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RESEARCH ARTICLE

How different successive elaboration methods affect *Hermetia illucens* meals? Macronutrients, *in vitro* protein digestibility, oxidative status and hygienic-sanitary quality

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Abstract

Insects are an emerging protein alternative in aquaculture. To obtain an easy-to-handle feed with an improved nutritional quality, longer shelf life and better hygienic-sanitary properties, the insects must go through a multi-step process including sacrifice, drying and defatting. This work aims to evaluate the effect of the interaction of different successive methods of processing of *Hermetia illucens* larvae proximate composition, digestibility, and microbial loading. The examined methods were slaughtering (blanching or freezing), drying (oven drying or freeze drying) and defatting (mechanical press or supercritical CO_2 extraction). Our results showed that the combination of freeze-drying and mechanical press defatting resulted in the highest residual fat content ($P < 0.001$), that could negatively affect the relative protein content of the meals. Despite this, with respect to *in vitro* amino acid digestibility, the least favorable results were obtained from the samples dried by lyophilization and defatted by supercritical CO_2 extraction ($P < 0.001$), independently of the slaughter method. Regarding the oxidative status, the slaughter by freezing conserved a higher level of antioxidant defenses *versus* the blanching method ($P < 0.001$), although only the freeze drying method ensured a lower level of lipid oxidation in this type of sacrificing method. The applied processes resulted in a decrease in the presence of pathogenic bacteria compared to the raw insect in all examined groups. The mesophilic aerobic microorganism content was heavily affected by the temperature applied ($P < 0.05$), while the enterobacteria were also affected by the lyophilization, in addition to temperature. *Salmonella* spp was eliminated from all meals, except for the combination of freezing (sacrifice), freeze drying (drying) and supercritical CO_2 extraction (defatting). Sulfite-reducing clostridia were not present in any of the prepared insect meals.

Keywords

defatting – drying – insect processing – slaughter – supercritical fluid extraction

1 Introduction

Aquaculture currently represents 52% of the total fish industry for human consumption and is continuously growing (FAO, 2022), postulating itself as a suitable source to meet human protein demand. Currently, in addition to the rather unsustainable fishmeal or by-products of other animal husbandry, new alternative sources of protein are emerging in aquaculture feed, such as single cell protein biomass (Agboola *et al.*, 2021; Delamare-Debouteville *et al.*, 2019, Shah *et al.*, 2018), new plant protein sources (Daniel, 2018) and insect meal (Tran *et al.*, 2022). Using insects as a source of protein in aquaculture has several advantages: high feed conversion efficiency of insects, their farming produces lower greenhouse gas emissions, less use of water and farmland and certain species can consume waste and by-products from the food industry (Van Huis, 2013).

Hermetia illucens (Linnaeus, 1758) larvae (Black Soldier Fly larvae, BSFL) is one of the eight insect species regulated for feed formulation in the European Union (EU Regulation 2017/893 from July 2017, 2021/1372 from August 2021 and 2022/1104 form July 2022). However, their inclusion in aquaculture diets is limited to formats such as processed animal protein, live larvae and hydrolyzed protein; furthermore, restrictions exist regarding the substrates allowed for its rearing. The protein content of the BSFL can reach up to 46.2% in dry matter, depending on the quality of the substrate and the larval stage (Liu *et al.*, 2017). Several researchers have obtained BSFL meal with an essential amino acid profile that met FAO/WHO standard requirements (FAO, 2011), both in quality and quantity (Fuso *et al.*, 2021; Huang *et al.*, 2019; Caligiani *et al.*, 2018). They can also accumulate a relatively high amount of fat (15-49% of their dry weight), which can be further used as animal feed complementation or biodiesel (Franco *et al.*, 2021).

In addition to the crude protein content, it is necessary to obtain information on bioavailability, which is the fraction of the ingested nutrient, in this case protein, that becomes available for use and storage in the body (Ojha *et al.*, 2021a). The bioavailability of the protein is dependent on the digestibility of the protein source, which depends largely on the degree of protein hydrolysis after digestion (Rodríguez-Rodríguez *et al.*, 2022). *In vivo* determination of protein digestibility is a long and expensive process, thus *in vitro* techniques are developed to mimic the conditions of digestive processes through proteolytic enzymes (Almeida *et al.*, 2015). For the determination of the protein hydrolysis grade, a rapid and accurate method is the measurement of compounds, such as trinitrobenzenesulfonic acid (TNBS), nihydrin or *o*-phtaldialdehyde (OPA), which react specifically with amino groups (Yi *et al.*, 2016). These *in vitro* methods allow comparison between different protein sources when used under the same experimental conditions.

To obtain an easy-to-handle feed with an improved nutritional quality, longer shelf life and better hygienic-sanitary properties, the insect must go through a multi-step process including sacrifice, drying and degreasing (Melgar-Lalanne *et al.*, 2019). These methods can influence the parameters of the final product, such as proximate composition, microbial load, and sensorial parameters such as color and taste, due to the different heat effects and biochemical changes in the insect (Ojha *et al.*, 2021b).

Various killing methods exist, such as freezing, blanching, steaming, grinding, asphyxiation by CO_2, among which the most frequent ones appear to be freezing and blanching. (Singh *et al.*, 2020). Blanching can reduce enzymatic browning and microbial load due to the heat effect. On the other hand, it can cause denaturalization of proteins and loss of vitamins and minerals when the larvae are immersed in water. Meanwhile, freezing does not reduce the load of most pathogens, neither prevents lipolysis by endogenous enzymes, thus browning can be expected (Larouche *et al.*, 2019).

Drying is one of the traditionally used technologies for increasing the shelf life of foods. Drying can be performed through traditional methods such as sun and oven drying and more modern methods such as freeze drying, microwave drying (Melgar-Lalanne *et al.*, 2019). Several authors found that a higher drying temperature caused darkening, which can be due to Maillard reaction, affecting the antioxidant capacity and the susceptibility to digestive proteolysis of the meal (Mshayisa and Van Wyk, 2021; David-Birman *et al.*, 2018). As for more modern technologies, the cost of freeze drying is relatively high (Lenaerts *et al.*, 2018) and results in a less complete and slower moisture removal compared to oven drying, although this difference is minimal, and it guarantees a better preservation of structure and nutrients according to Spranghers *et al.* (2017).

Partial or complete defatting of insect meal is a requirement for feed formulation and a protein-rich meal can be obtained (Mishyna *et al.*, 2018), also the separated fat can be used for other purposes as mentioned before. The effect of defatting on the insect meal nutritional properties has been investigated by various studies (Laurent *et al.*, 2022; Jeong *et al.*, 2021; Laroche *et al.*, 2019). A widely used technique is extraction; solvent

extraction methods are popular, but many organic solvents are toxic and harmful to the environment. Supercritical fluid extraction (SFE) is a relatively modern method that is based on the high diffusivity and low density of the supercritical state gases. Carbon dioxide is a popular solvent for SFE, as it is easily removed from the product, non-toxic, non-flammable, inexpensive and readily available (Ueno *et al.*, 2008; Doborganes Nodar *et al.*, 2002; Stahl *et al.*, 1980). The drawbacks of this method are high unit operation cost and extraction efficiency (Dobarganes Nodar *et al.*, 2002; Friedrich and List, 1982; Stahl *et al.*, 1980). Mechanical defatting techniques are also existing; Kim *et al.* (2022) reported that mechanical press method gave an excellent nutritional profile with high essential amino acid content, while being cheap, fast and ecofriendly; at these aspects this technique performed better than traditional solvent methods.

The combination of the before mentioned operations influences the nutritional, organoleptic and hygienic properties (Regulation (EC) No. 2073/2005) of the generated meals (Liceaga, 2021); thus, it is important to determine, in each case, the characteristics of the obtained meals and to design a protocol that results in a product that shows the best possible values.

In the present article, BSFL were processed through different pathways, including sacrifice (blanching vs. freezing), drying (oven vs. freeze drying) and defatting (mechanical press vs. supercritical fluid extraction). Proximate, digestibility, oxidative status and hygienic-sanitary aspects of the obtained meals were investigated.

2 Materials and methods

Sample preparation

The BSFL used in this experiment was supplied by Entomo Agroindustrial (Cehegin, Spain). The dried and defatted insect meals were prepared as described by Hurtado-Ribeira *et al.* (2023). 12-days-old larvae were sieved and washed prior to slaughter. For blanching as a devitalization method, larvae were submerged in 90 °C water (in a 1:10 w/v ratio) for 40 s, then immersed in cold water and drained. For freezing, larvae were frozen at –20 °C for 24 h. Each slaughtering procedure was performed in duplicate (8 kg per replicate). Half of the as prepared larvae were dried in a conduction oven at 65 °C for 24 h and the other half was freeze-dried (LyoBeta 15, Telstar, Terrassa, Spain) for 4 days with the condenser at –81 °C and a program of –20 °C for 2 h, followed by a gradual increase until reaching 20 °C which was maintained until the end of the procedure in the chamber. Later, each resulting batch was divided in half for the defatting procedures. For mechanical defatting the dried larvae were extracted using a screw-press expeller (InVIA, Barcelona, Spain) with a heating jacket that was set to the minimum temperature of 136 ± 13.1 °C. Supercritical CO_2 defatting was performed following the conditions described by Cantero-Bahillo *et al.* (2022) using a supercritical CO_2 extraction equipment (Model Thar SF2000, Thar Technology, Pittsburgh, PA, USA). The defatting was carried out at 450 bar, 60 °C, and a CO_2 flow of 130 g/min for 240 min. The process and the result of certain steps can be observed in Figure 1.

In the discussion part, abbreviations were used to describe the meals prepared by the different combinations of treatments.

Proximate analysis

The proximate analysis of the prepared BSFL meals was performed following the Methodology established by the Association of Official Analytical Collaboration International (AOAC, 2005). Dry matter and ash content were examined gravimetrically. Drying was carried out at 105 ± 1 °C until constant weight in a laboratory oven (AOAC #934.01), while the ash content was obtained using a muffle furnace at 500 °C ± 5 °C for 12 hours (AOAC #942.05). The crude protein content was determined by the Kjeldahl method (AOAC #954.01). To determine the exact protein content 4.76 (Janssen *et al.*, 2017) was used as conversion factor. The crude fat content was examined gravimetrically after Soxhlet extraction with diethyl ether (AOAC #920.39). The values of the neutral detergent and acid detergent fiber of the meals were determined using the van Soest (1991) method, while the analysis of the chitin content was performed using the method described by Woods *et al.* (2020).

Degree of hydrolysis

In vitro amino acid digestion was performed in two steps, simulating the gastric and intestinal digestive processes, following the modified method of Huang *et al.* (2019) to obtain the degree of hydrolysis (DH) of each step. Easily available commercial monogastric enzymes were used. The simulation of the digestion was performed using a water bath shaker set to 37 °C with constant motion. For the gastric phase digestion, 0.05 g of experimental BSFL meals were added to 4 mL solution containing 2 g/L NaCl and 7 mL/L HCl, with a pH

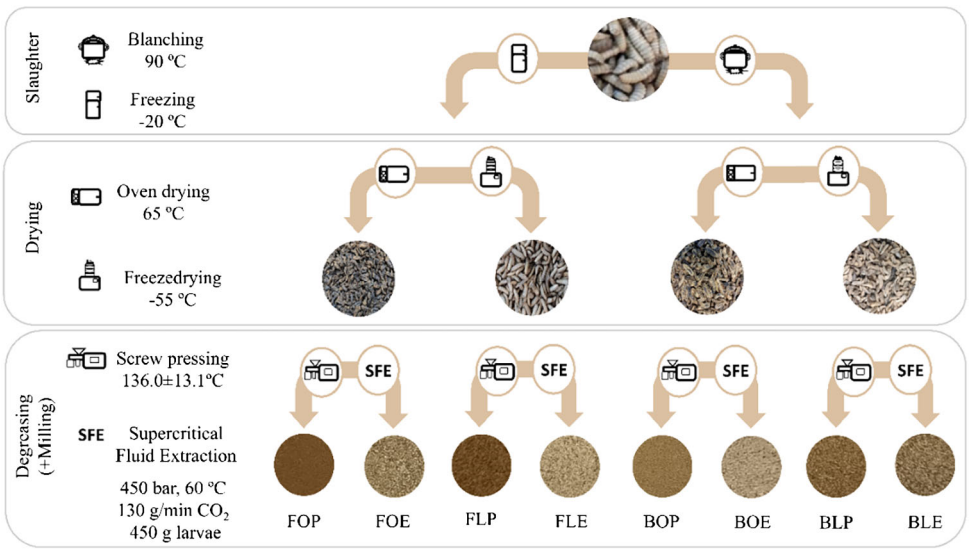

FIGURE 1 Black Soldier Fly Larvae meal preparation process diagram. At the abbreviations, the first letter stands for the devitalization method (F = Freezing; B = Blanching); the second letter stands for the drying method (O = Oven drying; L = Lyophilization) and the third letter stands for the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

adjusted to 2. Right before starting the digestion process, 3.2 mg/mL of fresh pepsin (porcine pepsin 2000 U/g, Merck 7190) was added to the mixture and the digestion was carried out for 3 hours. Subsequently, intestinal phase digestion was performed, for this purpose the pH of the gastric phase mixture was adjusted to 6-8 with NaOH and 2 mL of a solution containing 1.5 mg/mL of pancreatin (porcine pancreatin grade VI, SigmaP-1750) and 24 mg/mL of bile extract were added. The incubation was continued for 2.5 h under the same conditions described before. 100 μL of mixture supernatant was withdrawn at the beginning of the process and at the end of gastric and intestinal phase, and mixed with the same amount (100 μL) of 20% trichloroacetic acid solution in centrifuge microtubes. The resulting samples were chilled at 4 °C during 30 minutes and centrifuged at 12,000 × g for 15 minutes at 4 °C (Biocen 22 R, Ortoalresa, Madrid, Spain). The resulting supernatant, containing trichloroacetic acid soluble amino acid and protein fractions, was collected and examined spectrophotometrically, at 340 nm with a UV spectrophotometer (PowerWave XS, BIO-TEK Instruments, Winooski, VT, USA), according to the OPA assay (Church *et al.*, 1985) to obtain the amount of NH_2 groups released at each step of the digestion. The degree of hydrolysis was presented with respect to the total NH_2 groups present in the sample as displayed in the next equation:

$$DH\,(\%) = NH_{2acid} \text{or} NH_{2alkaline}/NH_{2total} \cdot 100(\%) \quad (1)$$

where $NH_{2\ acid}$ and $NH_{2\ alkaline}$ is the amount of NH_2 groups released at the end of the gastric or intestinal phase and $NH_{2\ total}$ is the total content of NH_2 groups of the sample. Different DH were defined according to whether it was the DH obtained in gastric (DA) or intestinal digestion (DB) or the sum of both (DAB).

To determine the total NH_2 group content of each sample, a complete hydrolysis of protein was performed, where 0.05 g of the meal from each treatment was heated in an oven at 110 ± 1 °C for 24 h with 2.5 mL of 6 N HCl. The samples were then diluted with distilled water in a ratio of 1:1, centrifuged at 12,000 × g and the supernatant was assayed with OPA method for the NH_2 group content. The results were used in the calculations of *in vitro* digestion and *in vitro* amino acid digestibility.

In vitro *amino acid digestibility*

The *in vitro* amino acid digestibility of the BSFL meal was determined by the direct method, briefly the supernatant obtained at the end of the intestinal digestive phase was hydrolyzed with 6 N HCl at 110 ± 1 °C for 24 h. The total NH_2 group content of the supernatant hydrolysate was quantified by OPA assay. The digestibility based on this assay method was calculated as follows:

$$\text{Digestibility}\,(\%) = NH_{2digestibility}/NH_{2total} \cdot 100(\%) \quad (2)$$

where $NH_{2\ digestibility}$ is the amino groups present in the supernatant after the digestion and $NH_{2\ total}$ is the total amino group content in 100 g dry material.

Trolox equivalent antioxidant capacity

Levels of Trolox equivalent antioxidant capacity (TEAC) were determined using the method described by Erel (2004), based on colour change produced by the reduction of previously oxidized ABTS (2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)) in a proportional way to the antioxidant compound present in the sample. Briefly, samples of BSFL meals were mechanically homogenized in 9 volumes of ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. Homogenates were centrifuged at 30,000 × g for 20 min at 4 °C and the supernatants were used for TEAC determination. The reaction mixture consisted of 0.4 M acetate buffer pH 5.8, 30 mM acetate buffer pH 3.6, 2 mM hydrogen peroxide 30 % and 10 mM ABTS. After 5 minutes of incubation, spectrophotometric lecture was performed at 595 nm. For quantification of TEAC, a standard curve of Trolox was used. TEAC levels were expressed as mmol of Trolox equivalent per kg of BSFL meal.

Lipid peroxidation

The analysis of lipid peroxidation levels (LPO) was performed according to the modified technique of Buege and Aust (1978). The procedure was carried out according to colorimetric detection based on the presence of thiobartituric acid reactive molecules (TBARS), such as malondialdehyde (MDA), a molecule derived from lipid peroxides. The supernatant of the samples previously processed as described in Section 2.5 was mixed with a solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, 80% (v/v) HCl 0.25 N and 0.01% (w/v) butylated hydroxytoluene. The mixture was heated to 100 °C during 15 min. After being cooled at room temperature and centrifuged at 1,500 g for 10 min, the absorbance was measured at 535 nm. MDA was used as standard curve. LPO levels were expressed as μmol MDA per kg of BSFL meal.

Hygiene and sanitary

Sulfite-reducing clostridia (*Clostridium perfringens*) were estimated using SPS (Sulfite Polymyxin Sulfadiazine) Agar (PanReac) and were incubated at 37 °C for 48 h. The potential presence of *Salmonella* spp. was investigated according to the following protocol: Pre-enrichment stage: Incubation of the sample at 37 °C for 18 h in buffered peptone water, where the meal to nutrient medium ratio was 1:9. Enrichment stage: Incubation in Rappaport-Vassiliadis broth, a selective medium, at 41.5 °C for 24 h, extendable to 48 h in case of initial negative results. Selective isolation stage: Seeding from the selection medium grown on Hektoen Agar selective medium and incubation at 37 °C for 24 h. Confirmation stage: Study of suspected colonies by biochemical tests (Kligler Agar, incubated at 37 °C for 24 h). The presence of enterobacteria colonies of adequate morphology was investigated in VRBG Agar plates, grown for 24 h at 37 °C. The presence of mesophilic aerobic microorganism (MAM) was investigated on PCA Agar plates, grown for 24 h at 37 °C.

Statistical analysis

Data are presented as mean ± S.E. and they were checked for normal distribution and homogeneity of variances and normalized, if necessary, by logarithmic transformation, and in those cases where adjustment could not be made, the Kruskal-Wallis test was applied. All statistical analyses were performed using SPSS version 28.0 for Windows software package (IBM, Armonk, NY, USA, 2022).

3 Results and discussion

Proximate analysis

The results of the proximate analysis of the prepared BSFL meals can be observed in Table 1. The moisture content was significantly influenced by the combination of slaughter and drying (SxD) and drying and defatting (DxDF) methods, although the interaction between slaughter and defatting (SxDF) did not show significant differences (Figure 2). Drying BSFL by freeze-drying resulted in a higher percentage of moisture in the meals compared to oven drying regardless of the slaughtering method. Other authors evaluated the effects of the freeze drying and oven drying on *Tenebrio molitor* L. larvae (Ribeiro *et al.*, 2024; Vlahova-Vangelova *et al.*, 2024; Selaledi *et al.*, 2021; Kröncke *et al.*, 2019), the results obtained in these studies showed higher moisture levels in *T. molitor* meals dried by freeze-drying compared to oven-drying. However, although oven drying generally gives lower moisture levels, the final result will depend on the different parameters applied (temperature, grinding, drying time, total mass to be dried) to each of the drying methods (Kröncke *et al.*, 2018). Blanching slaughtering method showed a higher moisture content compared to freezing in the freeze-dried larvae, this may be due to the increase in the initial moisture content when the larvae were immersed in boiling water (Ribeiro *et al.*, 2024).

The protein content of the meals was significantly influenced by the slaughtering, drying and defatting

TABLE 1 Results of proximate analysis of BSFL meals

	Moisture	Protein	Fat	NDF	ADF	Chitin	Ash
FOP	4.85 ± 0.48ab	56.96 ± 0.13cd	7.81 ± 0.20cd	38.51 ± 0.88e	9.93 ± 0.29d	6.06 ± 0.26b	17.35 ± 0.03c
FOE	5.70 ± 0.16b	57.30 ± 0.14cd	6.58 ± 0.28bc	30.71 ± 1.84cd	8.49 ± 0.18c	5.92 ± 0.00 ab	19.25 ± 0.14e
FLP	8.75 ± 0.90cd	54.28 ± 0.24b	11.69 ± 0.35e	16.44 ± 0.87a	7.59 ± 0.03abc	5.22 ± 0.15ab	16.84 ± 0.06b
FLE	8.37 ± 0.04c	56.20 ± 0.28c	6.15 ± 0.34b	21.64 ± 1.25ab	6.76 ± 0.33ab	5.44 ± 0.12ab	20.08 ± 0.07f
BOP	3.36 ± 0.04a	56.32 ± 0.27c	7.43 ± 0.03bcd	32.42 ± 1.82de	8.50 ± 0.41c	5.15 ± 0.18ab	16.50 ± 0.12b
BOE	4.74 ± 0.21ab	53.94 ± 0.39b	8.43 ± 0.21d	25.49 ± 1.45bcd	7.72 ± 0.18bc	5.20 ± 0.12ab	18.62 ± 0.09d
BLP	10.90 ± 0.50de	51.17 ± 0.26a	12.28 ± 0.67e	16.90 ± 2.24a	6.45 ± 0.15a	4.61 ± 0.14a	15.82 ± 0.08a
BLE	11.48 ± 0.08e	57.85 ± 0.27d	1.06 ± 0.18a	24.21 ± 1.74bc	7.23 ± 0.38ab	4.98 ± 0.58ab	20.39 ± 0.10f

Different letters indicate significant differences ($P < 0.05$) according to different methods of slaughtering, drying, and defatting. At the abbreviations the first letter stands for the devitalization method (F = Freezing; B = Blanching); the second letter stands for the drying method (O = Oven drying; L = Lyophilization) and the third letter stands for the defatting method (P = Screw pressing; E = Supercritical fluid extraction). NDF = neutral detergent fiber; ADF = acid detergent fiber.

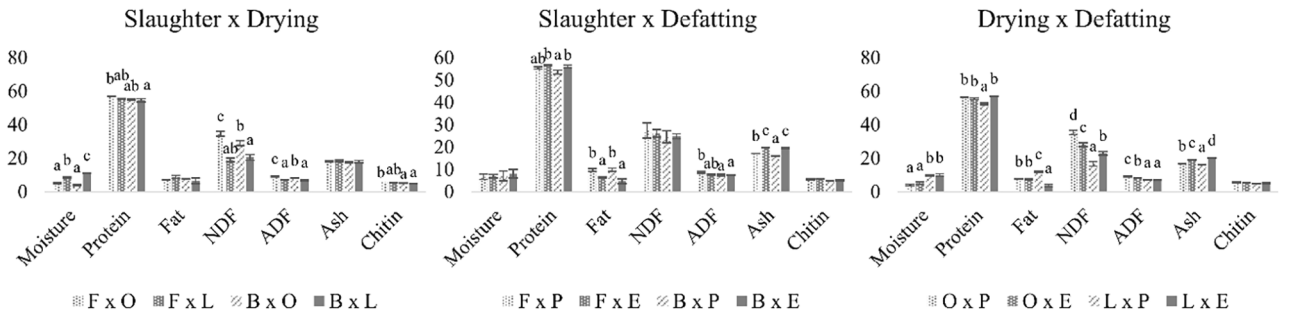

FIGURE 2 Representation of the interaction of pairs of processing methods, slaughtering (S), drying (D) and defatting (DF), for the proximate composition data for the different BSFL meals. Moisture values are expressed as g/100 g BSFL meals, the rest of the values are expressed as g/100 DM BSFL meals. Values in the columns are presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) within each parameter evaluated. The abbreviations stand for the devitalization method (F = Freezing; B = Blanching); drying method (O = Oven drying; L = Lyophilization) and the defatting method (P = Screw pressing; E = Supercritical fluid extraction). NDF = neutral detergent fiber; ADF = acid detergent fiber.

methods used, as well as by the interaction between the drying and defatting methods. Most of the combinations of treatments generated a meal with a protein content above 56%. The protein level decreased below this value for the treatments FLP, BLP and BOE, displaying the lowest protein content BLP with 51.2%. The highest protein content can be observed in BLE with 57.3%, where the residual fat content was the lowest. The results displayed an inverse tendency compared to the fat content, we assume that the lower protein content could be explained with the higher residual fat content of the samples, as were in the case of Laurent *et al.* (2022) and Jeong *et al.* (2021). SxDF and DxDF interactions also showed lower levels of protein in the meals with a higher percentage of fat. Regarding S × D interaction the protein content tended to increase when the larvae were slaughtered by freezing, with this value being significantly higher for the combination of freezing and oven drying. In addition to the observed trend regarding the influence of defatting, the slaughtering process may also affect the protein levels of insect meal, however, other studies carried out on BSFL showed no difference in protein levels of larvae killed by freezing or blanching (Rodríguez-Rodríguez *et al.*, 2024; Zhen *et al.*, 2020). In other insect species such as *Acheta domesticus*, a decrease in protein levels was observed in insects killed by blanching compared to freezing; this may be due to the partial loss of protein that is solubilized by the high temperatures in blanching method (Singh *et al.*, 2020). Oven drying could lead to increased levels of protein regarding freeze drying as observed in other studies carried out on insect meal (Kröncke *et al.*, 2019, Kröncke *et al.*, 2018). Another aspect that can affect the level of protein in the meal is the presence of non-protein nitrogen. Non-protein nitrogen accounts for 16-26% of the total nitrogen present in BSFL, which is made up of different nitrogenous components such as chitin, nucleic acids and nitrogenous products related to digestion (Janssen *et al.*, 2017). The loss of these nitrogenous compounds during the different processes of slaugh-

tering, drying or defatting could affect the conversion factor used to convert the total nitrogen, obtained in the Kjeldahl analysis, into protein resulting in under- or overestimation of the true protein content (Nguyen *et al.*, 2022).

The percentage of fat in the dry matter of the insect meal was significantly influenced by the defatting method ($P < 0.001$) and the interaction of slaughtering and drying with the defatting method (S1-3). The fat content ranged from 1.06 to 12.28 g/100 g of dried BSFL, found in BLE and BLP meals respectively. The combination of hot pressing with either of the slaughter method resulted in a higher fat content respect to SFE, although when we examine the interaction of the drying and defatting methods, only in the case of freeze drying can be observed a significant difference between the two defatting methods. This phenomena may be explained by the structure. Several articles reported that slow freezing (–20 °C), prior to freeze drying, can cause structural damage in cells of organic tissues, resulting in larger pores (Qui *et al.*, 2022). This porous structure can be conserved by the freeze drying method, facilitating the way of supercritical CO_2, thus improving the extraction, as Fornari *et al.* (2023) also suggested in a recent article. However, screw press can damage the structure and expose the material to higher temperature, hindering fat extraction.

Regarding the Neutral Detergent Fiber (NDF), the combination of S × D and D × DF had a significant effect on total NDF content, where oven drying resulted in higher values. Overall the following tendency can be observed within the two sacrifice methods: OP > OE > LE > LP. FOP gave the highest value (38.5%), while the lowest was detected in FLP (16.4%). This strong influence of the drying process may be related to the decrease in the solubility of the protein when drying the insects in an oven compared to freeze-drying, making it more difficult to solubilize in the neutral detergent solution and therefore increasing the NDF content (Kröncke *et al.*, 2018). The tendency was similar for the Acid Detergent Fiber (ADF), although it also showed a significant difference for the combination of S × DF where freezing as slaughter method showed slightly higher values compared to blanching. Cortazar-Moya *et al.* (2023) found that the defatting of insects resulted in a higher fiber content, which could be observed in our results, as the combination of freeze-drying with defatting by hot-pressing gave the highest residual fat values and the lowest fiber values. In the insect meals produced, the chitin content was only significantly influenced by the combination of slaughtering and drying method where meals obtained by freezing as slaughter method resulted in higher values. Also, it was observed that the chitin content of the meal processed by freezing-oven-pressing was higher than when the meal was produced by blanching-lyophilizing-pressing. All other combinations showed similar chitin content (4.61-6.06%).

The ash content of the manufactured meals varied significantly with the combination of S × DF and D × DF. Under the same sacrifice and drying conditions, samples treated with hot press resulted in a lower ash content than samples treated with SFE. The cause of this can be that during CO_2 extraction, part of the organic compounds were extracted resulting in a higher ash content (Krivonos and Belskaya, 2020). The highest values were obtained for BLE meal, reaching 20%.

Degree of hydrolysis

Gastric and intestinal hydrolysis were examined separately, as it can be seen in Figure 3, where the sum of their value is also presented as the total grade of hydrolysis. The values are expressed respect to the total amino acid content.

The combination of treatments applied to BSFL affected the gastric degree of hydrolysis (DA), higher DA value was observed for BOE, while the lower was found for the meal prepared by the combination of freezing, oven drying and SFE defatting (FOE). Statistically, DA was significantly influenced by the combination of S × D, S × DF and D × DF, where, in general, blanching resulted in higher values of protein hydrolysis in the S × D interaction, so as the defatting by SFE in D × DF interaction (Figure 4). In the case of the intestinal degree of hydrolysis (DB) and the sum of the gastric and intestinal degree of hydrolysis (DAB) the significant effects were the combination of S × DF and D × DF. In DB, the tendency was the opposite of what was observed in DA; the higher values were obtained by the hot pressing as defatting method in S × DF and D × DF interactions. DAB followed the tendency of DB. Regarding the oven-dried samples, the values of DAB were similar within each sacrifice method, being the blanched ones (35-37%) higher than the frozen ones (30-32%). Among the lyophilized samples, screw press defatting method resulted in a higher grade of hydrolysis than SFE. The lowest values were obtained for both sacrifice methods with the combination of freeze drying and SFE defatting, 26.9% for FLE and 25.2% for BLE. The increase of DAB when blanching killing method was applied regarding freezing method was observed in previous studies (Rodríguez-Rodríguez *et al.*, 2024; Leni *et al.*, 2019). This increase may be due to thermal

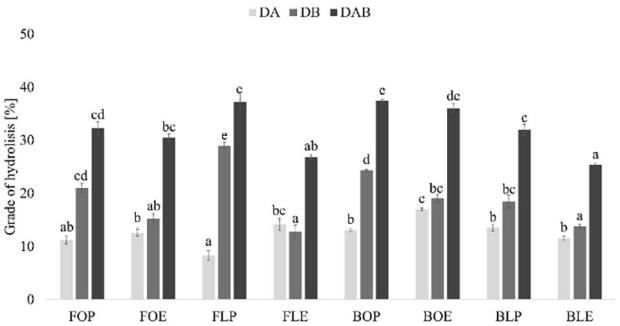

FIGURE 3 Grade of hydrolysis of the different BSFL meals, expressed in grams of liberated amino acid with respect to the total amino acid content of 100 g, where DA is the degree of gastric hydrolysis; DB is the degree of intestinal hydrolysis and DAB is the sum of gastric and intestinal degree of hydrolysis. Values in the columns are presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) according to different methods of slaughtering, drying and defatting. At the abbreviations, the first letter stands for the devitalization method (F = Freezing; B = Blanching); the second letter stands for the drying method (O = Oven drying; L = Lyophilization) and the third letter stands for the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

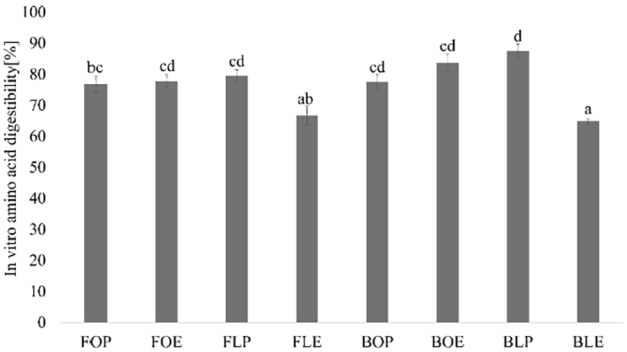

FIGURE 4 Representation of the interaction of pairs of processing methods, slaughtering (S), drying (D) and defatting (DF), of the analysis performed for the *in vitro* digestibility results, where DT is *in vitro* amino acid digestibility, DAB is the sum of acidic and alkaline digestibility, DA is acidic digestibility and DB is the alkaline digestibility of the different BSFL meals. Digestibility values are expressed as g of – NH_2 released respect 100 g of total – NH2. Values in the columns are presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) within each parameter evaluated. The abbreviations stand for the devitalization method (F = Freezing; B = Blanching); drying method (O = Oven drying; L = Lyophilization) and the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

inhibition of the enzymes responsible for the melanisation process in insects. This enzymatic process occurs in response to stress and is involved in the aggregation of proteins making them less susceptible to enzymatic hydrolysis (Leni *et al.*, 2019). This enzymatic process is behind the aggregation of the proteins making them less susceptible to enzymatic hydrolysis. When comparing the gastric and intestinal hydrolysis, the screw pressed samples had larger difference between them than the ones obtained by SFE defatting. This may be the result of the high temperature applied during the screw pressing, which can decrease protein solubility and hinder digestibility at the gastric environment, while increasing the same at the intestine tract, not affecting the overall digestibility as Zhang *et al.* (2022) explained.

In vitro *amino acid digestibility*

The *in vitro* amino acid digestibility can be observed in Figure 5.

The values are expressed with respect to the total amino acid content. Similarly to DB and DAB, the combination of S × DF and D × DF had a significant effect on the *in vitro* amino acid digestibility of the BSFL meals. Hot press as defatting method resulted in significantly higher DT values compared to SFE. It can be clearly seen that the combination of freeze drying and SFE defatting had the worst performance (66% for freezing and 64% for blanching) compared to the other combinations which were all above 70%, being the highest value 87.4% for the BLP sample. Also, the rest of the samples only had a slight difference between each other. Studies that provide information on amino acid digestibility levels are scarce, especially in relation to the impact of the combination of different drying and defatting methods on this parameter. In this sense, it is difficult to find an argument to explain this specific result, although previous studies support the hypothesis that different combinations of processing technologies lead to variations in both the composition and digestibility of the amino acid fraction (Mohd Zaini *et al.*, 2023).

Oxidative status

Lipid oxidation can directly influence the palatability of the feed and the health of the animals consuming it, affecting later also the quality of the fillet (Mouithys-Mickalad *et al.*, 2021). In order to determine the oxidative status, total antioxidant capacity as TEAC and lipid peroxidation levels were determined in the different BSFL meals and the obtained results are shown in Figure 6.

Results showed that insect meals obtained by the freezing method of slaughtering of animals tended to have higher levels of TEAC and LPO regarding blanching method. No significant influence of the D × DF interaction were observed for TEAC results (Figure 7). On

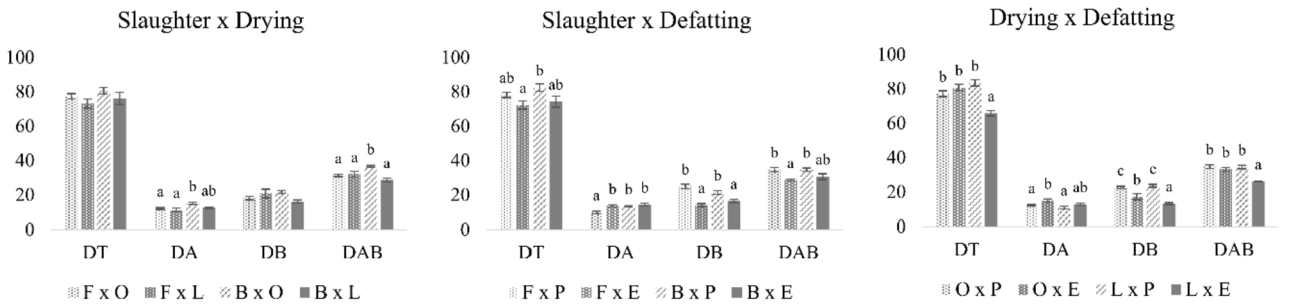

FIGURE 5 *In vitro* amino acid digestibility of the different BSFL meals, expressed in grams of liberated amino acid (at the endpoint of the *in vitro* digestion) after hydrolisis with respect to the total amino acid content of 100 g * Values in the columns are presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) according to different methods of slaughtering, drying and defatting. At the abbreviations, the first letter stands for the devitalization method (F = Freezing; B = Blanching); the second letter stands for the drying method (O = Oven drying; L = Lyophilization) and the third letter stands for the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

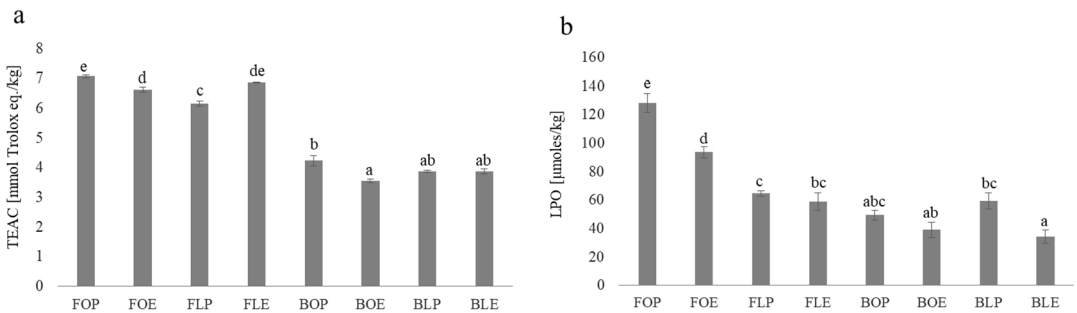

FIGURE 6 Trolox equivalent antioxidant capacity-TEAC (a) and lipid peroxidation-LPO (b) levels of the different BSFL meals. Values are mean ± S.E. (n = 4). Different letters indicate significant differences ($P < 0.05$) according to different methods of slaughtering, drying and defatting to obtain the experimental insect meal and based on one-way ANOVA followed by Tukey test. At the abbreviations, the first letter stands for the devitalization method (F = Freezing; B =Blanching); the second letter stands for the drying method (O = Oven drying; L = Lyophilization) and the third letter stands for the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

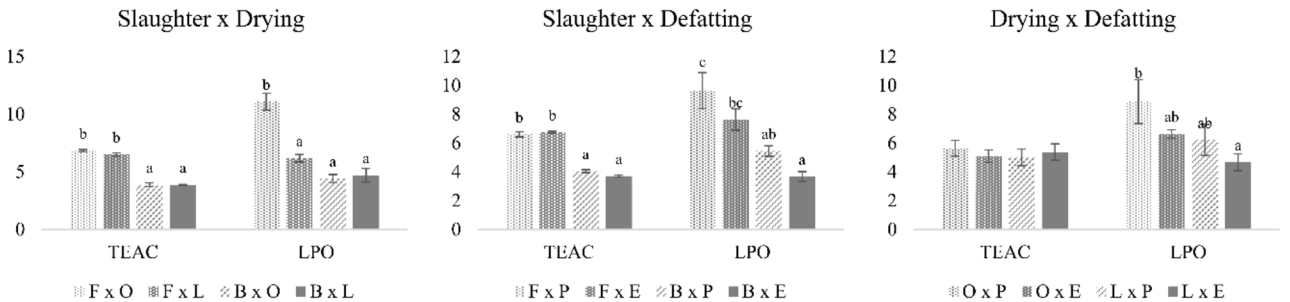

FIGURE 7 Representation of the interaction of pairs of processing methods, slaughtering (S), drying (D) and defatting (DF), of the analysis performed for Trolox equivalent antioxidant capacity (TEAC) and lipid peroxidation (LPO) levels of the different BSFL meals. TEAC values are expressed as mmol Trolox equivalents/kg of BSFL meals and LPO values are expressed as µmol of MDA/kg of BSFL meals. Values in the columns are presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) within each parameter evaluated. The abbreviations stand for the devitalization method (F = Freezing; B = Blanching); drying method (O = Oven drying; L = Lyophilization) and the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

the other hand, the use of oven for drying the insects increased lipid peroxidation, as can be seen in the S × D interaction, but only when the animals were slaughtered by freezing method, without found differences between the two drying methods in the blanching groups of insect meals. Regarding defatting methods of insects, levels of LPO were significantly higher when press was used instead of SFE, but only for FOP and BOP.

In general, results show that the processing techniques used to obtain BSFL meal, including slaughtering, drying and defatting, have influenced its final oxidative status. Therefore, as it was expected, freezing as slaughter method, as opposed to blanching, preserved their endogenous antioxidants, as observed in the higher TEAC values obtained for BSFL meals. It is known that thermal methods for insect processing affect significantly their antioxidant content, such as it has been observed for mulberry silkworm (*Bombyx mori* L.) when exposed to 90 °C water (Anuduang *et al.*, 2020). In that case, time was a decisive factor for the conservation of endogenous antioxidants and shorter exposure times to hot water resulted in higher total phenolic compounds and free radical scavenging capacity.

Notwithstanding, freezing as slaughter method benefits endogenous antioxidant preservation and the maintenance of final meal quality will be conditioned by the subsequent process. In this sense, during the second step of drying, once again, the use of thermal *versus* non-thermal procedures, such as oven or freeze-drying, is decisive. Our results showed that freeze-drying presented lower levels of thiobarbituric acid reactive substances, indicative of lipid oxidation, compared to oven-drying but only in those insect meals obtained by freezing process of slaughtering and not by blanching, in which no influence of drying was observed.

These results could be related to the ectotherms condition of BSFL and a slow freezing method of sacrifice would lead to a slowing down of the metabolism, but not to a total inhibition of enzymatic activity, as it has been described by several authors (Zhen *et al.*, 2020; Caligiani *et al.*, 2019; Larouche *et al.*, 2019; Leni *et al.*, 2019). Moreover, the slow freezing slaughter could also produce larger ice crystals that would induce damage in cell membrane, increasing the susceptibility to oxidation (Rusell and Gould, 2003). Then, during drying, the freeze drying method would keep the enzymatic activities that could contribute to the generation of oxidative damage diminished, while oven drying would imply a slow and progressive heating until reaching the final temperature of 65 °C, but during said heating an enzymatic activation would occur, which would lead to an increase in the appearance of oxidative damage as such observed in our results. The fact that this process is not observed when blanching is used as a sacrifice method could be due to different BSFL components that could be destroyed during heat treatment at 90 °C, inhibiting completely both the enzymes responsible for intervening in lipid oxidation, as well as other important components such as antioxidants as the aforementioned (Zhen *et al.*, 2020).

Hygiene and sanitary

Hygienic-sanitary quality of the prepared meals, as well as the raw insect, were investigated to give a better understanding of the overall effects of the treatments. The initial contamination of the BSFL was 5.92 log CFU/g for MAM and 5.32 log CFU/g for Enterobacteria and also sulphite reducing Clostridia and *Salmonella* spp. were present in the sample (Table 2). On MAM S × D and S × DF had significant effect, while on Enterobacteria S × D and D × DF had (Figure 8). As for the effect of sacrifice, blanching generally reduced more the microbial charge than the frozen method. This can be due to the heat effect (90 °C) of blanching which several groups already reported (Larouche *et al.*, 2019; Vandeweyer *et al.*, 2017). Examining the effect of drying, it is interesting to note that, in the case of MAM, the difference between the two drying methods was not significant. 65 °C drying temperature should be high enough to affect MAM viability, although Sadiq *et al.* (2016) investigated the heat resistance of several mesophilic aerobic bacteria strains and found that most of the examined species survived even 100-110 °C treatment for 30 min with little to no significant reduction in log CFU/ml. Lyophilization had similar effect on the MAM microbial load of the samples. This correlates with the findings of Bourdoux *et al.* (2018), who found that freeze-drying only lowered the mesophilic bacteria load by 0.60 log CFU/g. It was not the case for Enterobacteria, which showed an excessive sensibility to lyophilization in present experiment (complete elimination in all freeze-dried samples) and the values decreased significantly in most cases of the oven-dried meals also. This can be explained by the fact that the gram-negative bacteria are more affected by drying methods due to the structure of the cell surface (Miyamoto-Shinohara *et al.*, 2000; Pembrey *et al.*, 1999). Defatting method had a significant effect on MAM where was a considerate decrease in all the meals defatted by screw press. This can be explained by the high (136.0 ± 13.1 °C) temperature applied during the process. SFE treatment reduced

TABLE 2 Study of the hygienic-sanitary quality of BSFL meals; quantitative study of mesophilic aerobic microorganisms (MAM) and Enterobacteriaceae (both in CFU/g), and presence of *Clostridium perfringens* and *Salmonella* spp. in the samples

	MAM	Enterobacteria	*Salmonella*	*Clostridium*
Raw insect	5.92 ± 0.35d	5.32 ± 0.13c	+	+
FOP	1.61 ± 1.48b	0.73 ± 1.27b	–	–
FOE	3.29 ± 0.07c	2.17 ± 0.12b	+	–
FLP	1.4 ± 1.23ab	n.d.	–	–
FLE	3.53 ± 0.13c	n.d.	–	–
BOP	0.53 ± 0.92a	0.2 ± 0.35a	–	–
BOE	1.17 ± 1.02a	0.2 ± 0.35a	–	–
BLP	n.d.	n.d.	–	–
BLE	1.17 ± 1.02a	n.d.	–	–

* Values in the columns are presented as mean ± S.E. At the abbreviations, the first letter stands for the devitalization method (F = Freezing; B = Blanching); the second letter stands for the drying method (O = Oven drying; L = Lyophilization) and the third letter stands for the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

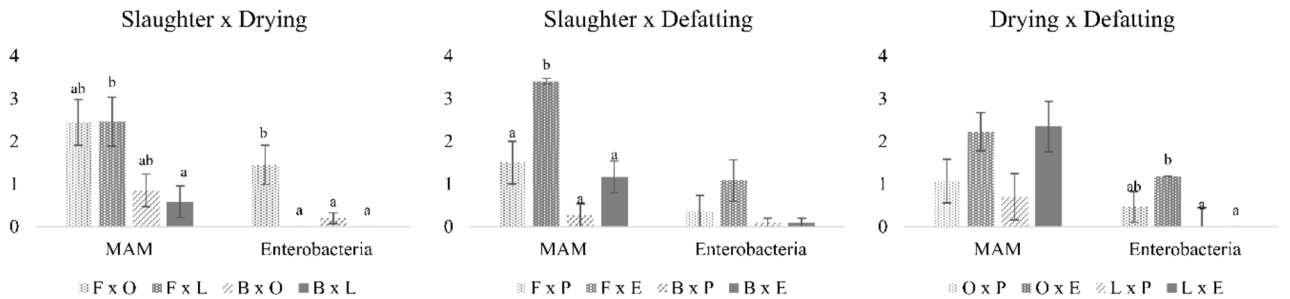

FIGURE 8 Representation of the interaction of pairs of processing methods, slaughtering (S), drying (D) and defatting (DF), of the analysis of the hygienic-sanitary quality of the different BSFL meals. Mesophilic aerobic microorganisms (MAM) and Enterobacteriaceae are expressed as CFU/g of BSFL meals. Values in the columns are presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) within each parameter evaluated. The abbreviations stand for the devitalization method (F = Freezing; B = Blanching); drying method (O = Oven drying; L = Lyophilization) and the defatting method (P = Screw pressing; E = Supercritical fluid extraction).

the microbial population less than screw press in both cases of MAM and Enterobacteria.

Bourdoux *et al.* (2018) found similar results, where mesophilic bacteria were only lowered by 0.11 log CFU/g, while Enterobacteria were reduced by 4.61 log CFU/g. The highest value for Enterobacteria was given by FOE (2.17 log CFU/g) which can be explained by the mild temperature treatment, reaching maximum 65 °C at the drying step and the limited sterilizing effect of the SFE defatting method. FOE was also the only processed meal which showed *Salmonella* spp. contamination, as *Salmonella* spp. needs 55-70 °C to significantly lower its charge depending on the species (Silva and Gibbs, 2012). As for the SFE, although Bourdoux *et al.* (2018) found that supercritical CO_2 drying at 80 bars, 40 °C and 150 minutes, reduced ~5 log CFU/g of three salmonella species, the official requirement is the absence of *Salmonell*a in 25 g sample, which was not fulfilled in the case of FOE. Sulfite-reducing clostridia (*Clostridium perfringens*) was successfully eliminated from all meals. *Clostridium* is highly resistant to extreme temperatures, it needs 70-80 °C to inactivation and 100-110 °C to the spore elimination (Byrne *et al.*, 2006). It tolerates well water scarcity and, being a gram-positive bacteria, freeze-drying as well. However, Dacal-Gutérrez *et al.* (2022) obtained a significant population decrease of a *Clostridium* species (*Clostridium botulinum*) by supercritical CO_2 treatment (at 10 MPa, 60 minutes), where the population decrease was 90% for 40 °C and 99.7% for 80 °C. Considering these findings, the most probable step of the elimination was the defatting process, where the screw press was above the tolerated temperature (136 °C ± 13.1°C) and the SFE circumstances were 45 MPa and 60 °C.

4 Conclusions and future perspectives

In this study the effect of successive processing steps, such as slaughtering, drying and defatting, was investigated on the proximate composition, *in vitro* digestibility, oxidative status and hygienic-sanitary properties of *Hermetia illucens* larvae. Although there are several research papers focusing on the separate effects of the processes, many of the combined effects still need further investigation as the resulted meal does have important changes in properties that are crucial for its further use as feed. It was found that the combined effect of the processing steps showed differences in proximate composition, more importantly in the protein and fat content of the meal. The *in vitro* digestibility only showed a significantly lower values when the samples were dried by lyophilization and defatted by SFE, independently from the slaughtering method. Regarding oxidative status, the freezing method of slaughter conserved a higher level of antioxidant defenses versus the blanching method, although only drying by lyophilization method ensured a lower level of lipid oxidation in this kind of sacrificing method. The applied processes resulted in a better hygienic quality compared to the raw insect in all the examined aspects, although hygienically speaking FOE was the least interesting treatment. The nutritional qualities of the prepared meals still need further *in vivo* experiments.

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