preliminary assessment of the quality and performance is needed. This evaluation is usually done by *in vivo* feeding trials, but *in vitro* digestion methods have been successfully used for gathering information regarding the nutritional value of the different feed components. Therefore, in this work, an *in vitro* digestion method was developed using black soldier fly (BSF) larvae meals. The method included seven experimental variables, namely: pH in stomach, pH in intestine, reaction time in stomach, reaction time in intestine, enzyme concentration in stomach, enzyme concentration in intestine and temperature. The experimental variables were screened by means of a fractional factorial design (2^{7-2}) that allowed to select the concentration of enzyme in the intestine and temperature (at 7 U and 37 °C, respectively) as the most influential factors on amino acid and mineral solubility in BSF larvae meal. Performing the *in vitro* digestibility at 37 °C with pH of the stomach and intestine at 2 and 9, reaction time in the stomach and intestine at 1 hour and 4 hours and concentration of enzyme in the stomach and intestine at 1 U and 7 U resulted in the highest overall nutrient solubility. This optimised *in vitro* digestion methodology was used to assess the nutritional stability of three batches of BSF meals delivered by the same producer. The *in vitro* digestion method was able to reveal differences between the three batches of BSF meals.

Keywords: accessibility, bioavailability, factorial design, feed, insect meal

1. Introduction

Rapid growth of the animal husbandry sector requires a continuous demand for sustainable feed resources. With a large pressure on land- and water-use by agriculture, and an already maximised output from most fisheries, there is a search for novel feed ingredients with a smaller impact on the global environment. Insect farming has emerged as a viable and sustainable source of high-quality raw materials for the animal feed industry. In addition to their nutritional qualities, the production of insect-based feed ingredients is also accompanied by certain environmental benefits due to the insects being production animals with exceptionally fast growth, easy reproduction, fast life-cycles and efficient conversion of low-grade organic matter to high-value protein and fat (Van Huis, 2020). In 2017, the European Commission (EC) allowed the use of insect meal processed from seven different insect species (i.e. black soldier fly (BSF; *Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*) in aquafeed (Commission Regulation (EU) 2017/893 (EC, 2017)). Furthermore, in August 2021, the EC adopted the decision to allow the use of processed insect meal in formulated pig and poultry feeds (EC, 2021). Insect meals have a great potential to supply the protein required for future animal feed production (DiGiacomo and Leury, 2019; Makkar *et al.*, 2014; Nogales-Mérida *et al.*, 2019). Therefore, there is a strong research effort between academia and industries

regarding insect processing, upscaling production and commercialisation (Hua *et al.*, 2019; IPIFF, 2019)

The BSF larvae is considered one of the most suitable insect species to be used as a feed ingredient (Belghit *et al.*, 2019a; Chia *et al.*, 2019; Van Huis, 2013; Liland *et al.*, 2021). Its larvae typically contain between 30-58% of protein (% of dry weight) and a balanced amino acid (AA) composition (Henry *et al.*, 2015). The BSF larvae are also a rich source of many macrominerals (e.g. phosphorus, P; magnesium, Mg; potassium, K) and microminerals (e.g. iron, Fe; zinc, Zn; manganese, Mn; selenium, Se) (Nogales-Mérida *et al.*, 2019; Shumo *et al.*, 2019).

A preliminary assessment of the quality and performance (i.e. digestibility, growth performance and feed conversion) of novel feed ingredients is necessary, before their inclusion in animal feed (Bryan *et al.*, 2018; Choi *et al.*, 2021; Glencross, 2020). The major criterion for determining the nutritional value of new ingredients is the apparent digestibility coefficient (ADC) of nutrients. The evaluation of nutrient ADCs is usually performed through *in vivo* feeding trials. This type of evaluation has been successfully used to study BSF in animal feed (Belghit *et al.*, 2018, 2019a; Biasato *et al.*, 2019; Caimi *et al.*, 2021). However, feeding trials can be lengthy, expensive and use large numbers of animals. *In vivo* experiments for estimating digestibility of feeds include either the total faecal collection approach or the index marker approach (NRC, 2011, 2012). The last approach requires the addition of inert markers to the feeds (e.g. yttrium oxide, chromic oxide), followed by a feed quantification and the collection of faecal samples. For these reasons, there is a general motivation for the development of methods for screening the performance of novel feed ingredients that are less time consuming and cheaper than *in vivo* trials, while still being reliable and biologically relevant (Bryan and Classen, 2020; Moyano *et al.*, 2015; Silva *et al.*, 2020; Zaefarian *et al.*, 2021). Nutrients need to be released from the dietary matrix into the aqueous or fluid phase of the chyme before becoming available for absorption. Thus, it is hypothesised that the digestibility of a nutrient is positively related to its aqueous solubility. This principle has been demonstrated with a high correlation between soluble phosphorus in different feed ingredients and *in vivo* digestibility of phosphorus from these ingredients in the feed of rainbow trout (*Oncorhynchus mykiss*) (Weerasinghe *et al.*, 2001). An *in vitro* digestion assay can be regarded as an experimental tool that simulates the digestive system of humans or animals. The principle is that a food with a known composition is incubated in the presence of specific enzymes. After a given reaction time, the final digestive products or nondegraded substrates are quantified (Moyano *et al.*, 2015). *In vitro* digestion methods can therefore obtain detailed information on the influence of the different dietary, physiological and environmental factors on the solubility of nutrients in the digestive system and thus give information that is important as part of a nutritional evaluation of novel feed ingredients (Bryan *et al.*, 2018, 2019; Morales and Moyano, 2010; Moyano *et al.*, 2015). *In vitro* digestion methods have been applied in several studies on protein and AA digestibility in monogastric animals, including salmonids (Bassompierre *et al.*, 1998; Márquez *et al.*, 2013; Morales and Moyano, 2010), poultry (Bryan and Classen, 2020; Bryan *et al.*, 2018; Zaefarian *et al.*, 2021) and pigs (Chen *et al.*, 2019; Graham *et al.*, 1989; Huang *et al.*, 2000). Fewer studies have been conducted to study mineral digestibility *in vitro*, and these are mostly confined to phosphorus (Lineva *et al.*, 2019; Morales and Moyano, 2010; Silva *et al.*, 2020; Weerasinghe *et al.*, 2001).

The aim of this study was primarily to determine the optimal experimental variables to study digestibility of proteins, solubility of AAs and minerals contained in a BSF larvae meal by means of a fractional factorial design. Secondly, we wanted to evaluate the selected optimal experimental variables on three different batches of BSF 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. 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). Nitric acid (HNO_3, trace select, ≥69% w/w) was purchased from Sigma-Aldrich (St. Louis, MO, USA). AccQ.Tag™ Ultra Derivatization Kit, AccQ.Tag™ Ultra Eluent A and Ultra Eluent B were obtained from (Waters, Milford, MA, USA). Pepsin (2,500 U, P7012, from porcine gastric mucosa) and trypsin (5,000 U, T0303, from porcine pancreas) were obtained from Sigma Aldrich (St. Louis, MO, USA). Chymotrypsin (1000 U, from porcine pancreas) was purchased from Creative Enzymes (Shirley, NY, USA) and protease (11 U, PT307, from porcine pancreas) was purchased from Elastin Products Company (Owensville, MO, USA).

Black soldier fly larvae meals

Four different batches of BSF larvae meals, namely BSF0, BSF1, BSF2 and BSF3, were provided by a commercial insect producer. The BSF0 meal was produced in May 2019 and used for a screening of the experimental variables. The remaining BSF meals (BSF1-BSF3) were produced in February, March and April 2020, respectively and used to assess the composition stability. Details on the chemical composition of the BSF larvae meals are presented in Supplementary Table S1.

In vitro digestion method and screening of the experimental variables

The experimental variables of the *in vitro* digestion method were pH in stomach (A), pH in intestine (B), reaction time in stomach (C), reaction time in intestine (D), concentration of enzyme in stomach (E), concentration of enzyme in intestine (F) and temperature (G) (Table 1). The endpoints were protein digestibility, and amino acid and mineral solubility. A two-level fractional factorial design (FFD) 2^{7-2} (32 experiments, described in Table 2) was used to establish optimal experimental variables that yield maximum solubility. The experimental codes +1 or -1 in Table 2 for the first five variables (A, B, C, D, E) were independently generated, and for the last two variables (F, G) by combining the independent variables as A×B×C×D and A×B×D×E, respectively.

The corresponding codes +1 or -1 are equivalent to the physical values of seven factors that were set at 2 or 4 (in pH units for A); 7 or 9 (in pH units for B); 1 or 2 (in hour units for C); 2 or 4 (in hour units for D); 1 or 7 (in U/mg units for E and F); and 12 or 37 (in °C for G). A total of 31 responses in duplicates (true protein + 16 AA + 14 minerals) were measured at every experiment in Table 2. The experimental procedure was as follows: the +1 or -1 pH units of the factors A and B were prepared by diluting 10 mM HCl and 100 mM NaOH in Milli-Q® water, respectively. Factors E and F were prepared by weighing appropriate amounts of pepsin that were dissolved in their corresponding acidic solution (factor A) and protease, chymotrypsin, trypsin that were dissolved in their corresponding alkaline solution (factor B).

An overview of the *in vitro* method can be seen in Figure 1. The 32 aliquots of 0.5 g of BSF0 were weighed in 13 ml polypropylene tubes with round bottom. The acidic solution dictated by factors A and E was added and monitored for 1 or 2 hours (factor C) at the level of temperature dictated by factor G (12 or 37 °C) and by keeping a continuous rotation (20 rpm). Immediately after, the alkaline solution dictated by factors B and F were added into the tubes and monitored for 2 or 4 hours (factor D) at 12 or 37 °C (factor G) by keeping a continuous rotation (20 rpm). At the end of the reaction times, the 32 samples were centrifuged (3,000×*g*) for 10 min at 4 °C, and the non-soluble (bottom layer) and the soluble (top layer) fractions were separated by transferring the soluble fractions into new tubes. The former and the latter fractions were submitted to AA and mineral analysis, respectively. All the samples were kept at -20 °C until further analysis.

Table 1. List of factors (A-G) and respective levels used in the experimental design.

Factors	Level (-1)	Level (+1)
A: pH in stomach	2	4
B: pH in intestine	7	9
C: reaction time in stomach (h)	1	2
D: reaction time in intestine (h)	2	4
E: [enzyme] in stomach (U/mg of protein)	1	7
F: [enzyme] in intestine (U/mg of protein)	1	7
G: temperature (°C)	12	37

True protein analysis

Insect species, including BSF larvae, contain high concentrations of non-protein nitrogen (N). By using the standard 6.25 N-to-protein factor, the protein content is often overestimated, as reported earlier (Belghit *et al.*, 2019b; Janssen *et al.*, 2017; Liland *et al.*, 2017). Therefore, the protein content of the BSF meal and the non-soluble digest fractions is presented as true protein, based on the sum of anhydrous AAs residues.

Amino acid analysis

The amino acid analysis of the BSF meals and the non-soluble fractions were carried out by an ultra-performance liquid chromatography (UPLC, Waters Acquity UPLC system, Milford, MA, USA), coupled with a UV detector. 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; Espe *et al.*, 2014). In brief, ground samples equivalent to 20 mg of protein were hydrolysed in 6 N HCl at 110 °C for 22 hours. Prior to hydrolysis, 3.125 mM Norvaline (Sigma-Aldrich, St. Louis, MO, USA) was added as internal standard, and 0.1 M Dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO, USA) was added as an antioxidant agent to protect methionine from degradation during acid hydrolysis. For a further protective aid, a layer of N_2 gas was put into the flasks for 30 s, and then the flasks were capped immediately. During acid hydrolysis, tryptophan and cysteine are destroyed and are therefore not reported in our results. After hydrolysis, the samples were cooled in cold water until room temperature was reached and centrifuged in a vacuum centrifuge to complete dryness. After centrifugation, the residues were diluted in deionised water and filtered through a syringe-driven filter. Prior to the instrumental analysis, a derivatisation agent (AccQ.TagTM, Waters, Milford, MA, USA) was added to each sample. Finally, amino acids were separated by UPLC (column: Waters Aquity UPLC BEH C18 1.7 µm (2.1×100 mm), flowrate 0.7 ml/min) and results integrated by Empower 3 (Waters, Milford, MA, USA).

Table 2. Experimental design and respective levels, 2^{7-2} fractional factorial design (resolution IV). The tested factors were A: pH in stomach, B: pH in intestine, C: reaction time in stomach (h), D: reaction time in intestine (h), E: [enzyme] in stomach (U/mg of protein), F: [enzyme] in intestine (U/mg of protein), G: temperature (°C). Coded factor levels are denoted in parenthesis as '-1' or '+1' preceded by the real factor setting.

Experiment	Factors						
	A	B	C	D	E	F=ABCD	G=ABDE
1	2 (-1)	7 (-1)	1 (-1)	2 (-1)	1 (-1)	7 (+1)	37 (+1)
2	4 (+1)	7 (-1)	1 (-1)	2 (-1)	1 (-1)	1 (-1)	12 (-1)
3	2 (-1)	9 (+1)	1 (-1)	2 (-1)	1 (-1)	1 (-1)	12 (-1)
4	4 (+1)	9 (+1)	1 (-1)	2 (-1)	1 (-1)	7 (+1)	37 (+1)
5	2 (-1)	7 (-1)	2 (+1)	2 (-1)	1 (-1)	1 (-1)	37 (+1)
6	4 (+1)	7 (-1)	2 (+1)	2 (-1)	1 (-1)	7 (+1)	12 (-1)
7	2 (-1)	9 (+1)	2 (+1)	2 (-1)	1 (-1)	7 (+1)	12 (-1)
8	4 (+1)	9 (+1)	2 (+1)	2 (-1)	1 (-1)	1 (-1)	37 (+1)
9	2 (-1)	7 (-1)	1 (-1)	4 (+1)	1 (-1)	1 (-1)	12 (-1)
10	4 (+1)	7 (-1)	1 (-1)	4 (+1)	1 (-1)	7 (+1)	37 (+1)
11	2 (-1)	9 (+1)	1 (-1)	4 (+1)	1 (-1)	7 (+1)	37 (+1)
12	4 (+1)	9 (+1)	1 (-1)	4 (+1)	1 (-1)	1 (-1)	12 (-1)
13	2 (-1)	7 (-1)	2 (+1)	4 (+1)	1 (-1)	7 (+1)	12 (-1)
14	4 (+1)	7 (-1)	2 (+1)	4 (+1)	1 (-1)	1 (-1)	37 (+1)
15	2 (-1)	9 (+1)	2 (+1)	4 (+1)	1 (-1)	1 (-1)	37 (+1)
16	4 (+1)	9 (+1)	2 (+1)	4 (+1)	1 (-1)	7 (+1)	12 (-1)
17	2 (-1)	7 (-1)	1 (-1)	2 (-1)	7 (+1)	7 (+1)	12 (-1)
18	4 (+1)	7 (-1)	1 (-1)	2 (-1)	7 (+1)	1 (-1)	37 (+1)
19	2 (-1)	9 (+1)	1 (-1)	2 (-1)	7 (+1)	1 (-1)	37 (+1)
20	4 (+1)	9 (+1)	1 (-1)	2 (-1)	7 (+1)	7 (+1)	12 (-1)
21	2 (-1)	7 (-1)	2 (+1)	2 (-1)	7 (+1)	1 (-1)	12 (-1)
22	4 (+1)	7 (-1)	2 (+1)	2 (-1)	7 (+1)	7 (+1)	37 (+1)
23	2 (-1)	9 (+1)	2 (+1)	2 (-1)	7 (+1)	7 (+1)	37 (+1)
24	4 (+1)	9 (+1)	2 (+1)	2 (-1)	7 (+1)	1 (-1)	12 (-1)
25	2 (-1)	7 (-1)	1 (-1)	4 (+1)	7 (+1)	1 (-1)	37 (+1)
26	4 (+1)	7 (-1)	1 (-1)	4 (+1)	7 (+1)	7 (+1)	12 (-1)
27	2 (-1)	9 (+1)	1 (-1)	4 (+1)	7 (+1)	7 (+1)	12 (-1)
28	4 (+1)	9 (+1)	1 (-1)	4 (+1)	7 (+1)	1 (-1)	37 (+1)
29	2 (-1)	7 (-1)	2 (+1)	4 (+1)	7 (+1)	7 (+1)	37 (+1)
30	4 (+1)	7 (-1)	2 (+1)	4 (+1)	7 (+1)	1 (-1)	12 (-1)
31	2 (-1)	9 (+1)	2 (+1)	4 (+1)	7 (+1)	1 (-1)	12 (-1)
32	4 (+1)	9 (+1)	2 (+1)	4 (+1)	7 (+1)	7 (+1)	37 (+1)

Amino acids were quantified using standards from Thermo Fisher Scientific (product number; 20088 Rockford, IL, USA).

Mineral analysis

The content of minerals in BSF meals and the soluble fractions were determined by inductively coupled plasma mass spectrometry (ICP-MS) after wet digestion in a microwave oven, as described by (Julshamn *et al.*, 2013) with some modifications. Briefly, the BSF meals (approx. 0.2 g) or the soluble fractions (1 ml) were digested in 69% nitric acid (2 ml) and 30% hydrogen peroxide (0.5 ml) using a microwave digestion system (UltraWAVE, Milestone, Sorisole, Italy). The digested samples were diluted with Milli-Q® water (Merck Millipore, Billerica, MA, USA) to 25 ml (BSF meal) or 10 ml (soluble fraction). Mineral concentrations in the samples were quantified by ICP-MS (iCapQ ICPMS, ThermoFisher Scientific, Waltham, MA, USA) equipped with an autosampler (FAST SC-4Q DX, Elemental Scientific, Omaha, NE, USA). Data were collected and processed using the Qtegra ICP-MS software (Thermo Scientific, version 2.10, Waltham, MA, USA).

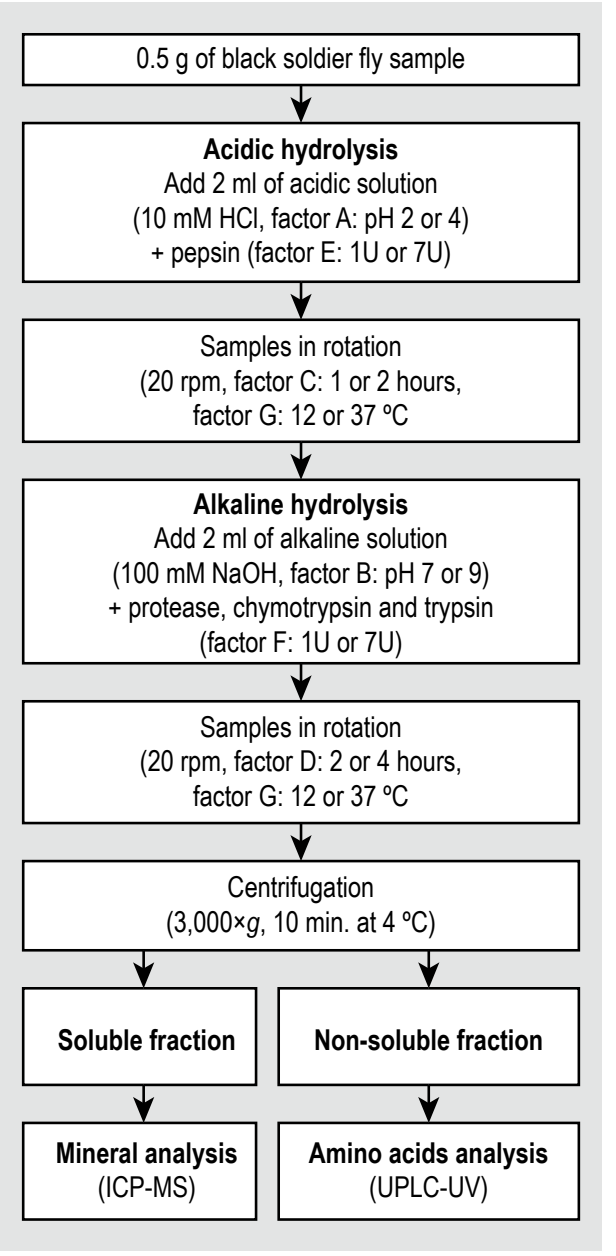

Figure 1. Flowchart of the *in vitro* digestion method. The method includes an acidic and alkaline hydrolysis. The non-soluble (bottom layer) and the soluble (top layer) fractions were submitted to amino acid and mineral analysis, respectively. ICP-MS = inductively coupled plasma mass spectrometry; UPLC-UV = ultra-performance liquid chromatography

Calculations and statistical analysis

The method for analysis of AA is validated do be used in solid samples. Therefore, $\%TP_{digestibility}$ and $\%AA_{solubility}$ were calculated indirectly by determining the concentration of AA in the non-soluble fraction (Equations 2 and 3). The $\%M_{solubility}$ is calculated by determining the concentration of minerals in the soluble fraction (Equation 4).

The true protein (TP) content was determined as the sum of amino acids residues as follows:

$$E_i = AA_i \times \left(\frac{AA_i\,(MW) - H_2O(MW)}{AA_i\,(MW)}\right) \quad (1)$$

Where AA_i represents the proportion of the single amino acid (g amino acid per 100 g of dry weight); MW is the molecular weight of a single amino acid.

The percentage of true protein digestibility ($\%TP_{digestibility}$) was determined as follows:

$$\%\,TP_{digestibility} = 100 - \left(\frac{[TP]_{in\ non-soluble\ fraction}}{[TP]_{in\ BSF\ meal}} \times 100\right) \quad (2)$$

The term $[TP]_{\text{in non-soluble fraction}}$ represents the concentration of TP that was not soluble after application of the *in vitro* digestion method and the term $[TP]_{\text{in BSF meal}}$ represents the concentration of TP determined in the BSF meal.

The percentage of amino acid solubility ($\%AA_{solubility}$) was determined as follows:

$$\%\,AA_{solubility} = 100 - \left(\frac{AA_{\text{in non-soluble fraction}}}{[AA]_{\text{in BSF meal}}} \times 100\right) \quad (3)$$

The term $[AA]_{\text{in non-soluble fraction}}$ represents the concentration of AA that were not soluble after application of the *in vitro* digestion method and the term $[AA]_{\text{in BSF meal}}$ represents the concentration of AA determined in the BSF meal.

The percentage of mineral solubility ($\%M_{solubility}$) was determined as follows:

$$\%\,M_{solubility} = \left(\frac{[M]_{\text{in soluble fraction}}}{[M]_{\text{in BFS meal}}} \times 100\right) \quad (4)$$

The term $[M]_{\text{in soluble fraction}}$ represents the concentration of minerals that were soluble after the application of the *in vitro* digestion method and the term $[M]_{\text{in BSF meal}}$ represents the concentration of minerals determined in the BSF meal.

The effects of the seven factors (A-G) on the $\%TP_{digestibility}$, $\%AA_{solubility}$ and $\%M_{solubility}$ were estimated by a multiple regression model of the form:

$$Y_i = b_0 + \sum_{i=A}^{G} b_i X_i + \sum_{i=A}^{G}\sum_{j=B}^{G} b_{ij} X_i X_j \quad (5)$$

The term Y_i represents the $\%TP_{digestibility}$, $\%AA_{solubility}$ or $\%M_{solubility}$, the coefficient b_0 represents the average response, the coefficients b_i and b_{ij} are the effect of a single factor and the interaction between two factors, respectively. The terms X_i and X_iX_{ij} represent the level of the single or interaction factor (±1). Equation 5 indicates that the higher the coefficients b_i and b_{ij} the greater their impact on the $\%TP_{digestibility}$, $\%AA_{solubility}$ or $\%M_{solubility}$ responses. One-way ANOVA and Tukey`s post hoc test at significance level of 95% were performed on the data obtained in this work, using GraphPad Prism (version 8.03, for Windows, GraphPad Software, La Jolla, CA, USA). Figures and graphs were obtained by using GraphPad Prism (version 8.03, for Windows, GraphPad Software, La Jolla, CA, USA).

3. Results

Evaluation of experimental variables to study true protein digestibility, amino acid and mineral solubility of BSF meal

The digestibility of TP, AA and solubility of minerals ($\%TP_{digestibility}$, $\%AA_{solubility}$ and $\%M_{solubility)}$ in BSF0, after applying the 2^{7-2} fractional factorial design are presented in Figures 2, 3 and Supplementary Tables S2 and S3. The lowest and highest $\%TP_{digestibility}$ were 53±1.3% and 76±0.2%, recorded in experiments 19 and 11, respectively (Table 1 and Figure 2A). The AA solubility ($\%AA_{solubility)}$ ranged from 40 to 80% (Figure 2B and Supplementary Table S2). The highest solubility for the EAA (Lys, 80±0.4%) and NEAA (Ala, 80±0.07%) was observed under the experiments 9 and 11, respectively (Figure 2B and Supplementary Table S2). While the lowest solubility value for EAA (Met, 40.5±0.6%) and NEAA (Tyr, 45±0.7%) was obtained under the experiment 19 (Figure 2B and Supplementary Table S2). In general, among the 32 experimental conditions tested, the solubility of AA in the tested BSF0 meal exhibited a maximum $\%AA_{solubility}$ at experiment 11.

The mineral solubility (%) varied considerably between the elements investigated (Figure 3). Regarding the macrominerals, the solubility of P ranged from 40 to 65% and the solubility of Mg ranged from 69 to 88% (Figures 3A and 3B). The range of solubility of microminerals such as Cu, Zn, Mn and Se were 25-48%, 6-18%, 6-38%, and 11-44%, respectively (Figures 3C-F). The highest solubility for P and Mg was obtained under experiment 11, with values of 65±0.9% and 87±1.2%, respectively. Their lowest solubilities were observed under experiment 2, with values of 41±0.4% and 70.3±0.2%, respectively (Figures 3A and B). Experiments 10 and 11 resulted in the highest solubility of Cu, Zn and Mn (≈48±0.1, 18±0.1 and 37±1.3, respectively) and experiments 2 and 3 gave the lowest solubility of these elements (≈25±1.3, 7.0±1.0 and 6.0±1.0, respectively, Figures 3 B-E). Unlike the solubility of P, Mg, Cu, Zn and Mn, where the highest solubility was seen in experiment 11, the highest solubility of Se was observed in experiment 22 (44±0.8%) (Figure 3F).

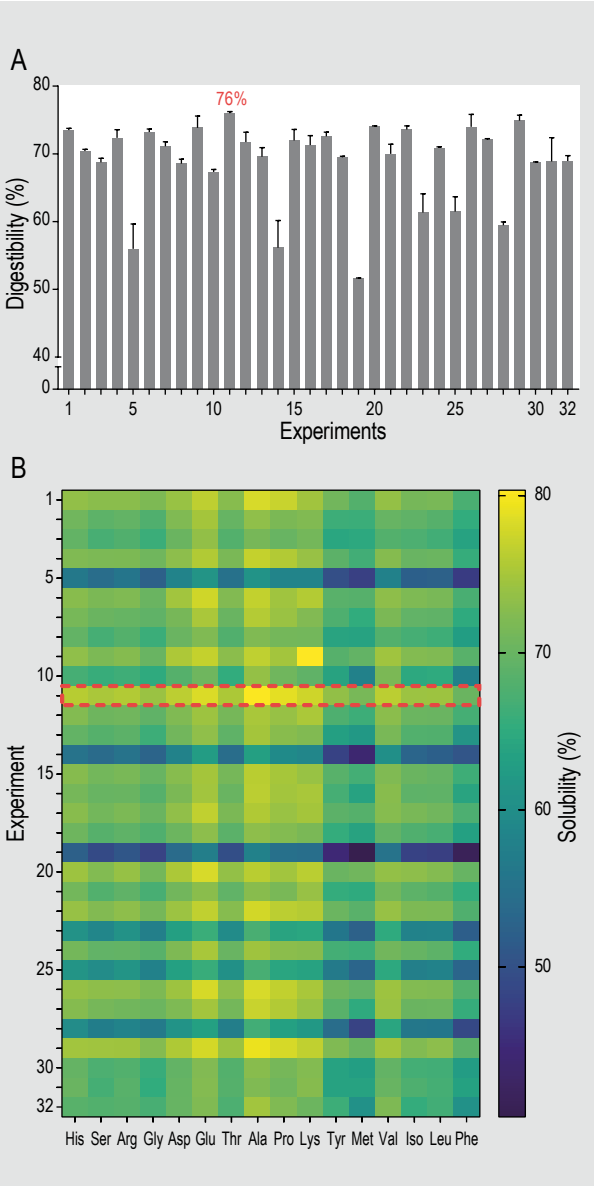

Figure 2. Overview of the digestibility of true protein (A) and solubility of amino acids (B) after applying the *in vitro* digestion method using BSF0. Experiments numbers are described in Table 1. Digestibility (%) data are presented as average ± standard deviation (n=2). The highest values for true protein digestibility and amino acids were 76±0.24 and 80.14±0.07% (alanine), respectively, recorded in experiment 11. The deeper colours represent the lowest solubility and the lighter colours represent the highest solubility in the respective sample (Figure 1B). His = histidine; Ser = serine; Arg = arginine; Gly = glycine; Asp = asparagine; Glu = glutamine; Thr = threonine; Ala = alanine; Pro = proline; Lys = lysine; Tyr = tyrosine; Met = methionine; Val = valine; Iso = isoleucine; Leu = leucine; Phe = phenylalanine.

The effect of the different factors (A-G) on $\%AA_{solubility}$ and $\%M_{solubility}$ is shown in Figures 4-5 and Supplementary Table S4. The only factors that significantly affected the digestibility of $\%TP_{digestibility}$ and $\%AA_{solubility}$ were the 'concentration of enzyme in the intestine' (F) and 'temperature' (G) ($P<0.05$) (Figure 4 and Supplementary Table S4). Enzyme concentration in the intestine of the *in vitro* digestion assay at 7 U/mg of protein led to higher solubility of all AA (except for Lys) compared to the digestion performed at 1 U/mg of protein ($P<0.05$). This is reflected by the positive value of the factor F (Figure 4 and Supplementary Table S4). A higher solubility of AA from BSF0 meal was also obtained at 12 °C compared to *in vitro* digestion performed at 37 °C, as reflected by the negative value of the factor G (Figure 4 and Supplementary Table S4). No significant effects on $\%TP_{digestibility}$ or $\%AA_{solubility}$ were reported for the factors 'pH in the stomach' (A), 'pH in the intestine' (B), 'reaction time in the stomach' (C), 'reaction time in the intestine' (D) or 'concentration of the enzymes in the stomach' (E) (Figure 4 and Supplementary Table S4).

The solubility of P, Mg, Cu, Zn, Mn and Se were also significantly affected by the factors F and G ($P<0.05$) (Figure 5). The factors F and G had a positive value for all minerals investigated in this study, which means that a higher solubility of minerals from BSF0 meal was achieved with the highest enzyme concentration in the intestine (7 U/mg of protein) and the highest temperature (37 °C) (Figure 5 and Supplementary Table S4). The solubility of P, Cu and Mn from BSF0 meal was higher after a reaction time of four hours compared to a two-hour reaction time in the alkaline hydrolysis step factor D (reaction time in

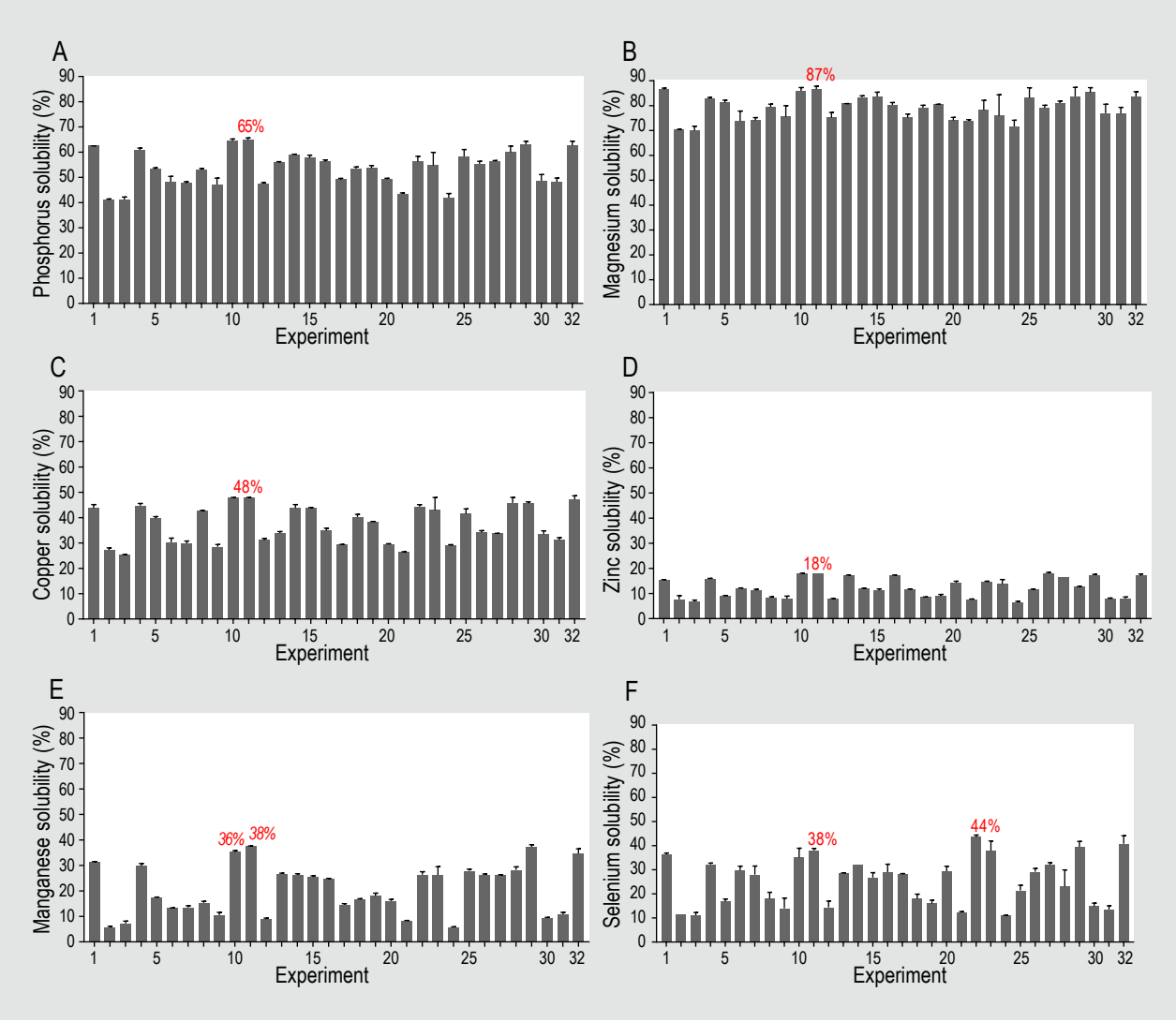

Figure 3. Solubility of phosphorus (A), magnesium (B), copper (C), zinc (D), manganese (E) and selenium (F) and after applying the *in vitro* digestion method using BSF0. The highest solubility (%) values were 65±0.9 (P), 87±1.2 (Mg), 48±1.2 (Cu), 18±0.05 (Zn) and 38±0.1 (Mn), respectively, recorded in experiment 11; 44±0.8 (Se) was recorded in experiment 22. Experiment number (x-axis) is described in Table 1. Solubility (%) data (y-axis) is presented as average ± standard deviation (n=2).

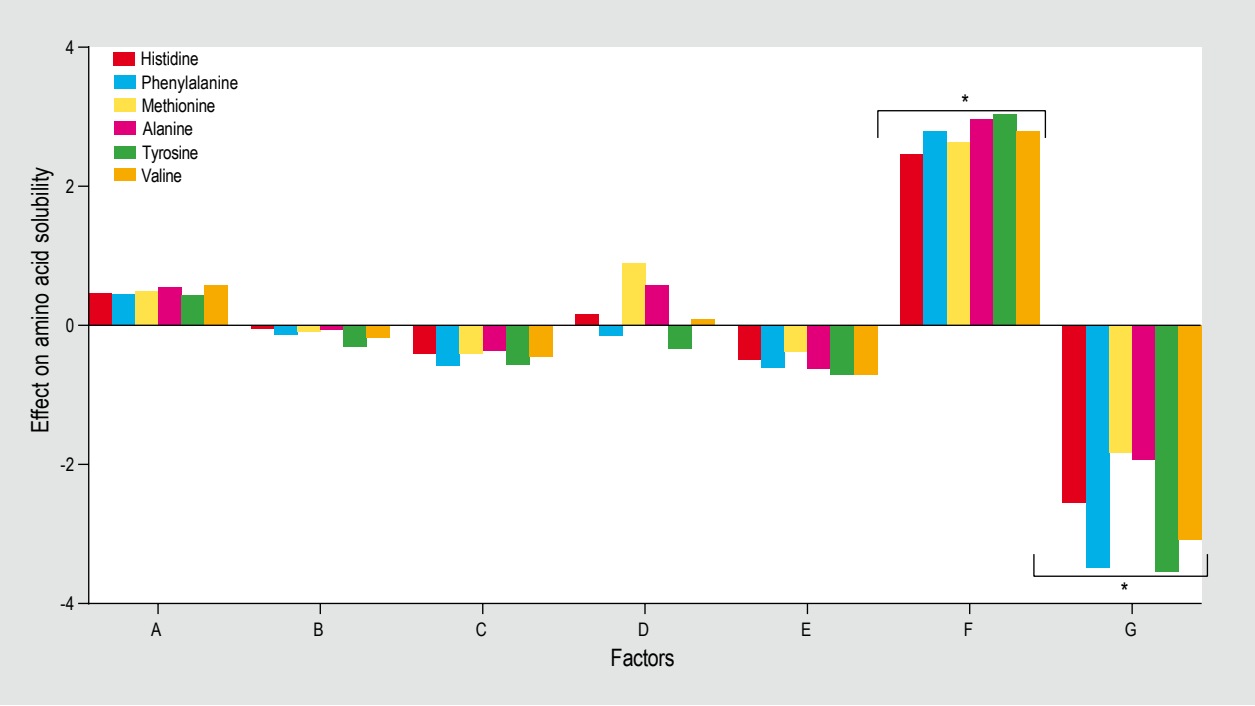

Figure 4. Effect of the different factors on amino acid solubility, represented by A = pH in the stomach; B = pH in the intestine; C = reaction time in the stomach (h); D = reaction time in the intestine (h); E = concentration of enzyme in the stomach; F = concentration of enzyme in the intestine; G = temperature (°C). * = *P*<0.05. A multiple regression model was used to estimate the significant effect of the main factors (A-G). Effects of the different factors for remaining amino acids are presented in Supplementary Table S4.

intestine, Figure 5 and Supplementary Table S4). This factor did not lead to statistically significant changes in solubility of Mg, Zn and Se (Figure 5). The digestion factors A, B, C and E did not affect the solubility of minerals in the BSF0 meal (Figure 5).

Application of the optimized *in vitro* digestion method for assessing amino acid and mineral solubility

After determining the optimal experimental variables, these were used to evaluate three different batches of BSF meals from the same producer (BSF1-3). The relative $\%AA_{solubility}$ varied between ≈30 and 39% among the three batches of BSF meal, being generally higher in BSF1 and lower in BSF2 (e.g. relative histidine solubility was 38.1±0.3% and 32.5±0.04% in BSF1 and BSF2, respectively). As can be seen in Figure 6, the solubility of non-essential AA (glycine, alanine and proline) was similar between BSF2 and BSF3 meal (≈34%) but significantly different from BSF1 (≈38%, P<0.05). For the essential AA, the solubility of AA significantly (P<0.05) differed between the three batches; as an example, the solubility of methionine was 20 and 10% higher in BSF1 than BSF2 and BSF3, respectively (Figure 6).

Similar to the results obtained for the AA, the relative solubility of minerals significantly (P<0.05) differed among the three batches of BSF meal (BSF1-3) (Figure 7). The relative solubility of P and Mg (%) was significantly higher in BSF1 (26.4±0.3% and 37.4±0.4%, respectively) than in BSF2 (22.9±0.4% and 30.4±0.03%, respectively) and BSF3 (22.4±0.04% and 30.8±0.2%, respectively) (Figure 7). The relative solubility of Zn (%) was significantly higher in BSF2 and BSF3 (P<0.05) than BSF1 (relative solubility was 6.6±0.1%, 7.4±0.2% and 7.8±0.1% in BSF1, BSF2 and BSF3, respectively). Furthermore, the relative solubility of Mn (%) was significantly (P<0.05) higher in BSF1 than BSF 2 and BSF3 meal, while the relative solubility of Se (%) was significantly (P<0.05) higher in BSF1 and BSF3 than BSF2 (P<0.05) (Figure 7).

4. Discussion

Optimal experimental variables to study true protein digestibility, amino acid and mineral solubility of BSF meal

Four main factors involved in the digestion of protein, AA and minerals were examined in the present study: pH (factors A and B), reaction time (factors C and D), concentration of the enzymes (factors E and F) and temperature (factor G). The digestive physiology, absorption and transport of nutrients in monogastric animals like pigs, poultry or fish are essentially similar to a larger extent (Krogdahl *et al.*, 2015; Zaefarian *et al.*, 2021). The experimental conditions for the *in vitro* digestion factors investigated in this study (i.e. pH, reaction time and concentration of the enzymes in the stomach and

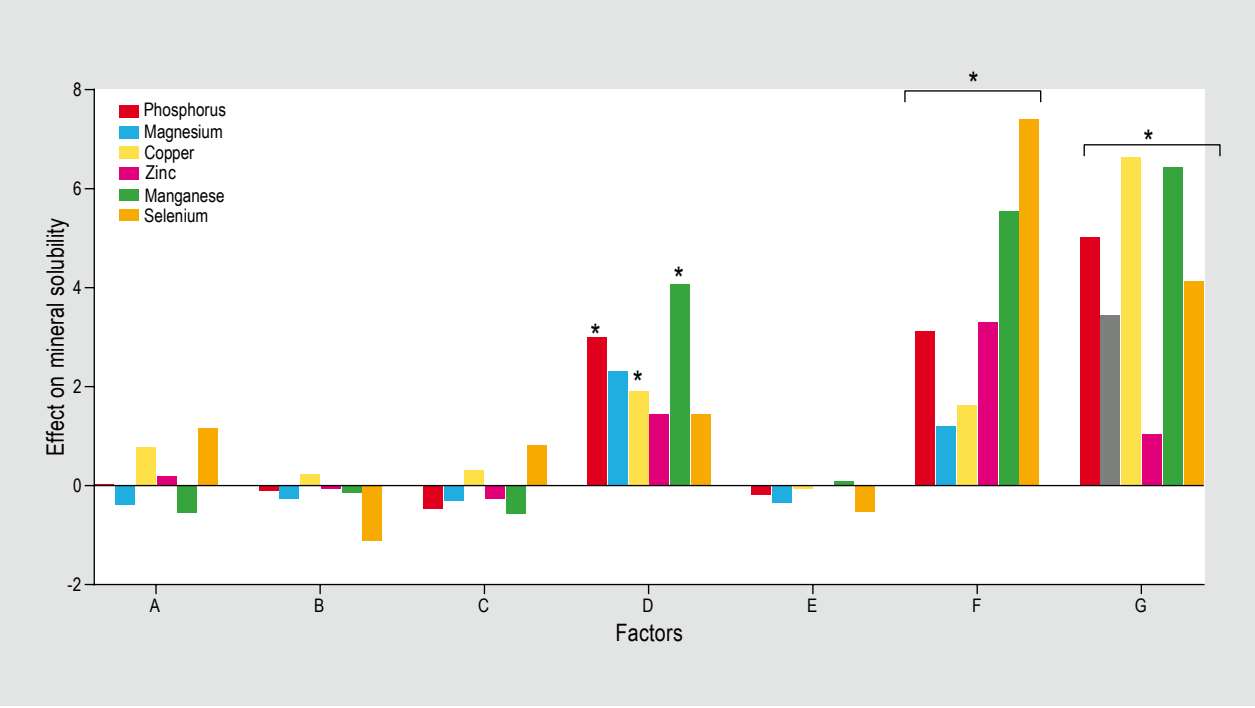

Figure 5. Effect of the different factors on mineral solubility, represented by A = pH in the stomach; B = pH in the intestine; C = reaction time in the stomach (h); D = reaction time in the intestine (h); E = concentration of enzyme in the stomach; F = concentration of enzyme in the intestine; G = temperature (°C). * = *P*<0.05. A multiple regression model was used to estimate the significant effect of the different factors (A-G). Effects of the different factors for remaining minerals are presented in Supplementary Table S4.

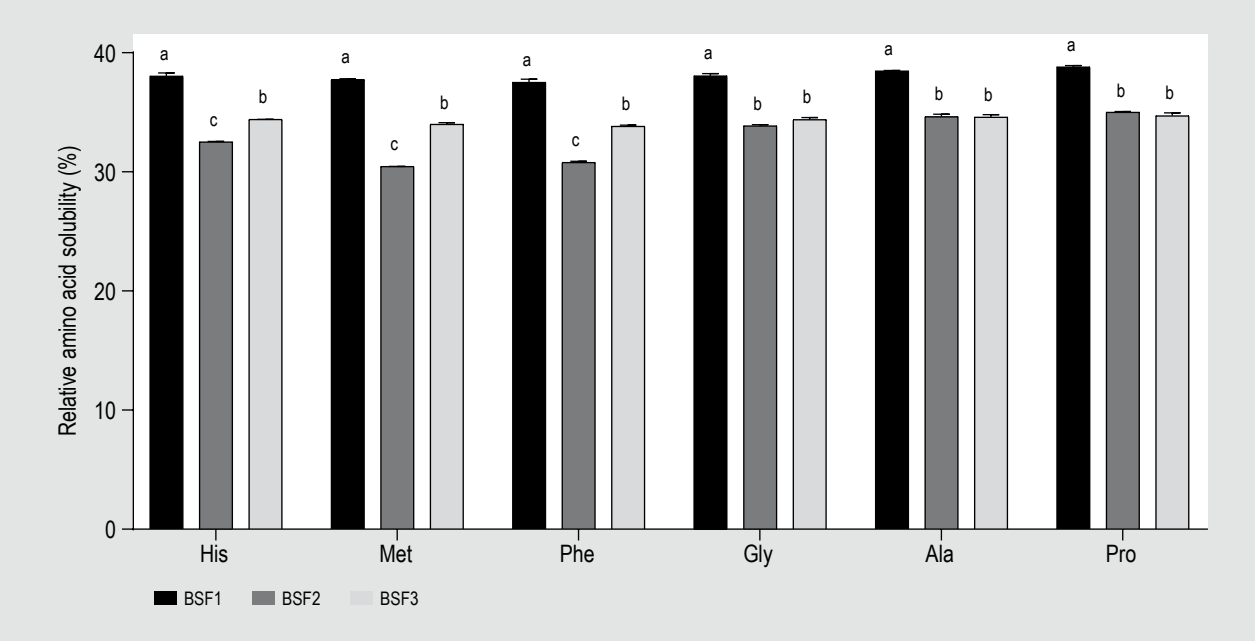

Figure 6. Relative amino acid solubility after applying optimised *in vitro* digestion method using three batches of BSF (BSF1-BSF3). Data are presented as average ± standard deviation (n=2). a, b, c represent significant effect between BSF meals (*P*<0.05, one-way ANOVA, followed by Tukey's HSD). His = histidine; Met = methionine; Phe = phenylalanine; Gly = glycine; Ala = alanine; Pro = proline.

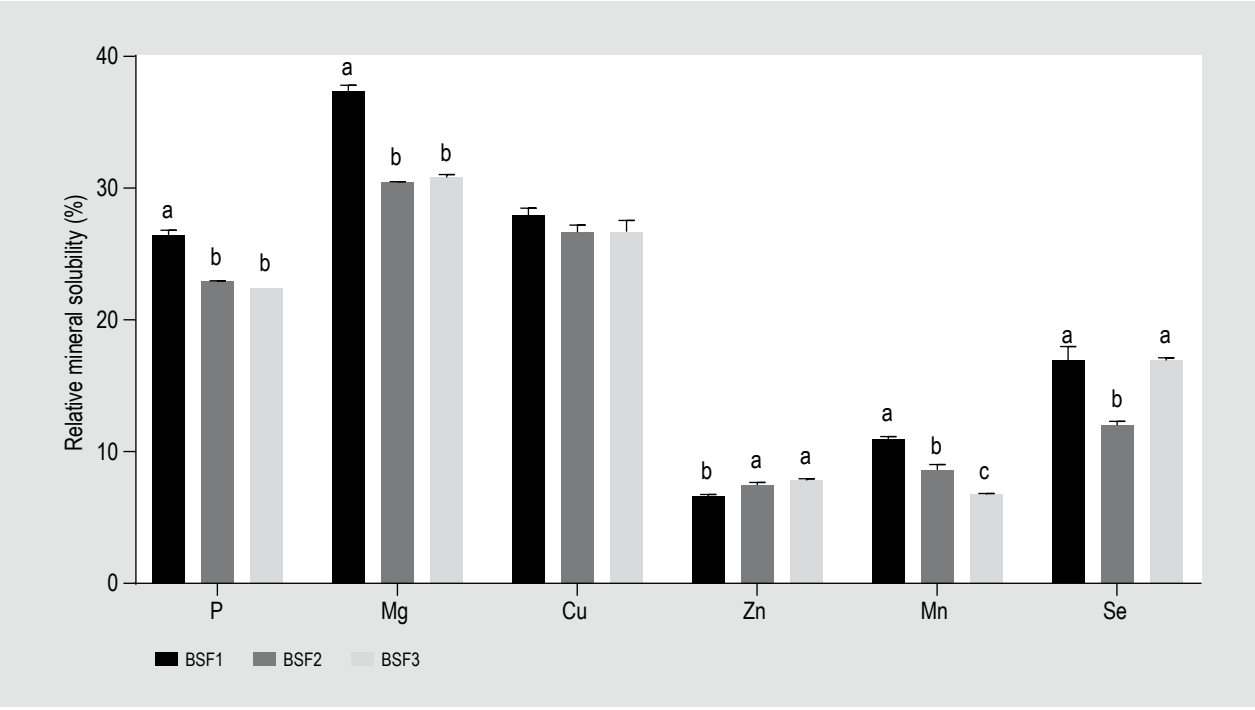

Figure 7. Relative mineral solubility after applying optimised *in vitro* digestion method using three batches of BSF (BSF1-BSF3). Values are means (n=2), with their standard deviation represented by vertical bars. a, b, c represent significant effect between BSF meals (*P*<0.05, one-way ANOVA, followed by Tukey's HSD). P = phosphorus; Mg = magnesium; Cu = copper; Zn = zinc; Mn = manganese; Se = selenium.

intestine) corresponded to the physiological digestion or digestive systems of monogastric animals (Dryden, 2008). Furthermore, two different temperatures were selected in this *in vitro* digestion method; 12 °C and 37 °C (factor G) based on the physiological temperature of salmonids and homeothermic species, respectively.

Our results show that only the factors 'concentration of enzyme in the intestine' (factor F) and 'temperature' (factor G) had a significant effect on $\%TP_{digestibility}$, $\%AA_{solubility}$ and $\%M_{solubility}$ of BSF meal. Gilannejad and colleagues, conducted a study to determine which *in vitro* digestion factors (pH, enzyme:substrate ratio and reaction time) led to the highest bioavailability of proteins and carbohydrates in gilthead seabream (*Sparus aurata*) gastrointestinal tract (Gilannejad *et al.*, 2018). The authors of this study used a model based on the response surface methodology and performed an *in vitro* digestion assay in a two-step hydrolysis (acidic and alkaline) by using a species-specific enzyme extract from gilthead seabream (Gilannejad *et al.*, 2018). Similar to our results, the factors most affecting nutrient solubility in their *in vitro* digestion method were related to the alkaline digestion, confirming the importance of this step in the complete nutrient breakdown (Gilannejad *et al.*, 2017, 2018; Zaefarian *et al.*, 2021).

In our trial, the highest solubility of both AA and minerals were obtained when the concentrations of enzyme in the alkaline hydrolysis (factor F) was set to 7 U/mg protein. Temperature, however, affected the solubility of AA and minerals differently. A higher solubility of AA was seen at 12 °C, while the highest solubility of minerals was obtained at 37 °C. The enzymes used in this study are derived from pig and the optimal temperature for the enzymes is expected be close to the physiological temperature of the organism from which the enzyme is derived (Bisswanger, 2014). The results obtained for optimal temperature for the solubility of minerals are consequently as expected. The reason why the solubility of AA was higher at a lower temperature might be due to a combination of the other factors involved. It is important to consider that factor G ('temperature') is generated by combining different factors (A×B×D×E) (Table 2). Thus, showing that an *in vitro* digestion method is a complex system that relies on a range of physical and biochemical processes where the digestion conditions influence each other (Dryden, 2008).

The factors 'concentration of enzyme in the intestine' (factor F) and 'temperature' (factor G) were the only factors which showed significant effects on protein digestibility and mineral solubility of the tested BSF meal (P<0.05). Therefore, these factors were the most relevant ones to be considered in the optimised *in vitro* digestion method. One of the goals of this study was to develop a method that allowed evaluation of both protein digestibility and mineral solubility equally well. Based on our results we set factor F to 7 U/mg protein and factor G to 37 °C in an optimal assay for AA and mineral *in vitro* digestibility. The factors

A-E did not show any statistically significant effects, but it was proved the importance to have an acidic digestion followed by an alkaline digestion. Thus, all the factors were included in the optimised method (experiment 11, Table 2).

Application of the optimised *in vitro* digestion method for assessing amino acids and minerals solubility

As discussed above, experiment 11 resulted in the highest overall nutrient solubility. Thus, the set of variables tested as experiment 11 was used to assess the nutritional stability of three batches of BSF meals (BSF1-BSF3) delivered by the same commercial producer.

An important factor to consider when performing an *in vitro* digestion method is the enzyme:substrate ratio, where an appropriate amount of enzyme is related to the concentration of the protein in each sample. In this study, achieving the enzyme:substrate ratio proved to be a challenging practice in the laboratory. Therefore, in this work, the enzyme concentration remained constant, despite the slightly differing protein concentrations between the three batches of BSF meals (i.e. BSF1 = 46.5%, BSF2 = 40.5%, BSF3 = 41.5%) (Supplementary Table S1). The relative soluble nutrients were calculated based on the soluble AA or mineral, adjusted for the concentration of protein in each BSF meal batch. As can be seen in Figures 6 and 7, the solubility of AA and minerals was different between the three batches. Several studies have shown that the processing of insect meals affect the protein quality and the *in vitro* digestibility of nutrients (Huang *et al.*, 2019; Manditsera *et al.*, 2019; Yi *et al.*, 2016). Manditsera *et al.* (2019) reported a decrease in protein *in vitro* digestibility and solubility of Fe, Mg, P, Se and Zn when boiling beetles compared to raw beetles. Processing methods like heating will affect the chemical structure of the proteins, which can influence their solubility and render them unavailable for enzymes (Lee *et al.*, 2019; Melgar-Lalanne *et al.*, 2019). Recently, Traksele *et al.* (2021) demonstrated that the protein of dried low-fat BSF larvae was more digestible in an *in vitro* digestion assay than the protein in larvae that were only dried. The authors of this paper speculate whether this difference in protein digestibility could be due the high amount of fat in the BSF larvae, which can induce an aggregation of the proteins in the larvae, resulting in a limited access of the enzymes to the protein complexes (Traksele *et al.*, 2021). However, the three batches of BSF meals used in the current study were produced by the same company and underwent the same processing. The differences observed can be due to the rearing substrate of the BSF larvae, which could have differed between the three batches. Interestingly, Galassi *et al.* (2021) observed differences in protein digestibility of BSF larvae reared on maize distillers, brewers' grains or by-product of soy milk, in an *in vitro* digestion model for monogastric animals. Another reason for the variation in protein and minerals availability observed between the three batches of BSF can be due to the chitin content, which may differ between the three batches due to the insect meal processing. Marono *et al.* (2015), observed a negative relationship between the protein digestibility and chitin content in yellow mealworm after in *vitro* digestion model for monogastric animals. However, the content of chitin was not measured in the BSF larvae meals of this experiment. It was therefore not possible to conclude if the chitin content affected the solubility of AA and minerals in the current *in vitro* study.

In general, it is known that both rearing and processing conditions will affect the outcome of the insect meal as final product. Thus, production methods in the insect farming process are to be evaluated carefully before implementing large-scale production. Additionally, it is of great importance for insect producers to assure the stability of batch-to-batch insect meals intended for animal nutrition. Accordingly, the method herein described could be used to investigate stability of batch-to-batch and the effect of the production methods on nutritional stability.

5. Conclusions

By using a fractional factorial design, an optimal combination of variables for an *in vitro* digestion method for measuring $\%TP_{digestibility}$, $\%AA_{solubility}$ and $\%M_{solubility}$ in a BSF larvae meal was found. *In vitro* digestion methods involve complex physical and biochemical processes so, it was a great advantage when screening the experimental variables by means of a factorial design. The highest overall nutrient solubility was recorded at condition 11 (Table 2), namely: pH in stomach and intestine (2 and 9, respectively), reaction time in stomach and intestine (1 and 4 hours, respectively), enzyme in stomach and intestine (1 and 7 U/mg, respectively) and temperature (37 °C).

The optimised *in vitro* digestion assay was then successfully used to evaluate the nutritional stability of three batches of BSF meals. The measured *in vitro* digestibility varied between the three evaluated BSF meals (BSF1-BSF3), indicating inconsistent product stability between production batches. This can be caused by both insect rearing conditions as well as the processing of the BSF meals.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2021.0197

Table S1. True protein content, amino acid (g/kg dry-weight basis) and minerals composition of black soldier fly (BSF0-3) larvae protein included in this study.

Table S2. Calculated percentages of true protein (%TP) and amino acids solubility (%$AA_{solubility}$) after applying the *in vitro* digestion method using BSF0 meal.

Table S3. Calculated percentages of minerals solubility (%$M_{solubility}$) after applying the *in vitro* digestion method using BSF0 meal.

Table S4. Effect of the main factors on true protein digestibility, amino acids and mineral solubility.

Acknowledgements

This study was supported by the Institute of Marine Research *(In vitro* digestibility of insect meals, 15551), the European Union's Horizon 2020 (SUSINCHAIN, 861976). Radhakrishnan Gopika thanks the fellowship provided by the Indian Council of Agricultural Research (ICAR) through the ICAR Netaji Subash International Fellowship. The authors thank David Dominguez and Nina Liland for providing valuable comments to this manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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