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Modulation of the lipolysis and subsequent antibacterial activity of the fat from black soldier fly (*Hermetia illucens*) by the combined selection of slaughtering, drying and defatting methods of the larvae

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ABSTRACT

The aim of this study was to evaluate the modulating effect of the processing of *Hermetia illucens* larvae on the antibacterial activity of the fat related to lipolysis to free fatty acids (FFAs), mainly as lauric acid. Blanching and freezing were compared for slaughtering, oven or freeze-drying for drying, and mechanical pressing or supercritical fluid for defatting. Freezing plus freeze-drying produced the highest FFAs content (21%), mainly as lauric acid (11%). In agreement, freezing plus freeze-drying was also the most effective method to obtain fats with higher antibacterial activity, regardless of the defatting method. The antibacterial activity was significantly more relevant for gram-positive bacteria, having a strain-dependent character. The most effective fat was bactericidal for *Listeria monocytogenes* and *Bacillus subtilis* and reduced *Staphylococcus aureus* growth. Therefore, it is possible to selectively modulate the antibacterial activity of the *H. illucens* fat due to lipolysis by the processing methods of larvae.

Industrial relevance: This study provides guidance to choose the best mode of processing of *H. illucens* larvae to selectively modulate the potential use of the lipid coproduct of this edible insect, either from the point of view of food use, which requires methods of processing to yield a high quality (low acidity), or from the point of view of antimicrobial applications, using methods of processing that selectively produce an antibacterial fat (high acidity). Furthermore, the best antibacterial activity demonstrated for fats of *H. illucens* of poor acid quality is an innovative approach for revalorization of such fats that would fail to meet the quality standard required for food applications.

1. Introduction

After the European approval of the edible species *Tenebrio molitor*, *Locusta migratoria, Acheta domesticus*, and *Alphitobius diaperinus* for human consumption in 2021 (European Commission, 2021a; European Commission, 2021b; European Commission, 2022; European Commission, 2023), other insect species are also under expectation, as *Hermetia illucens*, *Gryllodes sigillatus,* or *Apis mellifera* (Mancini et al., 2022). Additionally, most of these insect species are already authorized for feed production since 2017 for aquaculture, and since 2021 for poultry and pig feeding (van Huis, Rumpold, van der Fels-Klerx, & Tomberlin, 2021). Among these species, the larvae of *H. illucens* (black soldier fly larvae, BSFL) have gained great popularity in recent years due to its great potential as an alternative source of protein for food and feed, together with its great capacity for bioconversion of agri-food residues into high value products (Dörper, Veldkamp, & Dicke, 2021; Kaczor, Bulak, Proc-Pietrycha, Kirichenko-Babko, & Bieganowski, 2022; Mohan et al., 2022).

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Within the value products from BSFL, in addition to protein, fat also stands out. This is because the lipid fraction of BSFL can reach values in the range of 15–58%, mainly depending on substrate (Bellezza Oddon, Biasato, Resconi, & Gasco, 2022; Ewald et al., 2020; Suryati, Julaeha, Farabi, Ambarsari, & Hidayat, 2023; Wang & Shelomi, 2017). Additionally, this fat has an attractive and atypical fatty acid profile, since the medium-chain lauric acid is the major fatty acid of BSFL fat, reaching values closer to 50%. Palmitic acid, myristic acid, as well as oleic acid, and linoleic acid are also part of the fatty acid profile of BSFL (Cantero-Bahillo et al., 2022; Fornari et al., 2023; Franco et al., 2021). This makes BSFL fat quite similar to coconut oil or palm kernel oil (Nitbani, Tjitda, Nitti, Jumina, & Detha, 2022). Currently, these vegetable oils are the main sources to produce lauric acid-rich oils. The main interest on these oils is because lauric acid is a well-known fatty acid of interesting properties for food, feed, cosmetic, and pharmaceutical uses (Borrelli et al., 2021; Nitbani et al., 2022). Specifically, the strong antibacterial activity of lauric acid and its partial glycerides, is one of the most popular bioactivities, showing a broad antibacterial spectrum against a wide range of pathogens, including multidrug-resistant bacteria, linked to their ability to destabilize the bacterial cell membrane (Borrelli et al., 2021; Nitbani et al., 2022). However, specific studies about the antibacterial potential of BSFL fat, as alternative to the less sustainable vegetable oils, are still scarce (Dabbou et al., 2020; Lee, Yun, & Goo, 2022; Marusich, Mohamed, Afanasev, & Leonov, 2020).

Concerning the BSFL fat, it is important to note that the type of methodology used for the main processing steps of BSFL as slaughtering, drying, and final defatting, could affect the lipid composition of the derived fats. This is because since the initial process of slaughtering, an intense activity of lipase has been found in insects, being especially relevant in BSFL, which can result in an intense lipolysis of the lipids and a subsequent increase of free fatty acids (FFAs) and partial glycerides (Caligiani et al., 2019; Leong, Yap, & Kutty, 2022; Ravi et al., 2020). From the quality point of view of edible oils, this is a negative effect, since a high level of FFAs are conventionally related to poor quality and degradation of the fat, hence maximum acid values are usually limited for edible oils. Thus, thermal methods for slaughtering BSFL that inactivate the lipolytic enzymes, as the typical blanching used for many edible insects, have demonstrated to allow a proper quality and integrity of BSFL fat, in contrast with non-thermal methods, as freezing, which is a method that has demonstrated to keep the lipases active and that is also frequently used for edible insects (Leni, Caligiani, & Sforza, 2019; Leong et al., 2022; Ravi et al., 2020). However, from the antibacterial point of view of BSFL fat, we propose that those processing conditions that might favor the lipolysis of the fat to FFAs, mainly as lauric acid, would be of interest. However, clear conclusions about what factors modulate the magnitude of the lipolytic activity during slaughtering, and without compromising the rest of quality parameters of the fats and meals, remains unclear. Additionally, concerning the subsequent steps after slaughtering, as drying and defatting typically are, scarce information has been found on the impact of these processes on lipolysis of the BSFL fat to yield free lauric acid. This consideration is relevant, considering that non-thermal methods are being also used for such stages during the processing of BSFL, such as freeze-drying for drying or pressing and supercritical fluids for defatting (Hernández-Álvarez, Mondor, Piña-Domínguez, Sánchez-Velázquez, & Melgar Lalanne, 2021; Ojha, Bußler, Psarianos, Rossi, & Schlüter, 2021; van Huis, 2019). Nevertheless, thermal methods of drying, as oven-drying, are also among the most typically used for BSFL (Hernández-Álvarez et al., 2021; Ojha et al., 2021; van Huis, 2019). Therefore, the consideration of the three technological processes together (slaughtering, drying, and defatting), and the study of the interrelation between them as successive operations of a final process, should be approached to propose the best combination of processes that most efficiently would allow to produce the lipid coproduct with a high content on free lauric acid and hence, an expected antibacterial activity; or, on the contrary, to produce the lipid coproduct with the best lipid quality with the lowest acidity.

Therefore, the main aim of this study was the evaluation of the impact and interrelation of different slaughter, drying, and defatting conditions of BSFL on the FFAs content and antibacterial activity of the obtained fat. To perform this study, typical methods and conditions used for edible insect processing (Hernández-Álvarez et al., 2021; Ojha et al., 2021; van Huis, 2019), mainly those non-thermal and thermal methods, were compared. Thus, blanching and freezing were compared as slaughtering methods, each of them were followed by oven or freeze-drying as drying methods, and by mechanical pressing or supercritical CO_2 as defatting methods.

2. Materials and methods

2.1. Slaughtering of larvae

Larvae of BSF were reared by Entomo Agroindustrial (Cehegin, Murcia, Spain) using a typical substrate based on laying hen feed. The larvae were slaughtered by blanching or freezing, with each procedure being performed in duplicate (8 kg per replicate). Prior to slaughtering, the larvae were sifted and washed by immersing them in a basket in cool water and then drained. For blanching, the larvae were immersed in water at 90 °C at a ratio of 1:10 (*w/v*) of sample to water for 40 s. After that, the larvae were immersed in cool water and drained. For freezing, the larvae were frozen at −20 °C for 24 h. After each slaughtering procedure, each batch was divided in half to be dried by oven or freeze-drying.

2.2. Drying of larvae

Oven drying without internal ventilation was carried out at 65 °C for 24 h in a conduction oven in trays with a maximum thickness of larvae layer ≤1 cm. Freeze-drying was performed for 4 days using a 3-tray freeze drier (LyoBeta 15, Telstar, Terrassa, Spain) with a program of −20 °C for 2 h, followed by a gradual increase from −20 to 20 °C, and then maintained at 20 °C for the remainder of the time, with the condenser at −81 °C. After each drying procedure, each batch was divided in half to be defatted by either mechanical pressing or supercritical fluid extraction (SFE). The dried samples were stored at room temperature and defatted as soon as possible (within a period of 1–3 days of storage since drying).

2.3. Defatting of samples

For mechanical defatting, 450 g of dried larvae were extracted using a screw-press expeller (InVIA, Barcelona, Spain) with a heating jacket to allow the drainage of the oil. The larvae at room temperature, without previous preheating, were added to the press. The press was pre-heated at the minimum temperature (136.0 ± 13.1 °C), and the temperature of the press head was continuously monitored during pressing, reaching a mean maximum value of 140.4 ± 10 °C and a mean minimum value of 126.6 ± 11.9 °C, depending on the batches. The collected crude fat was centrifuged (Multifuge 3SR+ centrifuge, Thermo Scientific, Waltham, MA, USA) at 3400 ×*g* for 10 min to remove co-extracted solids.

Supercritical CO_2 defatting was performed by using a supercritical CO_2 extraction equipment (Model Thar SF2000, Thar Technology, Pittsburgh, PA, USA) and following the optimized conditions previously performed by Cantero-Bahillo et al. (2022) and scaled-up by Fornari et al. (2023). Prior to extraction, 500 g of dried larvae were ground in a knife mill (Grindomix GM 200, Retsch GmbH, Haan, Germany). The extraction cell (1350 cm^3) was loaded with 450 g of sample. The defatting was performed at 450 bars, 60 °C, and CO_2 flows of 130 g/min for 240 min.

2.4. Characterization of free fatty acids

FFAs were analyzed by GC–MS-FID according to Herrera, Navarro

del Hierro, Fornari, Reglero, and Martin (2019), previous derivatization by *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). This allowed to individually quantify the FFAs present in the obtained fat samples. Briefly, fat was mixed with BSTFA at 20 mg/mL and heated at 75 °C for 1 h, with shaking every 15 min. After that, samples were cooled at room temperature for 5 min and hexane was added to reach a final concentration of 10 mg/mL. Subsequently, samples were analyzed by a GC–MS (Agilent 7890 A, Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler (G4513A) a split/splitless injector, a flame-ionization detector and a triple-axis mass spectrometer detector (5975C). Lipid compounds were separated by using a HP-5MS column (30 m length x 0.25 mm internal diameter x 0.25 μm film thickness). Helium was used as carrier gas at a flow of 2 mL/min. Injector temperature was 260 °C while the mass spectrometer ion source and interface temperatures were 230 °C and 280 °C, respectively. Injection of samples (1 μL) were carried out in splitless mode. The analysis started holding the oven at 50 °C for 3 min and then increased at 15 °C/min until 310 °C, then held for 25 min. The mass spectra were obtained by electronic impact at 70 eV. The scan rate was 1.6 scans/s at a mass range of 30–700 amu. Identification of lipid compounds was performed by NIST MS Data library. For quantification of FFAs, calibration curves were obtained from the standards (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) of lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), linoleic acid (C18:2), and oleic acid (C18:1), which were also previously derivatized following the same procedure described for the samples.

2.5. Bacterial strains, growth media, and culture conditions

Antibacterial activity of BSFL fat was evaluated in six representative foodborne pathogen bacteria, gram-negative: *Escherichia coli* (*E. coli*) 25922 American Type Culture Collection (ATCC), *Salmonella enterica* (*S. enterica*) 14028 ATCC, *Pseudomonas aeruginosa* (*P. aeruginosa*) 9027 ATCC; and gram-positive: *Staphylococcus aureus* (*S. aureus*) 25923 ATCC, *Listeria monocytogenes* (*L. monocytogenes*) 13932 ATCC, and *Bacillus subtilis* (*B. subtilis*) 11774 ATCC. All bacteria strains were stored at −80 °C in Brucella Broth (BB) (Becton, Dickinson, & Co, Madrid, Spain) plus with 20% glycerol (*w*/w). The agar-plating medium consisted of Müeller-Hinton agar supplemented with 5% defibrinated sheep blood (MHB) (Becton, Dickinson, & Co), and liquid growth medium consisted of BB. Bacteria cultures were prepared as follows: the frozen stored strains were reactivated by inoculation in MHB and incubated on an orbital shaker (Infors HT, Biogen Científica, Madrid, Spain) under aerobic conditions at 37 °C for 24 h. After that, isolated colonies were inoculated into 10 mL of BB and incubated in the conditions described above following stirring (150 rpm) for 24 h until late exponential phase and used as experimental inoculum. These bacterial inoculum cultures $\sim 1 \times 10^8$ colony forming units (CFU/mL) were used for the antibacterial experimental assays.

2.6. Antibacterial activity

The antibacterial activity of BSFL fat was evaluated following the procedure described by Silvan et al. (2020). BSFL fat was solubilized in 10% (*v*/v) Tween-80 (2.5:1; v/v sample to solvent). Briefly, 1 mL of BSFL fat solution was transferred into different flasks containing 4 mL of BB (14% final concentration of fat, v/v). Then, bacterial inoculum (100 μL of $\sim 1 \times 10^8$ CFU/mL) was inoculated into the flasks under aseptic conditions. The culture was incubated under stirring (150 rpm) in aerobic conditions at 37 °C for 24 h. Bacterial growth controls were prepared by transferring 1 mL of sterile water to 4 mL of BB and 100 μL of bacterial inoculum. After incubation, serial decimal dilutions of mixtures were prepared in saline solution (0.9% NaCl) and plated (20 μL) onto fresh MHB agar. Plates were incubated in the conditions described above and the number of CFU was assessed after 24 h. All experiments were carried out in triplicate ($n = 3$) and results were expressed as CFU/mL.

2.7. Minimal inhibitory concentration

Minimal Inhibitory Concentration (MIC) of the samples processed by freezing, freeze-drying, and SFE, the most active fat against the gram-positive bacteria, was determined following the procedure described above. The fat solution was diluted in BB to obtain the desired final concentrations. MIC was defined as the lowest amount of fat that provoked a significant ($p < 0.05$) decrease in viability compared with the control growth after 24 h of treatment, expressed in Colony Forming Units (CFU)/mL (Silván et al., 2013). The dilution intervals for determination of MIC ranged from 12% to 0.09% (*v*/v).

2.8. Verification of antibacterial activity using pure standard of lauric acid and trilaurin

Antibacterial properties of pure standard of lauric acid were investigated against gram-positive bacteria (*S. aureus*, *L. monocytogenes*, and *B. subtilis*) following the procedures described above. Additionally, trilaurin (Sigma-Aldrich) was also tested as control for comparative purposes with the typical lipid form of lauric acid esterified as triglyceride. All analyses were carried out using the highest possible concentration equivalent to the present in the most active fat sample dilutable in the solvent used (sample first diluted in ethyl acetate), then brought to a final concentration in BB of 3 mg/mL (lauric acid) and 1.8 mg/mL (trilaurin). The original concentration in the most active fat was 19.9 mg/mL for lauric acid and 37.1 mg/mL for trilaurin.

2.9. Statistical analysis

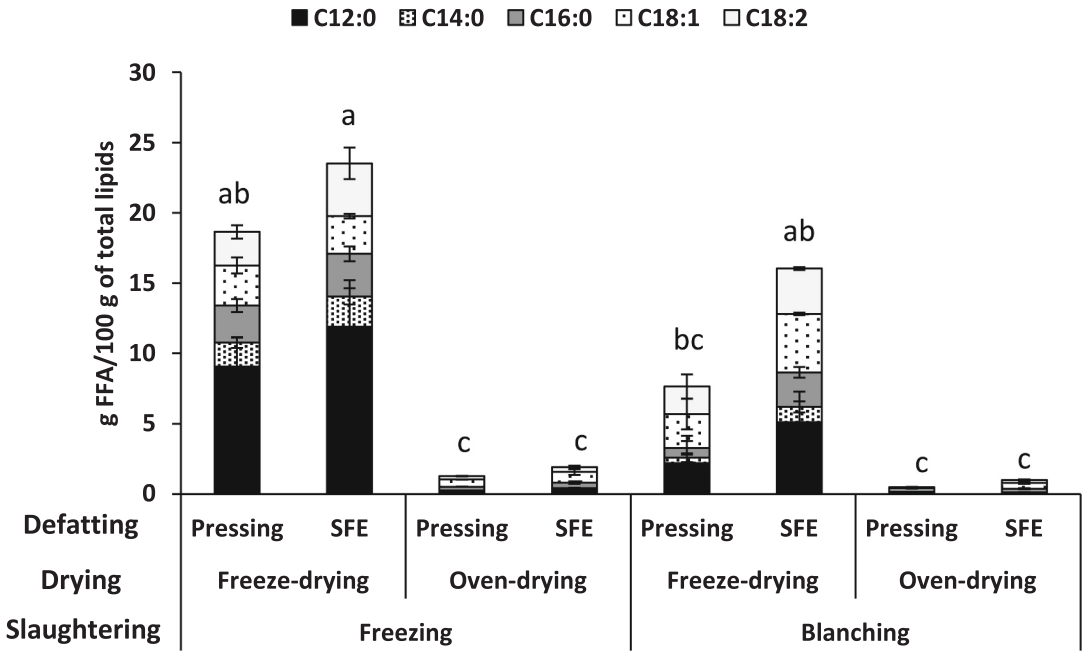
The effect of considered factors (slaughter, drying, and defatting procedures) and their respective interactions was evaluated by a three-way analysis of variance using the general linear model procedure of the SPSS 26.0 statistical package (SPSS Inc., Chicago, IL, USA). When the effect of any of the factors was significant ($p \leq 0.05$), differences between groups were analyzed by using the post-hoc Tukey's tests. Statistical analyses of antibacterial activity of each extract respect control group were performed by *t*-test ($p \leq 0.05$) (SPSS 26.0). Pearson correlation tests were conducted for additional analyses.

3. Results and discussion

3.1. Effect of the processing of BSFL on the free fatty acid content of the fat

The combination of different modes of slaughtering and drying of the BSFL produced dried larvae with different fat content. Thus, before defatting, the fat content was in the range of 18–29%, depending on the combined methods of slaughtering and drying (Hurtado-Ribeira et al., 2023). Those fats showed, in general, a similar global fatty acid profile, regardless of the treatments, with an average lauric acid content of 50.3 ± 2.1%, followed by palmitic acid (13.2 ± 0.9%), myristic acid (10.3 ± 0.3%), oleic acid (8.6 ± 0.6%) and linoleic acid (7.2 ± 0.6%), together with minor levels of other fatty acids as linolenic acid, stearic acid or palmitoleic acid.

After the processing of the larvae, and in order to evaluate its impact on the lipolysis of the fat, the FFAs content and FFA profile of the fat samples from BSFL derived from different modes of slaughtering, drying, and defatting was analyzed. As it can be observed in Fig. 1, the different modes of slaughtering and drying of BSFL produced fats with different content of total FFAs. Thus, in general, slaughtering by freezing and drying by freeze-drying caused, as independent factors, the highest production of FFAs. As expected, this FFAs profile mainly consisted of lauric acid (around 50% of total FFAs), since this was, in general, the major fatty acid of the whole BSFL fat. The content of the rest of FFAs



p	Total FFAs	C12:0	C14:0	C16:0	C18:1	C18:2
p (slaughtering)	0.013	0.004	0.003	0.003	0.688	0.283
p (drying)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
p (defatting)	0.051	0.124	0.086	0.007	0.055	0.027
p (slaughtering x drying)	0.028	0.005	0.005	0.010	0.104	0.546
p (slaughtering x defatting)	0.606	0.998	0.708	0.076	0.069	0.924
p (drying x defatting)	0.091	0.145	0.110	0.024	0.221	0.052
p (slaughtering x drying x defatting)	0.577	0.960	0.672	0.076	0.063	0.938

Fig. 1. Free fatty acids (FFAs) composition of BSFL fat after different modes of slaughtering, drying, and defatting. Different letters between the treatments mean significant differences in the total content of FFAs. The results (*p* values) of the statistical analysis of the effect of the three factors and their respective interactions on the FFAs profile are detailed.

was minor (in the range of 1.7–3.8%, depending on the fatty acid and treatment). Nevertheless, the release of palmitic acid, and myristic acid followed a similar pattern as lauric acid with the treatments ($p < 0.05$ for slaughtering and drying, according to Fig. 1). In case of oleic acid and linoleic acid, only freeze-drying caused a significant increase ($p < 0.05$), regardless of the slaughtering method ($p > 0.05$). Additionally, a significant interaction effect of both factors of slaughtering and drying on the total FFAs content was observed. Therefore, the slaughtering by freezing combined with drying by freeze-drying led to the most remarkable content of total FFAs (mean value of 21% of FFAs), which mainly consisted of lauric acid. On the contrary, regardless of the method of slaughtering, the larvae subjected to oven-drying were the ones that showed the lowest FFAs content (mean value of 1% FFAs). Therefore, the obtained results would confirm that lipolysis phenomenon took place for those samples that presented a high FFAs content, which were those samples derived from non-thermal methods of slaughtering and drying (freezing and freeze-drying). According to Leni et al. (2019), during the killing of BSFL by freezing, intense energetic metabolisms are activated, which includes lipolysis of glycerides by endogenous lipases. On the contrary, by blanching, the quick dying and the inactivation of enzymes would not allow such metabolic reactions and lower related metabolites were detected by Leni et al. (2019). This effect of enzyme inactivation due to the thermal method of blanching would explain the lower level of FFAs observed in the current study in the case of blanching followed by freeze-drying, compared to freezing followed by freeze-drying (Fig. 1). Thus, the short heat treatment of blanching could have partially inactivated the lipolytic enzymes, leading to slightly less lipolysis compared to slaughtering by freezing.

In agreement with our results, different studies have described the intense lipolysis of BSFL fat when non-thermal methods of slaughtering are used. As example, Leong et al. (2022) and Ravi et al. (2020) also recently indicated that the thermal slaughtering of BSFL would be necessary to avoid extensive lipolysis of the fat. Nevertheless, concise conclusions about the factors that modulate the magnitude of such lipolysis remains unclear. Additionally, the current study reveals that either the method of slaughtering, but also the drying method, can affect such lipolysis phenomenon, either individually, but also in interaction. This is a relevant finding since both processes are followed one after the other in the production of BSFL meal and they cannot be considered isolated. Finally, concerning the step of defatting of BSFL, a lack of significant effect on the content of total FFAs was observed (Fig. 1), although a trend to higher FFAs content for SFE compared to mechanical pressing was observed. Additionally, a lack of interaction of defatting with slaughtering or drying modes was observed. Thus, the effect of the defatting mode of BSFL on the lipolysis of the obtained fat could be considered negligible. Therefore, from the quality point of view, the best

method of processing of BSFL for keeping the integrity of the obtained fat is the combination of slaughtering by blanching with drying by oven-drying, regardless of the mode of defatting. On the contrary, for the rest of treatments, a subsequent refining process of the derived fat to remove the FFAs would be necessary in case that a high quality of the fat would be desired.

3.2. Effect of the processing of BSFL on the antibacterial activity of the fat

Despite the desirable low FFAs content from the quality point of view, those technological processes of BSFL that produced, in contrast, the highest lipolysis to free lauric acid would be preferred in case that a potential antibacterial oil would be desired to be obtained from BSFL. The potential of generating this product would be a way for revalorizing the fat from BSFL that is currently being obtained as a coproduct during the protein meal production, but which does not have a clear commercial use at present compared to the major interest on the protein meal. This is especially interesting when a clear standardized or preferred method of processing of BSFL has not yet been recommended or stablished, so nowadays thermal or non-thermal methods, as those used in the present study, are indistinctly being used and combined. Therefore, to confirm the hypothesis that specific methods of processing of BSFL may lead to an antimicrobial fat, we studied the effect of processing of BSFL on the antibacterial activity of the different obtained samples of fat against several foodborne pathogens. As shown in Table 1, the antibacterial activity of BSFL fat was significantly more relevant for gram-positive bacteria than for gram-negative. In fact, only the fat obtained after slaughtering by freezing, drying by freeze-drying, and defatting by SFE, with the highest content of total FFAs (Fig. 1), showed significant ($p < 0.05$) antibacterial activity against the gram-negative *P. aeruginosa* strain, reducing bacterial growth 1.42 log CFU/mL. In contrast, up to four different fats showed antibacterial activity against gram-positive bacteria and caused a greater degree of growth inhibition. Again, the fat obtained after slaughtering by freezing, drying by freeze-drying, and defatting by SFE was the most effective one, being bactericidal (< 1.48 log CFU/mL) for L. *monocytogenes* and *B. subtilis* and reducing the growth of *S. aureus* by 6.7 log CFU/mL.

The antibacterial capacity of fats was directly related to the concentration of lauric acid in the sample and was ordered as follow, in general: freezing/freeze-drying/SFE > freezing/freeze-drying/pressing > blanching/freeze-drying/SFE > blanching/freeze-drying/pressing. In any case, the most effective combination of factors to obtain fats with higher antibacterial activity was slaughtering by freezing and drying by freeze-drying, regardless of the method of defatting. This combination was also associated with the highest degree of lipolysis and therefore with a higher proportion of FFAs (Fig. 1). On the other hand, drying using oven-drying resulted in the least antibacterial fats, also presenting the lowest FFAs concentration. This relationship between the antibacterial activity and the FFAs content of the BSFL fats was effectively confirmed by evidencing a strong significant negative correlation between both variables, for both *S. aureus* and L. *monocytogenes*, as shown in Fig. 2a. Since lauric acid was the major FFAs of the samples, such correlation was also strongly significant for the antibacterial activity and the lauric acid content of the samples (Fig. 2b). Therefore, according to the response shown in Fig. 2, it seems that, when BSFL fat samples reach values >10% or > 20% of total FFAs, may cause an antibacterial activity closer to 50% for L. *monocytogenes* or *S. aureus*, respectively. We did not perform the correlation study for *B. subtilis*, since the CFU was under the detection limit (<1.48 $\log_{10}$ CFU/mL) for the four treatments that showed an antibacterial effect (Table 1). Furthermore, for other minor FFAs different to lauric acid, significant correlations were also observed with the antibacterial activity ($p < 0.05$), such as myristic acid ($r = -0.895$ for *S. aureus* and $r = -0.973$ for *L. monocytogenes*), palmitic acid ($r = -0.835$ for *S. aureus* and $r = -0.980$ for *L. monocytogenes*), or oleic acid ($r = -0.901$ for *S. aureus* and $r = -0.914$ for *L. monocytogenes*). Therefore, despite that these FFAs were present at minor concentrations during the bacteria culture assays, they may also contribute to the global antibacterial activity of the BSFL fats, especially for the most hydrolyzed samples that showed the major level of such minor FFAs, which were those mainly derived from freeze-drying (Fig. 1).

Since the fat from freezing, freeze-drying and SFE was the sample with the highest antibacterial activity, its minimum inhibitory concentration (MIC) against gram-positive bacteria was determined (Table 2). MIC was different for each strain but showed the high antibacterial capacity of the fat. L. *monocytogenes*, the least sensitive strain, had a MIC of 1.5% of fat, nine times lower than the initial concentration (14%), while the MIC of *S. aureus* (0.37%) and *B. subtilis* (0.18%) were 38 and 78 times lower, respectively, than the initial concentration. Although MIC values depend on a number of variables such as experimental conditions, type of solvent, or analytical method (Yoon, Jackman, Valle-González, & Cho, 2018), the fat from BSFL has been shown to be highly effective against a large number of microorganisms (Dabbou et al., 2020; Lee et al., 2022; Marusich et al., 2020). Previous studies have shown that medium-chain fatty acids are usually more active against gram-positive than against gram-negative bacteria (Yoon et al., 2018). Gram-positive bacteria have a cell wall formed by a thick peptidoglycan layer, while gram-negative bacteria also have an outer membrane composed of lipoproteins, lipopolysaccharides, and phospholipids that hinders the entry of FFAs into the cell (Saviane et al., 2021). Among FFAs, lauric acid has been shown to be a potent inhibitor of the growth of several gram-positive bacteria, such as *S. aureus* (Kim, Lee, Park, Kim, & Lee, 2022) and L. *monocytogenes* (Zhou, Velliou, & Hong, 2020), in

Table 1
Antibacterial activity of *Hermetia illucens* fats (14%), obtained by diferent processes of slaughtering, drying and defatting, on the viable counts of gram-negative and gram-positive bacteria after 24 h of treatment. Results are expressed as log CFU/mL ± SD ($n = 3$).

Bacteria	Control growth	Freezing				Blanching			
		Freeze drying		Oven drying		Freeze drying		Oven drying	
		Pressing	SFE	Pressing	SFE	Pressing	SFE	Pressing	SFE
Gram-negative									
Escherichia coli	9.10 ± 0.30	9.51 ± 0.07	8.49 ± 0.20	10.23 ± 0.09	10.10 ± 0.05	10.26 ± 0.05	9.25 ± 0.10	9.45 ± 0.05	10.15 ± 0.03
Salmonella spp.	10.04 ± 0.07	9.58 ± 0.17	9.62 ± 0.05	10.40 ± 0.03	10.34 ± 0.03	10.33 ± 0.03	10.40 ± 0.05	10.25 ± 0.06	10.20 ± 0.06
Pseudomonas aeruginosa	10.05 ± 0.04	10.23 ± 0.06	8.63 ± 0.09*	10.52 ± 0.02	10.64 ± 0.03	10.70 ± 0.03	10.29 ± 0.06	10.68 ± 0.06	10.50 ± 0.04
Gram positive									
Staphylococcus aureus	9.27 ± 0.10	6.75 ± 0.18*	2.58 ± 0.06*	9.82 ± 0.05	9.38 ± 0.04	6.69 ± 0.23*	7.30 ± 0.05*	9.81 ± 0.09	9.50 ± 0.02
Listeria monocytogenes	9.37 ± 0.10	4.36 ± 0.10*	< 1.48*	9.36 ± 0.04	8.84 ± 0.11	8.96 ± 0.08	4.34 ± 0.05*	9.32 ± 0.02	9.29 ± 0.08
Bacillus subtilis	8.79 ± 0.10	< 1.48*	< 1.48*	9.79 ± 0.02	8.97 ± 0.05	< 1.48*	< 1.48*	9.90 ± 0.03	9.12 ± 0.02

Bactericidal effect is expressed as "< 1.48". CFU detection limit was 1.48 log CFU/mL (30 CFU per plate).
Control growth is the bacterial growth in BB medium without *Hermetia illucens* fat.
* Values marked with asterisk indicates significant differences compared to the control growth by *t*-test ($p < 0.05$).

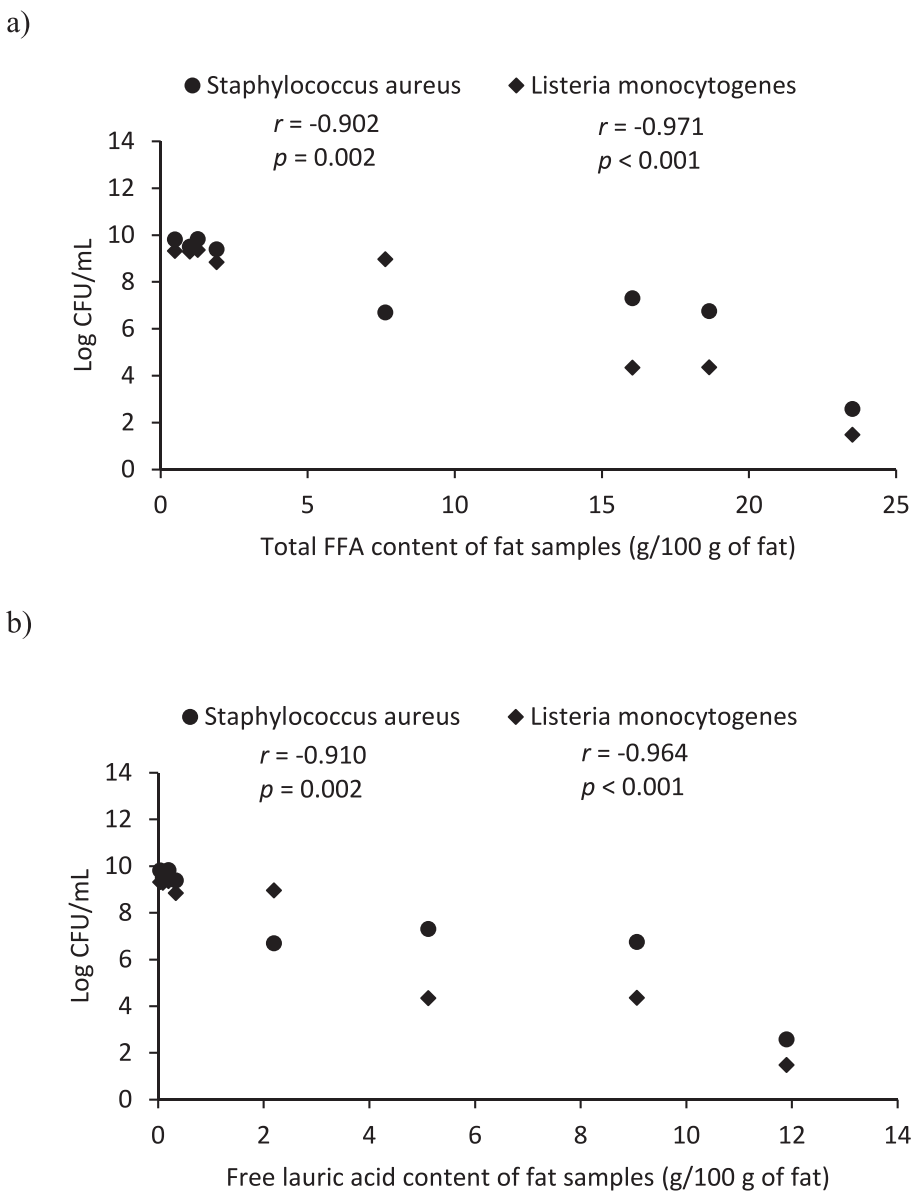


Fig. 2. Correlation between antibacterial activity and content of total FFAs (a) or free lauric acid (b) of the fat samples of BSFL. The Pearson test coefficients and significance values are shown.

Table 2
Determination of Minimal Inhibitory Concentration (MIC) of BSFL fat after freezing, freeze-drying and SFE against gram positive bacteria after 24 h of treatment. Results are expressed as log CFU/mL ± SD ($n = 3$).

Bacteria	Control growth	12%	6%	3%	1.5%	0.75%	0.37%	0.18%	0.09%
Staphylococcus aureus	9.56 ± 0.12	2.56 ± 0.13*	4.76 ± 0.07*	6.87 ± 0.07*	7.69 ± 0.17*	7.53 ± 0.03*	8.29 ± 0.11*	9.39 ± 0.11	9.42 ± 0.08
Listeria monocytogenes	8.68 ± 0.10	< 1.48*	< 1.48*	3.23 ± 0.07*	6.65 ± 0.08*	8.37 ± 0.03	8.57 ± 0.06	8.62 ± 0.03	8.63 ± 0.04
Bacillus subtilis	9.07 ± 0.04	< 1.48*	< 1.48*	< 1.48*	< 1.48*	4.57 ± 0.08*	5.06 ± 0.10*	7.61 ± 0.12*	8.91 ± 0.05

Bactericidal effect is expressed as "< 1.48". CFU detection limit was 1.48 log CFU/mL (30 CFU per plate).
Control growth is the bacterial growth in BB medium without *Hermetia illucens* fat.
* Values marked with asterisk indicates significant differences compared to the control growth by *t*-test ($p < 0.05$).

agreement with the observed results in the present study. The main target of lauric acid is the cell membrane. Due to its amphipathic properties, lauric acid interacts with the membrane creating pores of variable size that, if they remain in time, cause cell death (Yoon et al., 2018). Additionally, the antibacterial activity of other FFAs, as those found at minor proportions in BSFL fats, has been also evidenced by other authors (Kim et al., 2022; Marusich et al., 2020). As example, Kim et al. (2022) showed that lauric acid, but also myristic acid, were active fatty acids against *S. aureus*, and a synergistic effect between both fatty acids was suggested. Furthermore, it should be remarked that partial glycerides also produced during the lipolysis, as lauric acid monoglyceride derivative (monolaurin), are also known for a high antibacterial activity, even higher compared to that obtained for lauric acid (Borrelli et al., 2021). Therefore, the contribution of this lipid form to

the whole antibacterial activity of the hydrolysed BSFL fats should be also considered.

In the present study, we mainly focused on the free form of lauric acid, in order to simultaneously show the negative impact of the technological processing of BSFL on the quality of the fats due to release of FFAs, as well as the positive impact on the antibacterial activity due to such FFAs, mainly as lauric acid. Therefore, in order to delve into the direct impact of lauric acid on the antibacterial activity, we directly evaluated its effectiveness on gram-positive bacteria (Table 3). Additionally, for comparative reasons, we evaluated the role of trilaurin in the observed behaviour, which is the most common form in which lauric acid is found in the fat from BSFL in absence of lipolytic conditions. Furthermore, both lauric forms were also tested combined, at equivalent proportions close as those found in the most active fat sample, in order to test a potential interaction effect of both major lipid forms of lauric acid. The results obtained confirmed that lauric acid was bactericidal in all cases, both by itself and mixed with trilaurin, while trilaurin itself had no significant effect on the growth of L. *monocytogenes* and *B. subtilis*. Similarly, Kabara, Swieczkowski, Conley, and Truant (1972) also evidenced the antibacterial effect of lauric acid for up to 10 susceptible microorganisms, while trilaurin did not show inhibitory activity at the tested concentrations. Therefore, since triglycerides are the major lipid forms of the least active samples of BSFL (those showing minor FFAs content), this would agree with the lack of antibacterial activity observed for these BSFL fat samples, which were those mainly derived from oven-drying (Fig. 1). Nevertheless, it should be remarked that trilaurin was shown to be bactericidal for *S. aureus* (Table 3). This behaviour is consistent with the fact that many strains of *S. aureus* are able to produce and secrete into the extracellular medium a potent lipase which belongs to the glycerol ester hydrolases and which is an important virulence factor of this pathogen (Jaeger et al., 1994). This lipase will contribute to enrich the media with free lauric acid and other partial glycerides with strong antibacterial activity, such as monolaurin (Borrelli et al., 2021).

Therefore, according to all these findings, and considering the remarkable results of the antibacterial activity of the BSFL fat samples that were simultaneously those of worst quality from the acid value point of view, further studies would be of interest in order to stablish whether other parameters of lipid quality would be also affected, such as lipid oxidation, physicochemical properties, nutritional value or organoleptic attributes, since free acidity might affect these parameters. This would provide a more complete information of the entire potential of this BSFL fat in case that not only the antibacterial activity of this fat is desired. Concerning lipid oxidation, we recently evidenced, for the same samples studied in the current study, that the oxidative quality of the most antibacterial sample (freezing, freeze-drying, and SFE) was comparable to the rest of treatments, and its stability was even superior to those derived from slaughtering by blanching (Hurtado-Ribeira et al., 2023), which demonstrates an additional positive attribute of quality for this antibacterial fat. Concerning the nutritional effect of a partially hydrolyzed fat of BSFL, it is interesting to remark that the high content of FFA of an oil would not be an issue in case of its use in animal feeding, since this fat could be considered similar to the known as “acid oils” or “fatty acid distillates”, which are oils rich in FFA derived from the refining of edible vegetable oils (Varona et al., 2021a; Varona et al., 2021b). These oils are allowed in animal feeding as alternative to conventional oils to increase the dietary energy of feeds, due to lower cost and suitable nutritional performance (Varona et al., 2021a, 2021b). In addition to this potential use for high acidity BSFL fats, the demonstrated antibacterial activity in the present study, would give an extra value to this product for being used in animal feeding, due to the current interest on the development of additives that replace the use of antimicrobials in the diets. In fact, this is the case of the current claimed strategy of using medium-chain fatty acids in animal feeding, being the forms of lauric acid within the most popular (Borrelli et al., 2021; Jackman, Boyd, & Elrod, 2020; Rebucci et al., 2021; Rolinec et al., 2020; Suryati et al., 2023).

Table 3
Antibacterial activity of lauric acid and trilaurin on the viable counts of gram-positive bacteria after 24 h of treatment. Results are expressed as log CFU/mL ± SD (n = 3).

Samples	Control growth	Lauric acid	Trilaurin	Lauric acid + Trilaurin
Staphylococcus aureus	9.44 ± 0.05	< 1.48*	< 1.48*	< 1.48*
Listeria monocytogenes	8.52 ± 0.04	< 1.48*	7.67 ± 0.02	< 1.48*
Bacillus subtilis	8.93 ± 0.10	< 1.48*	8.80 ± 0.12	< 1.48*

Bactericidal effect is expressed as “< 1.48”. CFU detection limit was 1.48 log CFU/mL (30 CFU per plate).

* Values marked with asterisk indicates significant differences compared to the control growth by *t*-test ($p < 0.05$). Control growth is the bacterial growth in BB medium without lauric acid or trilaurin.

4. Conclusions

The methods of slaughtering and drying of BSFL affect the quality of the derived fat from the FFAs point of view. Thus, slaughtering by freezing and drying by freeze-drying cause, as independent factors, the highest lipolysis of the fat to FFAs, mainly as lauric acid. Additionally, due to the interaction of both steps, the slaughtering by freezing combined with drying by freeze-drying produces the BSFL fat with worst quality. In contrast, regardless of the method of slaughtering, the drying by oven-drying is the best method to keep the integrity of the fat. The final step of defatting of BSFL does not affect the lipolysis of the fat. Therefore, from the quality point of view, the best method of processing of BSFL is the combination of slaughtering by blanching with drying by oven-drying, regardless of the mode of defatting. In contrast, from the antibacterial point of view, those technological processes that produce the highest lipolysis are preferred, that is, slaughtering by freezing, drying by freeze-drying, and defatting by SFE. This specific mode of processing of BSFL produces a fat with a relevant antibacterial activity against gram-positive bacteria. Lauric acid seems to be the FFA mainly involved in the antibacterial activity of the BSFL fat.

Therefore, considering that standardized or preferred methods of processing of BSFL have not been still clearly recommended or stablished, these findings provide guidance to choose the best mode of processing of BSFL for fully utilizing the potential value that the obtained lipid coproduct of BSFL can offer, either from the point of view of food use, which requires methods of processing to yield a high quality (low acidity), or from the point of view of bioactivity applications, using methods of processing that produce an antibacterial fat (high acidity). This bioactive fat could be used for different applications in animal feeding, foods, pharmaceutics, or cosmetics. Nevertheless, concerning the method of production, the economic cost of the combination of freezing, freeze-drying and SFE should be considered, especially the cost of freeze-drying and SFE. Thus, only in case of a reaching a profitable revalorization of the fat or demonstrating additional advantages for the main obtained product as the protein meal is, the cost of this process would be worth. In any case, regardless of the mode of processing, the best antibacterial activity demonstrated for fats of poor acidity quality can be remarked as an interesting approach for revalorization of such BSFL fats that fail to meet the quality standard required for food applications.

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CRediT authorship contribution statement

Raúl Hurtado-Ribeira: Investigation, Data curation, Writing – review & editing, Visualization. **Jose Manuel Silvan:** Conceptualization, Methodology, Formal analysis, Investigation. **Tiziana Fornari:** Conceptualization, Methodology, Resources. **Luis Vázquez:** Methodology, Investigation, Writing – review & editing, Supervision. **Adolfo J. Martinez-Rodriguez:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization. **Diana Martin:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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