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Black Soldier Fly Larvae Grown on Hemp Fiber: Nutritional Composition and Production of Potential Bioactive Peptides

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Abstract: Black soldier fly larvae (BSFL) represent a way of converting organic substrates into valuable biomolecules, and are potentially exploitable as feed and food. In the present work, BSFL grown on retted hemp fiber were chemically analyzed to evaluate their nutritional profile. Chemical analysis revealed BSFL biomass to be an interesting source of proteins (40% on dry matter) rich in essential amino acids. In addition, larval biomass contained 12% fat, mainly composed of saturated fatty acids, and β-sitosterol and campesterol were found to be the most abundant among sterols. A total of 9% of the larval biomass was composed of chitin. The investigation extended to the enzymatic hydrolysis of proteins, leading to the identification of potential bioactive peptides. Peptidomics analysis coupled with in silico tools unveiled promising antioxidant, ACE-inhibitory, and DPP-IV-inhibitory properties within the protein hydrolysates. These findings revealed the potential of BSFL grown on retted hemp fiber as a source of dietary compounds as well as bioactive molecules which can be exploited as functional ingredients in the feed and food sectors.

Keywords: *Hermetia illucens*; novel protein; enzymatic hydrolysis; insect; waste valorization

Citation: Leni, G.; Del Vecchio, L.; Dellapina, C.; Moliterni, V.M.C.; Caligiani, A.; Cirlini, M. Black Soldier Fly Larvae Grown on Hemp Fiber: Nutritional Composition and Production of Potential Bioactive Peptides. *Macromol* **2024**, *4*, 135–149. https://doi.org/10.3390/macromol4010007

Academic Editor: Dimitrios Bikiaris

Received: 11 February 2024
Revised: 28 February 2024
Accepted: 14 March 2024
Published: 16 March 2024

1. Introduction

Climate change and continuous population growth are driving the search for alternative feed and food sources, which are more sustainable than conventional ones [1]. In this context, insects have been proposed as a novel protein source to be introduced into the food chain, since they have a well-balanced nutritional profile and are good sources of energy, protein, fat, vitamins, and minerals. In addition, insects can be reared on organic side-streams, such as agri-food wastes and by-products, perfectly meeting the Sustainable Development Goals of the Union Nations and the European Green Deal policies [2,3].

Black soldier flies (BSFs, *Hermetia illucens*) have been suggested to be among the most promising insect species for food and feed purposes, not only for their nutritional characteristics, but also for their capacity to develop and grow on a large spectrum of different organic substrates [4]. Furthermore, BSFs boast an impressive protein content which ranges from 32% to 58% of dry matter (DM), depending on the substrate of growth, and contain all the essential amino acids necessary for human consumption [5]. Hence, they constitute an appropriate protein reservoir for both the food and feed sectors. Although BSFs offer these advantages, their utilization as a human food source faces significant hurdles due to the considerable obstacle posed by consumer acceptance [6]. Therefore, integrating insect protein into food items, like meat substitutes, shows great potential as a means to enhance consumer acceptance of insect protein [7]. In this direction, several strategies have been developed to separate protein from BSF larvae [8]. Among the

different technologies, enzymatic hydrolysis represents a valid strategy, since the mild conditions of extraction applied allow us to preserve the protein's nutritional quality [8]. In addition, enzymes have been demonstrated to be able to release bioactive peptides which are encrypted in the insect proteome, and which exhibit different bioactivities (i.e., antioxidant, anti-angiotensin-converting enzyme, anti-dipeptidyl peptidase-IV, anti-glucosidase, anti-lipase, anti-lipoxygenase, anti-cyclooxygenase, anti-obesity, and hepatoprotective activities) [9]. Thus, incorporating protein hydrolysates from insects into food formulations can be considered a promising approach to the development of functional foods.

In the present work, prepupae larvae of black soldier flies (BSFL) reared on retted hemp fiber are characterized in terms of their chemical composition to evaluate their nutritional profile for future food and feed purposes. To the best of our knowledge, retted hemp fiber has never been applied before as substrate for insect cultivation. Since the growth substrate may influence the molecular composition of larvae, especially of their main characteristic components, such as fats and proteins, enzymatic hydrolysis is performed in order to extract and recover these latter compounds in form of peptides. In this direction, the obtained hydrolysates are characterized by shotgun peptidomics and analyzed in silico for the presence of potential bioactive peptides, which could be exploited as functional ingredients in insect-based meat substitutes.

2. Materials and Methods

2.1. Chemicals

Methanol (VWR Chemicals, France), dichloromethane (Honeywell chemicals, Seelze, Germany), hydrochloric acid (Carlo Erba, Milan, Italy), sulfuric acid, and hexane (Sigma-Aldrich, St. Louis, MO, USA) were used for the different experiments. Potassium and sodium chloride, potassium hydroxide, potassium sulphate, and ammonium hydroxide solution as well as the internal standards DL-norleucine, tricosanoic acid methyl ester (99%), 5-α-Cholestan-3-β-ol, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and trifluoroacetic anhydride (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amberlite 200 was obtained from Fluka (Buchs, Switzerland).

2.2. Black Soldier Fly Sampling and Preliminary Treatments

BSFL were provided by Azienda Agraria Sperimentale Stuard (Parma, Italy). BSFL were initially fed for 15 days with a starter feed composed of chicken feed. After that period, larvae were transferred to three bins containing retted hemp fiber and maintained for 6 days, in a temperature- (28–35 °C) and humidity (65%)-controlled room. To evaluate weight gain, fifty larvae of each replicate were appropriately weighed at time 0 and after 6 days of growth on the retted hemp fiber, recording an average weight gain of 50.87 ± 7.21%. Thereafter, BSFL were separated from the hemp fiber, killed by blanching them in boiling water for 40 s, and stored at −20 °C until analysis.

2.3. Proximate Composition of Larval Biomass

Moisture and ash were determined according to AOAC standard procedures [10,11]. Crude fat was determined according to the Folch method, using dichloromethane instead of chloroform, as proposed by Cequier-Sánchez et al. [12]. Total nitrogen was determined with a Kjeldahl system (DKL heating digestor and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA), and total protein was determined by assessing total amino acid composition (Section 2.6), assuming an equimolar amount of Asn/Asp and Gln/Glu. Chitin was determined according to Luparelli et al. [13].

2.4. Determination of Fatty Acid Profile by GC-MS

A total of 100 mg of extracted fat was dissolved in 8 mL of hexane and vigorously mixed for one minute with 3 mL of 5% KOH in methanol to extract the fatty acid methyl esters (FAME). Two phases were obtained and 900 µL of the upper phase including FAME was mixed with 100 µL of tricosanoic acid methyl ester as the internal standard (3000 ppm

in hexane). This procedure was repeated in triplicate. For GC-MS analyses, 1 µL of final solution was injected onto a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, MA, USA) equipped with a Supelcowax fused-silica capillary column (Supelco, Bellafonte, PS, USA; 30 m × 0.25 mm × 0.25 µm film thickness) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, MA, USA). Helium was the carrier gas. The injector temperature was set to 240° and the injection was performed in spilt mode (split ratio = 1/20). Oven temperature increased from 80 °C (3 min) to 240 °C at 20 °C per minute, maintaining this final temperature for 9 min. The MS acquisition mode was full scan (from 41–500 m/z). The content of each single fatty acid was calculated in relation to the concentration of the internal standard, after calculating the response factors using the Supelco®37 Component FAME Mix.

2.5. Determination of Sterols and Other Compounds of Unsaponifiable Fraction

A total of 500 mg of BSFL extracted fat and 20 mL of a 2.2 N potassium hydroxide solution in ethanol/water (8:2 v/v) were placed into a 250 mL flask. Saponification was carried out for 60 min, maintaining the mixture at the boiling-point temperature. After cooling, 20 mL of distilled water was added, and the solution was transferred to a separating funnel and 20 mL of ethyl ether was added. This procedure was repeated twice. Extracts were pooled and washed with distilled water, until the wash gave a neutral reaction. The organic extract was collected, dried with anhydrous sodium sulphate, filtered, taken to dryness, and the residue weighed. A total of 100 µL of internal standard (500 ppm, 5-α-Cholestan-3-β-ol solution in hexane) was added to the evaporated extracts and dried with a nitrogen flow. For derivatization, 100 µL of acid-catalyzed transmethylation reagent BSTFA was added, and the reaction was performed for 60 min at 60 °C. A total of 1 µL of silylated solution was injected on a GC-MS system (Thermo Scientific Trace 1300 gas-chromatograph; Thermo Scientific, Waltham, MA, USA) equipped with a ZB-5MS capillary column (Phenomenex, Torrance, CA, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, MA, USA). An initial oven temperature of 80 °C was used for 2 min, with a gradient of 15 °C/min until 280 °C, which was maintained for 20 min. The injector temperature was set at 240 °C and the injection was performed in split mode with a split ratio of 1/20, and the acquisition mode was full scan (40–550). All the compounds of interest were quantified based on the response of the internal standard (5-α-Cholestan-3-β-ol).

2.6. Amino Acid Analysis

For the amino acid profile, 0.5 g of ground BSFL were mixed with 6 mL HCl 6 M and hydrolyzed for 23 h at 110 °C. To the hydrolyzed residue, 1mL of DL-Norleucine 500 ppm was added as an internal standard and mixed for 10 min. Afterward the sample was filtered, evaporated, and dissolved in 20 mL of distilled water. A purification column filled with 5 mL cation exchange resin (Amberlite 200, Fluka Chemie, Buchs, Switzerland), previously regenerated with 2 N HCl (20 mL), and washed with distilled water, was used to retain amino acids. A total of 15 mL of 2N NH_3 was used to recover amino acids from the resin, and then, samples were evaporated to dryness under vacuum. In order to promote amino acid esterification, the residue was dissolved in 2 mL 1N HCl in 2-propanol and kept at 90 °C for 1 h. Then, the sample was evaporated, dissolved in 1 mL of dichloromethane, and treated with 0.5 mL of trifluoroacetic anhydride at 60 °C for 30 min. Then, nitrogen flow was used to evaporate the solvent. The samples dissolved in dichloromethane were injected (1 µL) and analyzed with a TRACE 1300 gas-chromatograph (Thermo Scientific, Waltham, MA, USA) coupled to a Thermo Scientific Trace IQS mass spectrometer (Thermo Scientific, Waltham, MA, USA) using a ZB-5MS capillary column of 30 m × 0.25 mm, f.t. 0.25 µm (Phenomenex, Torrance, CA, USA). Helium was used as a carrier gas (1 mL/min). The injector temperature was set to 280 °C and the injection was performed in split mode (ratio: 1/20). The oven temperature was increased from 60 °C to 280 °C at 20 °C/min after an initial hold at 60 °C for 3 min. The final temperature was maintained for 8 min. The

Ms condition were as follows: ion source temperature of 230 °C; acquisition mode: full scan m/z 41–500. Quantification was performed by using calibration standards. An amino acid score was calculated for each essential amino acid comparing them to the FAO/WHO reference pattern using the following Formula (1):

$$\text{Amino acid score} = \frac{\text{mg of AA in 1 g of protein}}{\text{mg of AA in 1 g of the FAO/WHO reference pattern}} \quad (1)$$

2.7. Protein Hydrolysis and Chemical Characterization of the Hydrolysate

The enzyme-assisted extraction of proteins from BSFL was performed in triplicate with the commercial protease, primarily composed of subtilisin A, obtained from *Bacillus licheniformis* (≥2.4 U/g; EC Number 3.4.21.62). For the hydrolysis, 5 g of ground larvae were mixed with 45 mL of Na_2HPO_4 10 mM (pH 6.5) and 1% of enzyme. Hydrolysis was performed at 60 °C and under agitation with a magnetic stirrer. In addition, two different times of reaction were tested and compared, specifically, 3 and 15 h. Next, the hydrolysates were heated at 95 °C for 5 min for enzyme inactivation and centrifuged (Eppendorf, 5810/5810 R, Milano, Italy) at 2683× *g* at 4 °C for 30 min. The supernatant was separated from the pellet and lyophilized with a LIO-5PDGT freeze-dryer (5pascal, Milano, Italy). Supernatants after lyophilization (hereafter called hydrolysates) were characterized for humidity, total N, ash, and lipid content, as previously described in Section 2.3.

2.7.1. High-Resolution Mass Spectrometry Analysis (μHPLC-LTQ-OrbiTRAP)

The dry protein hydrolysates were reconstituted with 50 μL of 0.2% formic acid solution for mass spectrometry analysis. High-resolution mass spectrometry was performed on the samples for peptide identification using a μHPLC DIONEX Ultimate3000 coupled to an LTQ-Orbitrap XL from Thermo Fisher Scientific. Column: Jupiter C18 4 μ, Proteo 90 Å 150 × 0.30 mm, Phenomenex; eluent A: water + 0.1% formic acid; eluent B: acetonitrile + 0.1% formic acid; flow: 5 μL/min, gradient: 0–4 min from 100% A to 95% A, 4–60 min from 95% A to 50% A, 60–62 min from 50% A to 10% A, 62–72 min 10% A, 72–74 min from 10% A to 95% A, 74–90 min 95% A; analysis time (min): 90; column temperature (°C): 30; injection volume (μL): 5; acquisition time (min): 0–75; ionization mode: ESI+; scan range (m/z): 200–1800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS + p res = 30,000 or (250.0–2000.0); (ion trap) ITMS + c Dep MS/MS, most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s): 30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000. Protein identification was performed by PEAKS software (Version 11; Bioinformatics Solutions Inc., Waterloo, ON, Canada) against the INSECTA (UniProt) protein database. Positive hits for protein identification were arbitrarily set for all those peptides identified in both replicates, with a score (−10lgP) > 20 and ppm in the range of ±6, thus reducing the probability of false positives.

2.7.2. In Silico Data Analysis for Putative Bioactive Peptides

The overall bioactivity of the released peptides was predicted by PeptideRanker (provider: University College Dublin, Ireland; http://distilldeep.ucd.ie/PeptideRanker/ (accessed on 18 September 2023)). This server is able to predict the probability that a peptide will be bioactive based on a novel neural network [14]. The PeptideRanker tool is able to rank a set of peptides and, based on function–structure models, to assign a score within the range of 0–1 for the predicted probability that the peptide will be bioactive. The higher the score, the higher the probability that the peptide has the predicted bioactivity. Only peptides with a score higher than 0.75 were selected and assessed for further investigation.

The BIOPEP-UWM database (available at https://biochemia.uwm.edu.pl/biopep-uwm/ (accessed on 18 September 2023)) was used to identify the presence of already known bioactive peptides and as a tool for the evaluation of encrypted bioactive moieties. The CAMP (available online at http://www.camp.bicnirrh.res.in/index.php (accessed on 18 September 2023)) webtool was used to identify the presence of potential antimicrobial peptides, considering as putative antimicrobial peptides only those with a positive hit for all three CAMP algorithms (RFC: Random Forest classifier; SVM: Support Vector Machine; ANN: Artificial Neural Network).

2.8. Data Analysis

All experiments were carried out in triplicate. Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The data were subjected to one-way analysis of variance (ANOVA) with Tukey's Post Hoc test to determine the differences between the two protein hydrolysates. Peptidomic data were analyzed by MetaboAnalyst 5.0 (available online at www.metaboanalyst.ca (accessed on 18 September 2023)) [15]. In particular, imputation was performed for missing values, which were then substituted with LoDs (1/5 of the minimum positive value of each variable). Furthermore, the data were normalized by median, Log transformed, and then auto-scaled. The processed data were then subjected to hierarchical cluster analysis in order to identify similarities and differences among the two peptidomic profiles.

3. Results

3.1. Black Soldier Fly Larva Composition

In the present work, BSFL grown on retted hemp fiber were chemically analyzed, in order to determine their nutritional composition in terms of protein, lipid, ash, and chitin content (DM of 20.08 ± 0.43%). The resulting lipid fraction was 12.41 ± 0.81% on DM, in agreement with other studies that reported a range of 12–40% for BSFL grown on organic waste materials [16,17]. As far as the protein content was concerned, BSFL contained 39.88 ± 2.97% of proteins expressed on DM. This value was close to the data obtained by Yakti et al. [18], who grew BSFL on substrates containing hemp wastes and determined an average protein content of 37% on DM. Furthermore, similar results were also obtained by Surendra et al. [4], who reported that BSFL developed on fruit and vegetable wastes contained proteins in amounts which ranged from 31 to 41% on DM. Finally, ash and chitin were also quantified and were found to contain about 16.56 ± 0.65% and 9.42 ± 0.69% on DM, respectively. These results were in line with the data reported by Luparelli et al. [13] and Fuso et al. [19], who thoroughly investigated the nutritional profile of BSFL grown on by-products of the agri-food sector.

In order to thoroughly examine the nutritional profile of BSFL grown on hemp fiber, the fatty acid profile was analyzed, and the results are reported below in Figure 1.

BSFL grown on retted hemp fiber were mainly composed of saturated fatty acids, representing 63% of the whole lipid profile. More specifically, lauric acid (C12:0) was the most abundant fatty acid (25.84 ± 0.85%), followed by oleic acid (C18:1; 15.25 ± 0.25%), palmitic acid (C16:0; 15.03 ± 0.03%), linoleic acid (C18:2; 13.77 ± 0.20%), and myristic acid (C14:0; 11.88 ± 0.13%). Overall, the total mono- and polyunsaturated percentages were higher than the value reported by Boukid et al. [20], who analyzed BFSL grown on agro-industrial by-products, including apricot and brewer's spent grain, which contained, on average, 23% unsaturated fatty acids. Also, Gao et al. [21] analyzed the changes in the fatty acid profile of BSFL grown on wheat bran and obtained a total percentage of saturated fatty acids of about 62.28 ± 2.54%, in line with the results obtained here and reported in Figure 1.

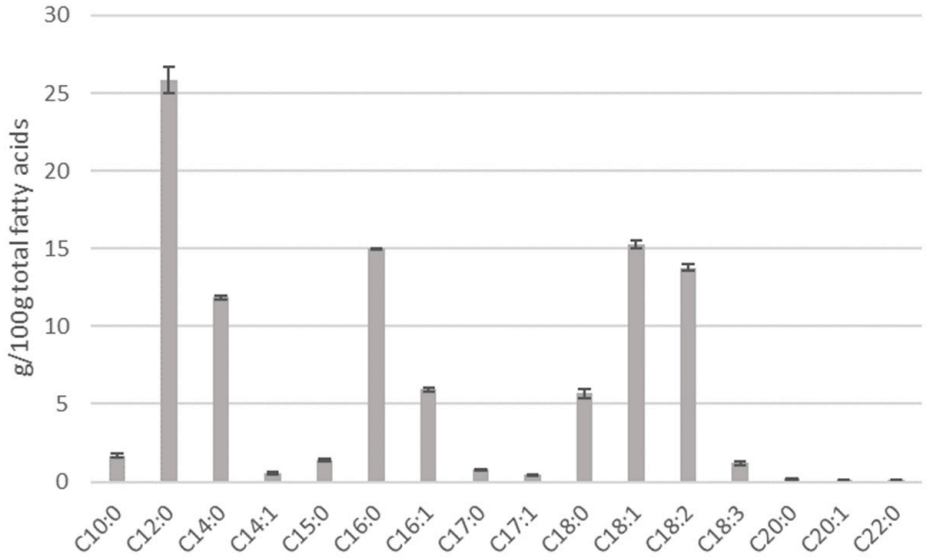

Figure 1. Fatty acid profile of BSFL grown on retted hemp. The results are expressed as relative percentage (g/100 g of fatty acids) and are the means of three different analyses.

The profile of sterols and policosanols was also determined, and the results are shown below in Figure 2.

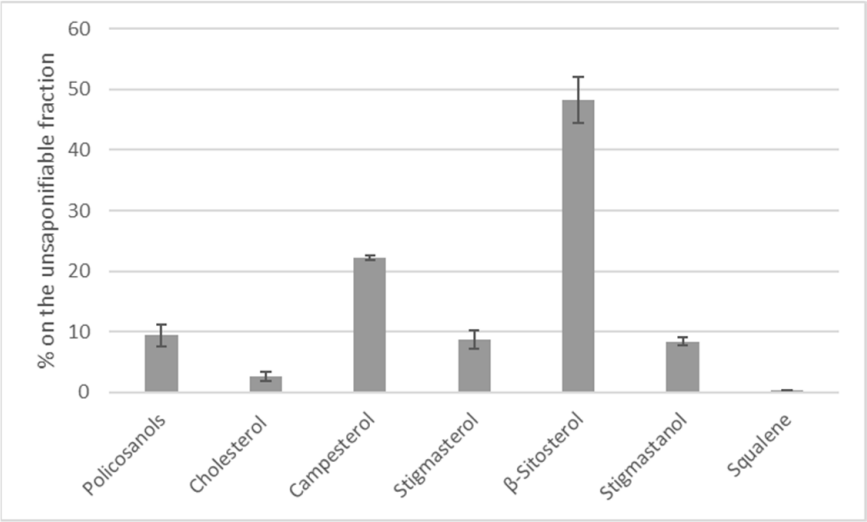

Figure 2. Sterol and policosanol content of BSFL fat. The data are expressed as relative percentages of the unsaponifiable fraction (g/100 g of unsaponifiable compounds) and are the means of three different replicates.

The main components of the BSFL sterol fraction were β-sitosterol (48.25 ± 3.73 g/100 g of unsaponifiable compounds) and campesterol (22.18 ± 0.34 g/100 g of unsaponifiable compounds), which are phytosterols commonly found in plants. This trend is consistent with a previous study in which BSFL were grown on vegetable residues (50% corn flour for zootechnical use, 40% wheat bran, 10% alfa-alfa flour) [22]. The combination of high phytosterol concentration and low cholesterol content make BSFL fat an interesting phytosterol reservoir, and thus, a potentially good LDL cholesterol reducer [23]. Although their real effectiveness is still controversial, the presence of policosanols and sterols in BSFL could contribute to a better lipid profile than other common animal fats [22]. Other elective constituents of the unsaponifiable fraction are policosanols (9.41 ± 1.86 g/100 g of unsaponifiable compounds), which have several positive effects: they reduce the risk of atherogenesis

because they restrict platelet aggregation, and reduce LDL cholesterol levels through the activity of HMG-CoA reductase, which inhibits endogenous cholesterol biosynthesis [24].

In order to complete the nutritional characterization of BSFL grown on retted hemp fiber, the protein fraction was analyzed in terms of essential and non-essential amino acids, and the results are reported below in Figure 3.

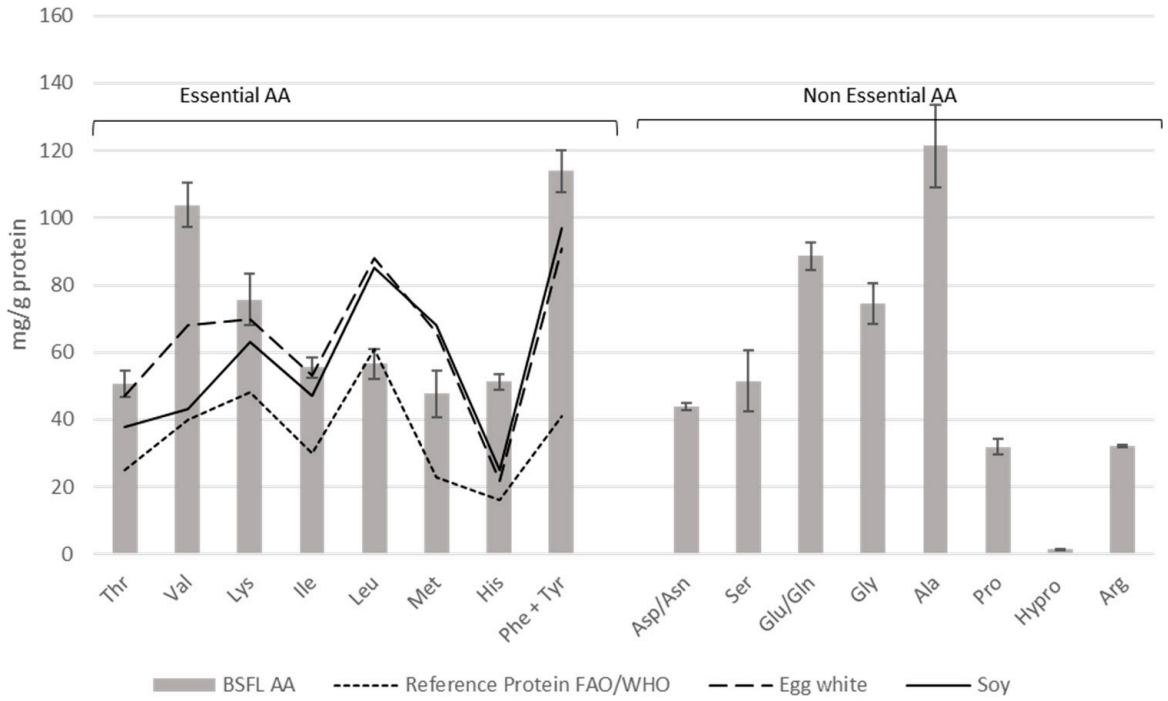

Figure 3. BSFL total amino acid profile expressed as mg/g protein (grey bars), compared with the FAO/WHO standard for human nutrition [25], egg white, and soy proteins (black lines) [26]. The results are expressed as means ± standard deviation of three replicates analyzed.

BSFL reared on retted hemp fiber contained a good-quality protein profile in terms of essential amino acid content, which represented 55% of the total amino acids detected. Valine (103.83 ± 13.24 mg/g protein) was the predominant essential amino acid, followed by lysine (75.67 ± 7.58 mg/g protein) and phenylalanine (56.94 ± 3.12 mg/g protein). Among the non-essential amino acids, alanine represented the most abundant amino acid (121.29 ± 12.4 mg/g protein), followed by glutamic acid/glutamine (88.57 ± 4.2 mg/g protein) and glycine (74.4 ± 6 mg/g protein). The amino acid scores reported in Table S1 show that all the essential amino acids exceeded the requirement proposed by the FAO/WHO for human nutrition [25]. As an exception, leucine was detected in BSFL in a concentration which was slightly lower than the recommendation proposed for humans, and it was identified as the limiting amino acid (amino acid score of 0.7). The amino acid profile was also compared with those of egg white and soy, two important sources of animal and vegetable proteins [26]. Valine, lysine, histidine, and phenylalanine plus tyrosine were detected in BSFL in amounts higher than the quantity reported in egg white and soy, while leucine and methionine presented lower concentrations. Generally, our samples had a slightly higher amount of essential amino acids than those reported by other authors who reared BSFL on agri-food waste substrates [19,27]. This difference could be clearly directly related to the different nutritional properties of the growth substrates used in the trials [28].

3.2. Protein Hydrolysates from BSFL and Peptide Characterization

In the present work, enzymatic hydrolysis was performed as a method for recovering and extracting insect proteins in the form of peptides. In particular, a commercial protease from Bacillus licheniformis (PBL) was selected for producing protein hydrolysates after 3 and 15 h of hydrolysis. Both hydrolysates produced were characterized in terms of their proximate composition and peptidomic profile. The results of the proximate composition are reported below in Table 1.

The two hydrolysates presented a similar compositional profile, and no significant differences were identified in terms of protein, lipid, and ash content ($p > 0.05$). Both were characterized by a high protein content, with percentages exceeding 60% on dry matter basis. On the contrary, both showed a low lipid content (lower than 2% on DM), pointing out how this technology represents a valid strategy for recovering and extracting proteins from BSFL. In particular, it was calculated that the enzymatic hydrolysis performed for 3 h allowed us to recover on average 62% of total proteins, while a yield of the 78% was obtained after 15 h of hydrolysis.

Table 1. Proximate composition of BSFL hydrolysates obtained with the protease from *Bacillus licheniformis* after 3 and 15 h of hydrolysis.

	Hydrolysis Time	
Composition%	**3 h**	**15 h**
Proteins	63.96 ± 1.80 [a]	64.23 ± 2.27 [a]
Lipid	1.15 ± 0.27 [a]	0.41 ± 0.1 [a]
Ash	22.31 ± 1.23 [a]	20.33 ± 0.62 [a]

The values are expressed on dry matter basis and are the results of three different replicates. The results are expressed as the mean ± standard deviation. Values followed by the same letters within one row are not significantly different ($p < 0.05$).

In order to characterize and thoroughly explore the peptidomic profile of both hydrolysates, a high-resolution mass spectrometry analysis was performed with an LTQ-Orbitrap instrument. A total of 499 and 463 peptides were identified in the 3- and 15-h hydrolysates, respectively. In order to reduce the presence of false positive identifications and focus the attention only on the most confident hits, only the peptides detected in all the replicates were considered. Furthermore, data filtering was performed on the peptidomic results by arbitrarily setting the cut-off at 20 for the score and at ±9 ppm for mass accuracy. After data filtering, the number of peptides was reduced to 207 for the 3 h hydrolysate and to 102 for the 15 h hydrolysate (complete results are reported in Table S2 of Supplementary Materials). As shown by the Venn diagram (Figure 4A), the two different hydrolysates shared 80 peptides.

The lower number of peptides identified in the longer hydrolyzation time could be related to the possible higher release of small peptides and free amino acids, which are too small to be identified with the peptidomic approach used. This hypothesis seems to be confirmed by comparing the molecular weight (MW) distribution of peptides released during the two different hydrolysis times (Figure 4B). A total of 46% of the identified peptides within the 3 h hydrolysate had an MW ranging between 1000 and 1500 Da, 39% between 1500 and 2000 Da, 12% between 2000 and 2500 Da, and finally, 2% had an MW higher than 2500 Da. On the other hand, the 15 h hydrolysate was composed of a higher percentage of peptides with a smaller MW: 5% lower than 1000 Da, 61% between 1000 and 1500 Da, and 22% between 1500 and 2000 Da. After 15 h of hydrolysis, no peptides with an MW higher than 2500 Da were identified. The different time of hydrolysis did not only affect the number of peptides identified and their length, but also their abundance. In fact, Figure 4C reports the dynamic changes in the relative abundances of the 80 peptides identified in both protein hydrolysates after 3 and 15 h of hydrolysis.

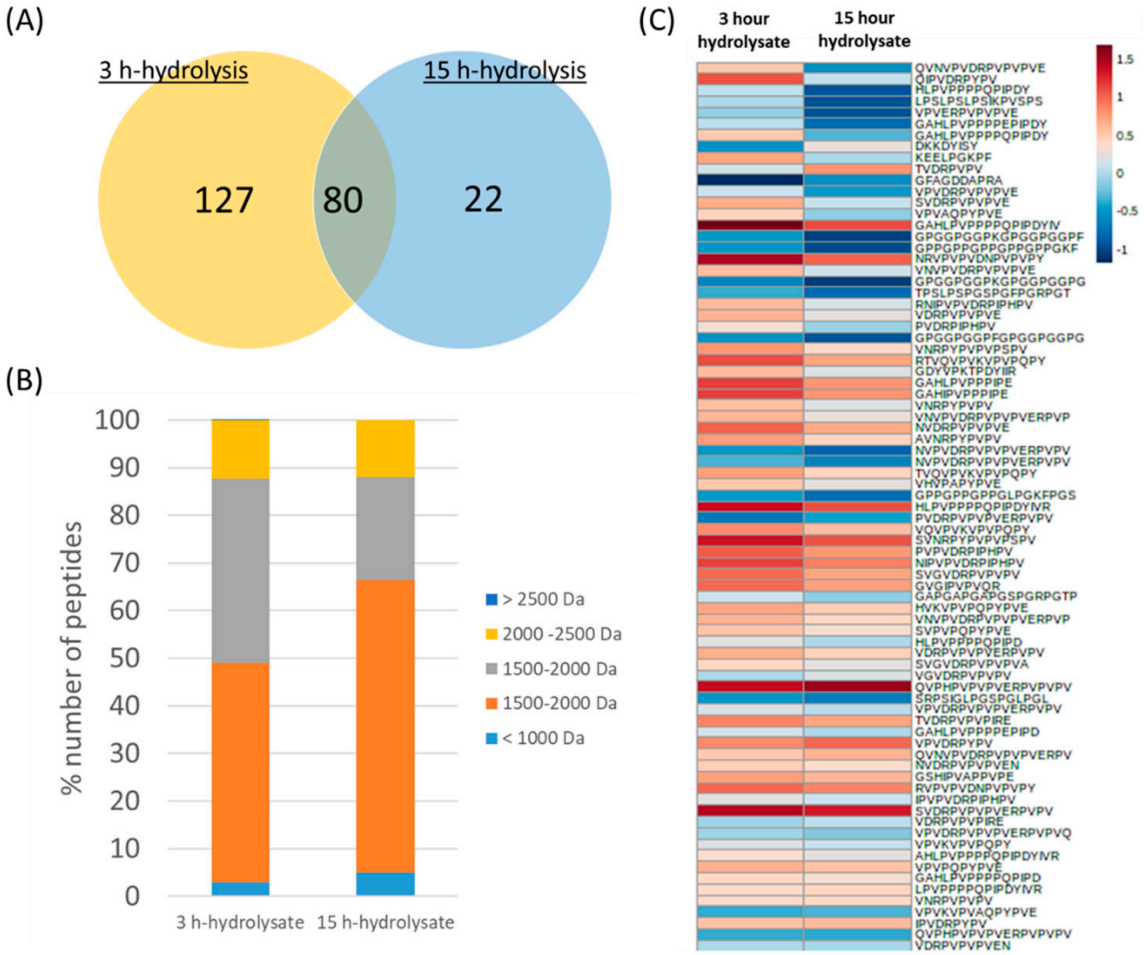

Figure 4. Venn diagram of the number of peptides identified in BSFL hydrolysate after 3 and 15 h of reaction (**A**). The molecular mass distribution of peptides identified in protein hydrolysates (**B**). Heatmap of the relative abundance of the 80 peptides identified in both protein hydrolysates (**C**).

The identified peptides were mapped on 32 proteins for the 3 h hydrolysate and 12 proteins for the 15 h hydrolysate (Table 2).

The identified peptides belonged to proteins with a wide spectrum of function: ATP, calcium ion and RNA binding proteins, enzymatic, hemolymphatic, muscular, and structural constituent of cuticle. In addition, 29 peptides belonging to proteins with an undefined function were identified, as well. More than the 80% of the peptidome of both hydrolysates was associated with hypothetical proteins, underlining that the database used for identification does not yet report complete information on the BSFL proteome, as also suggested in our previous work [29], even though the database is constantly being updated. For both hydrolysates, the identified peptides mainly mapped enzymatic and cuticular proteins, with more than 60% of whole identified proteins belonging to these two functional groups. Proteins belonging to the ATP, calcium ion and RNA binding groups were identified only in the 3 h hydrolysates. In general, the proteins which were identified in both hydrolysates were mapped by a higher number of peptides in the 3 h hydrolysate than in the 15 h hydrolysate. The lesser number of peptides identified for the same protein in the 15 h hydrolysate confirmed the increased proteolytic activity and the release in solution of free amino acids or di-tripeptides undetectable by the peptidomic approach applied here.

Table 2. The main proteins identified in hydrolysate extracts obtained at two different times.

Accession	Protein	Function	Average Normalized Peptide Area
3 h of hydrolysis			
A0A7R8V1D0	Hypothetical protein	Structural constituent of cuticle	1.369
A0A6J1S563	Larval cuticle protein 65Ag1-like	Structural constituent of cuticle	1.172
A0A7R8V0P4	Hypothetical protein	Structural constituent of cuticle	1.172
A0A7R8UKB6	Hypothetical protein	Structural constituent of cuticle	0.760
A0A7R8YSD0	Hypothetical protein	Structural constituent of cuticle	0.592
A0A0L7LRD1	Putative cuticle protein	Structural constituent of cuticle	0.339
A0A7R8YZ13	Hypothetical protein	Structural constituent of cuticle	0.307
A0A7R8URU7	Hypothetical protein	Unknown	0.274
A0A7R8URQ8	Hypothetical protein	Enzyme	0.211
A0A7R8V147	Hypothetical protein	Structural constituent of cuticle	0.173
B0WR94	Hypothetical protein	Unknown	0.001
A0A087ZMI4	Putative actin-related protein	Muscular	−0.011
A0A7R8UJC0	Hypothetical protein	Hemocyanin	−0.055
A0A7R8V0P3	Hypothetical protein	Structural constituent of cuticle	−0.088
B6ZCL2	Elongation factor 1 alpha	RNA binding	−0.132
A0A7R8UKG1	Catalase	Enzyme	−0.144
A0A7R8URW5	Hypothetical protein	Muscular	−0.249
A0A7R8YKR5	Hypothetical protein	Enzyme	−0.335
A0A7R8V4G4	Fructose-bisphosphate aldolase	Enzyme	−0.364
A0A310SDR1	ATP synthase subunit beta	Enzyme	−0.380
A0A7R8UM91	Hypothetical protein	Structural constituent of cuticle	−0.453
A0A7R8YNH0	Hypothetical protein	Structural constituent of cuticle	−0.462
A0A7R8YSW8	Hypothetical protein	Hemolymphatic	−0.521
A0A7R8YVB4	Hypothetical protein	Ca^{2+} binding	−0.811
A0A7R8UHR0	Hypothetical protein	Hemolymphatic	−0.833
A0A7R8UR83	Hypothetical protein	Unknown	−0.970
A0A7R8Z362	Hypothetical protein	ATP binding	−1.118
A0A2Z5REH8	Pyruvate kinase	Enzyme	−1.141
A0A7R8YT79	Hypothetical protein	Enzyme	−1.183
A0A023EQM6	Fructose-bisphosphate aldolase	Enzyme	−1.476
B4QW45	Fructose-bisphosphate aldolase	Enzyme	−1.476
A0A1J1IKW3	CLUMA_CG013677	Enzyme	−1.768
15 h of hydrolysis			
A0A7R8V1D0	Hypothetical protein	Structural constituent of cuticle	1.120
A0A6J1S563	larval cuticle protein 65Ag1-like	Structural constituent of cuticle	0.802
A0A7R8V0Q1	Hypothetical protein	Structural constituent of cuticle	0.802
A0A7R8URU7	Hypothetical protein	Unknown	0.279
A0A7R8URQ8	Hypothetical protein	Enzyme	0.154
A0A7R8V147	Hypothetical protein	Structural constituent of cuticle	0.021
A0A7R8UHR0	Hypothetical protein	Hemolymphatic	−0.099
A0A087ZMI4	Putative actin-related protein	Muscular	−0.103
A0A7R8YSD0	Hypothetical protein	Structural constituent of cuticle	−0.105
A0A7R8V0P3	Hypothetical protein	Structural constituent of cuticle	−0.383
A0A7R8UM91	Hypothetical protein	Structural constituent of cuticle	−0.996
A0A067RLP1	Catalase	Enzyme	−1.043
A0A7R8V1D0	Hypothetical protein	Structural constituent of cuticle	1.120

The table reports information about UniProt accession number, protein name, functionality, and average normalized peptide area. The order of the proteins follows their abundance, from the most to the least abundant.

3.3. Prediction of Potential Bioactive Peptides

Bioactive peptides are peptides which are encrypted in the amino acid sequence of proteins and usually consist of about 3–20 amino acid residues [30]. They derive from food proteins, becoming active once released by enzymatic hydrolysis by peptidases during food processing and/or during gastrointestinal digestion. Bioactive peptides can exert several beneficial effects, like preventing diseases or modulating physiological systems, once they are absorbed in the human or animal body [9], depending on their aminoacidic sequence and molecular structure. In this context, proteolysis performed by commercial proteases, such as PBL, could release peptides which could exert beneficial effects on human health. With the application of in silico tools, bioactive peptides from various sources can be predicted, allowing the screening of novel bioactive sequences. In previous studies, computational tools revealed the presence in BSFL of sequences with antioxidant, anti-ACE, and anti-DPP-IV activities [9]. In the present work, for the first time, this approach has been applied in the identification of potential bioactivities among the peptides released by PBL from BSFL.

The identification of putative bioactive peptides in our BSFL hydrolysates was performed by the Peptide Ranker^{TM}, BIOPEP-UWM and CAMP$_{R4}$ web tools, as also proposed by Mooney et al. [14] and Capriotti et al. [31], and the results are reported below in Table 3.

Table 3. Potential bioactive peptides identified after 3 and 15 h of hydrolysis.

Peptide	UNIPROT Accession	Protein Function	Peptide Ranker	Antimicrobial Activity			Encrypted Bioactive Properties		
				SVM	RFC	ANN	ACE Inhibitor	DPP IV Inhibitor	Antioxidative
3 h hydrolysis									
GPPGPPGPPGPPGPPGKFPL	A0A7R8UM91	Cuticular	0.982	N	Y	Y	X	X	X
GPGGPGGPKGPGGPGGPF	A0A7R8UM91	Cuticular	0.971	Y	Y	Y	x	x	
GPPGPPGPPGLPGKFPGST	A0A7R8UM91	Cuticular	0.969	N	Y	Y	x	x	X
NIYPVPDPRFPFL	A0A1J1IKW3	Enzymatic	0.917	Y	N	Y	X	X	X
ISPPPPLVSIPVGGIL	A0A7R8YVB4	Ca^{2+} binding	0.916	Y	Y	Y	X	X	
GNIYPVPDPRFPFL	A0A1J1IKW3	Enzymatic	0.908	Y	N	Y	X	X	
IISPPPPLVSIPVGGIL	A0A7R8YVB4	Ca^{2+} binding	0.906	Y	Y	Y	X	X	
GKPGPAGPPGPPGPL	A0A7R8UM91	Cuticular	0.892	N	Y	Y	X	X	X
GFPFDRPIDL	A0A7R8YSW8	Hemolymphatic	0.885	N	N	N	X	X	
SRPSIGLPGSPGLPGL	A0A7R8UM91	Cuticular	0.870	N	N	N	X	X	
GAHLPIPPPIPDYILR	A0A7R8YNH0	Cuticular	0.841	N	N	Y	X	X	X
SRPSLGLPGSPGSPGLPGL	A0A7R8UM91	Cuticular	0.840	N	N	N	X	X	
PPPPQPIPDYIVR	A0A7R8V147	Cuticular	0.834	Y	N	N	X	X	
GAHLPIPPPIPDYIL	A0A7R8YNH0	Cuticular	0.799	N	N	Y	X	X	X
KNPFDEPGKPGNL	A0A7R8Z362	ATP binding	0.798	N	N	N	X	X	X
GSRFFDDLPL	A0A7R8UJC0	Hemolymphatic	0.789	N	N	N	X	X	X
15 h hydrolysis									
GPPGPPGPPGPPGPPGKFPL	A0A7R8UM91	Cuticular	0.982	N	Y	Y	X	X	X
GPGGPGGPKGPGGPGGPF	A0A7R8UM91	Cuticular	0.971	Y	Y	Y	X	X	
GPPGPPGPPGLPGKFPGST	A0A7R8UM91	Cuticular	0.969	N	Y	Y	X	X	X
GPGGPGGPGGKGPGGPL	A0A7R8UM91	Cuticular	0.963	Y	Y	Y	X	X	
SRPSIGLPGSPGLPGL	A0A7R8UM91	Cuticular	0.870	N	N	N	X	X	
GAPGAPGSPGRPGSPIRPS	A0A7R8UM91	Cuticular	0.804	N	Y	Y	X	X	X

RFC: Random Forest classifier; SVM: Support Vector Machine; ANN: Artificial Neural Network. N and Y define negative and positive responses to all three CAMP algorithms, respectively. X indicates the positive hits for potential bioactivities.

The Peptide Ranker tool was first used to direct the further analysis only towards the most confident hits. In fact, this program assigns a rank to the likelihood of a peptide sequence being bioactive (0.0 being highly unlikely, 1.0 being highly likely). The cut-off was set to 0.75 and the program identified 16 and 6 putative bioactive peptides for samples subjected to 3 h and 15 h of hydrolysis, respectively. Among these peptides, four were reported in both samples (GPPGPPGPPGPPGPPGKFPL, GPGGPGGPKGPGGPGGPF, GPPGPPGPPGLPGKFPGST, GPGGPGGPGGKGPGGPL,

and SRPSIGLPGSPGLPGL). The 22 peptides selected by Peptide Ranker belonged to proteins with cuticular, hemolymphatic, enzymatic and binding functions, and were then selected for the further elaboration. The BIOPEP-UWM database was then used to identify among these peptides those for which the bioactivity was already reported in the literature. The results did not provide positive hits for any peptides included in the search analysis. However, potential bioactive moieties could still be encrypted in the peptide sequences generated by PBL and could potentially be released during further gastrointestinal digestion after exopeptidase or endopeptidase activity. BIOPEP-UWM allowed us to detect encrypted amino acids sequences which could exert antioxidant, angiotensin converting enzyme (ACE), or dipeptidyl peptidase IV (DPP-IV) inhibitory activity. ACE plays an important role in the regulation of blood pressure, and specific inhibitors are used as pharmaceuticals to treat hypertension, congestive heart failure, and myocardial infarction [32]. DPP-IV is involved in glycemic regulation, playing a central role in the processing of incretin. Therefore, inhibitory peptides may be used as food ingredients to improve glycemic regulation in Type 2 diabetics [33]. All the selected peptides were found to be potential sources of ACE and DPP-IV amino acid sequences by BIOPEP-UWM. In addition, 50% of them also provided positive results regarding oxidative properties.

Finally, the potential antimicrobial activity of these peptides was evaluated with the CAMP webtool, which will be useful in identifying potential antimicrobial peptides based on their primary structure and amino acid sequences [34]. Positive results were considered for those peptides which provided positive hits for all three CAMP algorithms (Artificial Neural Network (ANN), Support Vector Machine (SVM), and Random Forest (RF) (Table 3). In the 3 h hydrolysate, the potential AMPs were GPGGPGGPKGPGGPGGPF, ISPPPPLVSIPVGGIL, and IISPPPPLVSIPVGGIL, belonging to cuticular and Ca^{2+}-binding proteins. GPGGPGGPKGPGGPGGPF was also detected in the 15 h hydrolysate and resulted the only potential AMP after 15 h of hydrolysis. In conclusion, both hydrolysates were demonstrated to be good reservoirs of potential bioactive peptides with antioxidant and ACE- and DPP-IV-inhibitory activities, and could be exploited as functional ingredients for food and feed purposes.

4. Conclusions

Recently, edible insects have been proposed as a novel source of dietary proteins to be introduced in the feed and food sectors. In the present work, BSFL reared on retted hemp fibers showed a high content of proteins, rich in essential amino acids. The lipid fraction, even though it was composed of a high ratio of saturated fatty acids, may represent an interesting phytosterol reservoir. In order to further valorize the harvested BSFL biomass as a novel source of proteins for the food sector, and overcoming the insect neophobia which is particularly spread among Western consumers, enzymatic hydrolysis was performed and two different times of reaction were tested. Enzymatic hydrolysis was revealed to be a valid bio-tool for the extraction of proteins (64% on DM on average) in the form of peptides. Peptidomic characterization of protein hydrolysates revealed that different times of reaction affected the number of peptides identified, their length, and also their relative abundance. Both hydrolysates, assessed for the potential presence of bioactive peptides by computational analysis with in silico tools, were demonstrated to be a good reservoirs of bioactive peptides with antioxidant and ACE- and DPP-IV-inhibitory activities. In conclusion, this work shows that BSFL grown on retted hemp and subjected to enzymatic hydrolysis could be a good potential source of functional ingredients for the feed as well as the food sector. Given these premises, further analysis should thoroughly investigate by, using vitro methods, the presence of these putative bioactivities. Furthermore, future studies should focus on the formulation of insect-based meat analogues, with the subsequent evaluation of their functional and sensory properties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/macromol4010007/s1. The amino acid score of BSFL grown on retted hemp fiber is reported in Table S1. The interactive plot data with a complete list of peptides identified by high-resolution mass spectrometry in the BSFL hydrolysates produced after 3 and 15 h of hydrolysis is reported in Table S2.

Author Contributions: G.L.: conceptualization, methodology, formal analysis, investigation, writing—original draft, review, and editing. L.D.V.: formal analysis, investigation, writing—original draft, review, and editing. C.D.: formal analysis, investigation, writing—original draft, review, and editing. V.M.C.M.: conceptualization, investigation, writing—review and editing. A.C.: methodology, investigation; writing—review and editing. M.C.: conceptualization, supervision, methodology, investigation, writing—original draft, review, and editing, project administration. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by CATERPILLAR (Textile hemp for the production of functional food and protein biomasses for animal feed) research project, financed by the Emilia-Romagna region as part of the Rural Development Program 2014–2020, measure 16.1.01, "Operational Groups of the Agricultural European Innovation Partnership", Focus Area 2A (Act n. 7038 of 27 April 2020).

Data Availability Statement: The data are contained within the article or Supplementary Materials.

Acknowledgments: The authors would like to thank Marco Errani (Azienda Sperimentale Stuard, Parma, Italy) for projecting and providing the retting system located at the "StuardLab" (Parma, Itlay) and Andrea Faccini for conducting the LTQ-Orbitrap analyses at the Interdepartmental Centre for Measures "G. Casnati" of the University of Parma (Parma, Italy).

Conflicts of Interest: The authors declare no conflicts of interest.

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