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RESEARCH ARTICLE

Black soldier fly larvae efficiently bioconvert the organic fraction of municipal solid waste thanks to the functional plasticity of their midgut

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Abstract

The saprophagous larvae of *Hermetia illucens* show promising potential as effective agents for bioconverting organic waste and by-products into valuable biomass. Their capability to efficiently transform organic substrates, varying in nutrient content, origin, texture, and moisture, is strictly correlated with the morphofunctional complexity of their digestive system and the associated microbiota. In addition, post-ingestion mechanisms are set in motion to regulate the midgut activity depending on the feeding substrate, thus ensuring the nutritional requirements of the larvae. This study aims to assess the capability of *H. illucens* larvae to grow and biotransform the organic fraction of municipal solid waste (OFMSW). In particular, the larvae were reared on substrates mimicking the OFMSW with different nutrient content, and their growth and bioconversion performance, as well as the quality of larval and pupal biomass were evaluated. Our results demonstrate that the nutritional composition of the rearing substrates had minimal impact on the efficiency of the bioconversion process and on the protein, lipid, and chitin content of the insect biomass. This was due to a fine transcriptional regulation of genes encoding enzymes involved in protein, polysaccharide, and lipid digestion in midgut cells. As a consequence, larvae reared on OFMSW with the lowest protein, starch, and lipid content exhibited a significant increase in protease, amylase, and lipase activities. Overall, this study highlights the value of *H. illucens* larvae in valorising the OFMSW, demonstrating that variations in its composition do not significantly affect the quality of the bioconversion process. These findings hold significant practical implications as current strategies for OFMSW treatment remain unsatisfactory and innovative approaches are needed to enhance bio-waste recycling and reduce the environmental impact of waste management.

Keywords

bioconversion indexes – digestive enzymes – *Hermetia illucens* – insect midgut – organic waste valorisation

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1 Introduction

The larvae of the black soldier fly (BSFL), *Hermetia illucens* (Diptera: Stratiomyidae), are attracting increasing attention for the reduction and bioconversion of organic waste, providing a sustainable and efficient alternative to conventional applications for waste management. In fact, these saprophagous larvae can grow on different organic wastes, side streams, and by-products such as fruit and vegetable waste, okara, maize distillers, brewery and winery by-products, and food waste (Bava *et al.*, 2019; Ceccotti *et al.*, 2022; Meneguz *et al.*, 2018; Singh *et al.*, 2021), and bioconvert them into insect proteins, lipids, and other macromolecules of biotechnological interest, such as chitin and antimicrobial peptides (Barbi *et al.*, 2019; Jayanegara *et al.*, 2020; Nguyen *et al.*, 2018; Shin and Park, 2019; Tettamanti and Bruno, 2024). The impressive growth performance of BSFL on these substrates can be in part attributed to their peculiar mouthparts (Bruno *et al.*, 2020; Lievens *et al.*, 2023) but, above all, to their highly efficient digestive system and the associated microbiota (Bonelli *et al.*, 2019; Bonelli *et al.*, 2020; Bruno *et al.*, 2019a; Cifuentes *et al.*, 2020; Ijdema *et al.*, 2022; Tettamanti *et al.*, 2022). Digestion and nutrient absorption occur in the midgut, a complex organ that in BSFL is divided into three regions (anterior, middle, and posterior), each showing peculiar morphofunctional features (Bonelli *et al.*, 2019; Bonelli *et al.*, 2020). All these features allow the insect to process and exploit substrates with different size, texture, and nutritional value, ensuring their efficient reduction and biotransformation.

In the last fifteen years, great efforts have been spent in the development of circular value chains based on BSFL-mediated bioconversion that, starting from microbiologically safe organic by-products, lead to obtaining insect proteins that can be used as feedstuff for fish, poultry, and pigs (Lu *et al.*, 2022; Mohan *et al.*, 2022). These entrepreneurial initiatives, fostered in many countries by national and international policies on sustainable development, are essentially implemented in the framework of the feed sector and are strongly limited by the nature of the insect feeding substrate, in compliance with the regulations on the use of insects as feed (European Commission, 2017; IPIFF, 2024). For this reason, research has increasingly moved towards non-conventional applications of BSFL taking, on one hand, a glance at "not-safe" substrates such as animal manure, sewage sludge, slaughterhouse waste, and various contaminated biomasses (Arnone *et al.*, 2022; El-Dakar *et al.*, 2021; Lalander *et al.*, 2019; Leni *et al.*, 2019) and exploring, on the other hand, the use of the so obtained larvae for alternative, non-feed purposes, such as the production of bioplastics, biomaterials, and biodiesel (Ishak and Kamari, 2019; Setti *et al.*, 2020; Tettamanti and Bruno, 2024). In this scenario, the management of the municipal solid waste (MSW) is of paramount importance. In fact, MSW generated globally amounts to 2 billion tonnes/year (Kaza *et al.*, 2018), half of which is represented by food waste (900 million tonnes), the so-called organic fraction of the MSW (OFMSW), produced from households, food service, and retail (United Nations Environment Programme, 2021). Although an appropriate management and disposal of this impressive amount of waste is mandatory to reduce its impact on the environment – noteworthy 10% of greenhouse gases globally produced are associated with unconsumed food (United Nations Environment Programme, 2021) – OFMSW is currently valorised through composting and anaerobic digestion (Li *et al.*, 2013; Palansooriya *et al.*, 2023; Xu *et al.*, 2018), but neither is entirely satisfactory. In fact, composting needs large land areas, and factors such as temperature, moisture, pH, aeration rate, C/N ratio, and waste composition highly affect the process efficiency (Gao *et al.*, 2010; Kumar *et al.*, 2010; Palansooriya *et al.*, 2023; Sasaki *et al.*, 2003). On the other hand, anaerobic digestion represents a more advantageous solution since it allows the recovery of both energy and digestate, and is thus theoretically cost-effective. However, bacteria responsible for the process are strictly dependent on factors such as temperature, quality and size of the waste, and pH, thus raising costs and posing concerns on the economic sustainability of this application (Harirchi *et al.*, 2022).

An efficient valorisation of the OFMSW by using BSFL could provide an innovative, eco-friendly, and valuable opportunity in the coming years. However, many aspects on the growth performance of BSFL on this waste are still unclear and fragmentary, and the optimization of mass rearing needs to be further investigated. In fact, only a few studies have addressed the bioconversion of OFMSW through BSFL, exploring potential benefits and limitations in the digestion and degradation of this substrate (Diener *et al.*, 2011), or investigating the quality of the resulting rearing residue (frass) as fertiliser (Addo *et al.*, 2022).

The present study aims to investigate the growth and bioconversion efficiency of BSFL reared on substrates that mimic the OFMSW and to get insights into the role of the midgut – the organ involved in digestion and absorption of nutrients – in the capability of the larvae to exploit and bioconvert OFMSW with different

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chemical composition and thus different nutrient content. This knowledge is essential to set the stage for the development of new and innovative biotechnological approaches for OFMSW treatment and valorisation in line with the principles of the circular economy model.

2 Materials and methods

Insect rearing

BSFL were reared according to Pimentel *et al.* (2017). Briefly, eggs were collected in a humid chamber containing "s-OFMSW" or "modified s-OFMSW" (where "s" stands for surrogate), two experimental diets that reproduced the composition of the OFMSW. In detail, both feeding substrates were formulated with bread, pork meat, mozzarella cheese, banana, pear, apple, carrot, and potato (see Supplementary Table S1 for details), taking into consideration the variability in the composition of the OFMSW (Fisgativa *et al.*, 2016) and the need to perform the experiments under reproducible and standardised conditions. The chemical composition of the two substrates was determined as described in the paragraph *Determination of the chemical composition of substrates and insects* and shown in Table 1. All the ingredients were finely minced and mixed before feeding the larvae. Six days after hatching, batches of 300 larvae were placed in 16 × 16 × 9 cm plastic containers and fed with the two experimental diets. The boxes were kept in the dark at 27 ± 0.5 °C and 70 ± 5% relative humidity until insects reached the appropriate developmental stage (i.e. last larval instar and 4-day-old pupae) for the analyses. Flies were maintained at 29 ± 0.5 °C, 70 ± 5% relative humidity, with a 12:12 h light:dark photoperiod as reported in Bruno *et al.* (2019b).

Evaluation of larval growth rate, substrate reduction, and bioconversion efficiency

To monitor insect growth, 25 larvae were randomly sampled from the substrate every two days, washed in tap water, wiped dry, and weighed, starting from the 6th day post hatching (this time point was considered the beginning of the bioconversion process) until 25% of insects reached the pupal stage (this time point was considered the end of the bioconversion process), as reported in Bonelli *et al.* (2020).

The growth performance of the larvae and the efficiency of the bioconversion process were evaluated through the following indexes:

$$\text{substrate reduction (D)} : \big((W - R)/W\big) \times 100 \quad (1)$$

where W is the total amount of rearing substrate provided to the larvae, R is the rearing residue (frass) at the end of the bioconversion process;

$$\text{waste reduction index (WRI)} : (D/t) \quad (2)$$

where t indicates the time (days) spent by the larvae on the rearing substrate;

$$\text{efficiency of conversion of ingested food (ECI)} : \big[(B_{fin} - B_{ini})/(W - R)\big] \times 100 \quad (3)$$

where B is the total amount of the insect biomass at the end (B_{fin}) and at the beginning (B_{ini}) of the bioconversion process;

$$\text{relative growth rate (RGR)} : (B_{fin} - B_{ini})/(t \times B_{fin}) \quad (4)$$

$$\text{survival rate (SR)} : (I_{fin}/I_{ini}) \times 100 \quad (5)$$

where I_{fin} is the number of insects at the end of the bioconversion process and I_{ini} at the beginning of the bioconversion process;

$$\text{nitrogen conversion efficiency (NCE)} : \big[(N_{ins} \times B_{fin})/(N_w \times W)\big] \times 100 \quad (6)$$

where N_{ins} is the nitrogen content of insects at the end of the bioconversion process and N_w is the nitrogen content of the rearing substrate provided to the larvae.

All the indexes were calculated on dry matter. To calculate the total amount of dry matter of the rearing substrate, frass, and insects, samples were frozen at –20 °C for 24 hours and then dried at 60 °C overnight. Before drying, insects were removed from the rearing substrate, accurately washed, wiped dry, and then frozen.

All the experiments (analysis of the larval growth rate and calculation of indexes) were conducted in triplicate.

Determination of the chemical composition of substrates and insects

After collection, samples (rearing substrates, larvae, and pupae) were dried at 60 °C overnight, finely minced, and then analysed to determine the chemical composition. The analyses were conducted at LaChi Laboratory, University of Padova (Legnaro, Italy). Crude protein, crude lipid, crude fibre, nitrogen-free extract, and ash in the rearing substrates were determined following the protocols of AOAC International (Horwitz, 2000; Latimer, 2016). In particular, crude protein content was determined by using a nitrogen-to-protein conversion

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factor (Kp) of 6.25 (Janssen *et al.*, 2017). Hemicellulose, cellulose, lignin, starch, and free glucose and fructose content were determined as reported in Bonelli *et al.* (2020). Insects were analysed for crude protein, crude lipid, nitrogen-free extract, and ash following the protocols of AOAC International (Horwitz, 2000; Latimer, 2016). In particular, crude protein content was determined by considering a Kp of 5.6 (Janssen *et al.*, 2017). Chitin was calculated by using Van Soest acid detergent fibre method (Stelmock *et al.*, 1985).

Amino acids (except tryptophan) were calculated after acid hydrolysis of the samples and pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, separated by reversed-phase (RP) HPLC and analysed by UV detection following a method adapted from European Pharmacopoeia (2003). Tryptophan was determined following a method adapted from the Commission Directive 2000/45/EC (European Commission, 2000). Briefly, samples were subjected to basic hydrolysis with barium hydroxide at 105 °C for 24 hours and then neutralised with HCl, filtered, and analysed by RP-HPLC.

For all the analyses, at least 20 g of substrates and 15 g of insects (both larvae and pupae) were collected and at least two analytical replicates per sample were conducted.

Isolation of midgut tissues and midgut juice

Midgut tissues were isolated in cold phosphate buffered saline (PBS) (in mM: 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.76 KH_2PO_4, pH 7.4) from actively feeding last instar larvae after anaesthetization on ice. Samples for enzymatic assays were collected as reported previously (Bonelli *et al.*, 2019). Briefly, anterior, middle, and posterior midgut were isolated, the epithelium was separated from the lumen content and frozen in liquid nitrogen until use for aminopeptidase N (APN) activity assays (see section *Measurement of aminopeptidase N activity in midgut homogenates and in brush border membranes*). All the other enzymatic assays (see section *Enzymatic assays on midgut juice*) were performed on midgut juice. The latter was obtained as follows. The peritrophic matrix (PM) with the enclosed intestinal content from the anterior, middle and posterior regions was isolated and gently blotted on filter paper. The samples (i.e. midgut contents) were placed into microcentrifuge tubes and homogenised with a pestle using a motor homogeniser and vigorously vortexed. Samples were then centrifuged at 15,000 × *g* for 10 min at 4 °C and supernatant (midgut juice) was collected and stored at –80 °C until use. For Real-Time qPCR (RT-qPCR) experiments (see section *RNA extraction and RT-qPCR*) anterior, middle, and posterior midgut tissues were isolated in sterile conditions and only the central part of each region was collected to avoid any possible contamination of RNA deriving from contiguous regions. Collected samples were immediately put in RNA later™ stabilization solution (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –80 °C until use.

Samples for enzymatic assays contained pools of midgut tissues or midgut juice from at least 10 larvae. Samples for RT-qPCR contained pools of midgut tissues from at least 3 larvae.

RNA extraction and RT-qPCR

Total RNA was extracted from midgut tissue samples using PureLink™ RNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Purity and integrity of RNA were checked by electrophoresis on 1% (w/v) agarose gel (1 g of agarose in 100 ml of 40 mM Tris-acetate, 1 mM EDTA, pH 8.3), while quantification was performed by NanoDrop™ One (Thermo Fisher Scientific). cDNA was then obtained by reverse transcription of 1 μg of total RNA using LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA). Relative expression analysis of selected BSFL digestive enzymes genes (i.e. genes encoding trypsin-like, chymotrypsin-like, aminopeptidases N-like, α-amylase-like, and lipase-like enzymes) was performed by RT-qPCR on cDNA, using *HiRPL5* (*Hermetia illucens* Ribosomal Protein L5) as housekeeping gene (Bonelli *et al.*, 2019). All primers designed for RT-qPCR experiments (Supplementary Table S2) were previously tested by standard PCR with DreamTaq™ Green DNA Polymerase (Thermo Fisher Scientific) applying the following thermal conditions: 95 °C for 2 min, 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and final extension of 72 °C for 10 min. All PCR products were sequenced and confirmed by outsourced Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). RT-qPCR experiments were conducted in biological and technical triplicate using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and applying the following thermal conditions: 95 °C for 30 s, 40 cycles at 95 °C for 5 s, 60 °C for 30 s, followed by melt curve analysis under instrument conditions (CFX Connect Real-Time PCR Detection System, Bio-Rad Laboratories). Relative expression was expressed for each replicate as Normalised Relative Quantity (NRQ) ± SEM (Hellemans *et al.*, 2007) using the housekeeping gene expression for the normalization.

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Enzymatic assays on midgut juice

After thawing, samples of midgut juice were recentrifuged at 15,000 × *g* for 10 min at 4 °C and supernatant was used for the enzymatic assays. This step was necessary to guarantee that any food particle or PM residue in the midgut juice stored at –80 °C (see section *Isolation of midgut tissues and midgut juice*), was removed from the specimen to avoid interference with absorbance measurements. Protein concentration was determined by Coomassie Plus™ Protein Assay Reagent (Thermo Fisher Scientific), using bovine serum albumin (BSA) as standard.

Total proteolytic, α-amylase, and lipase activity were measured in midgut juice samples from anterior, middle, and posterior region. In particular, total proteolytic activity was assayed with azocasein (Merck KGaA, Darmstadt, Germany) as substrate, as reported previously (Bonelli *et al.*, 2019; Bonelli *et al.*, 2020). The assay was performed at pH 6.0 for the anterior, pH 5.0 for the middle, and pH 8.5 for the posterior midgut using Universal Buffer (UB) (Bonelli *et al.*, 2019), thus mimicking the pH in the lumen of each midgut region. Alpha-amylase activity was assayed with starch as substrate at pH 6.9, measuring its hydrolysis by the amount of maltose released (Bonelli *et al.*, 2019). Proteolytic and amylolytic activities were measured at their optimum of temperature (i.e. 45 °C), according to previous studies (Bonelli *et al.*, 2019). Lipase activity was assayed using 4-nitrophenyl acetate (Merck KGaA) as substrate and measuring its hydrolysis by the release of 4-nitrophenol (De Caro *et al.*, 1986). The assay was performed in 96-multiwell plates (200 µl final reaction volume). Different volumes of midgut juice were diluted to 196 µl with UB at pH 6.2, and then 4 µl of 225 mM 4-nitrophenyl acetate dissolved in absolute ethanol were added (4.5 mM final substrate concentration). Samples were subjected to continuous absorbance readings at 405 nm for 60 min at 42 °C in a microplate reader (Infinite M Plex, Tecan Group Ltd., Männedorf, Switzerland). One unit (U) of lipase activity was defined as the amount of enzyme that releases 1 µmol of 4-nitrophenol per min per mg of sample proteins (molar extinction coefficient, ε: 6.0 mM^{-1} cm^{-1}). Chymotrypsin and trypsin proteolytic activity in midgut juice from the posterior midgut was assayed with N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAPPpNA, Merck KGaA) and N-Benzoyl-D,L-Arg p-nitroanilide hydrochloride (BApNA, Merck KGaA) as substrates, respectively, in UB at pH 8.5 (Bonelli *et al.*, 2019). All the experiments were conducted at least in triplicate using samples obtained from larvae of at least two independent rearing batches.

Measurement of aminopeptidase N activity in midgut homogenates and in brush border membranes

After thawing, tissue samples were homogenised with a microtube pestle using a motor in ice-cold homogenisation buffer (100 mM mannitol, 10 mM Hepes-Tris, pH 7.2) (1 ml/100 mg tissue) and the suspension was filtered through 3 layers of gauze to remove debris (e.g. tracheae). To obtain brush border membranes (BBM), the homogenate of the posterior midgut was added with $CaCl_2$ (to a final concentration of 10 mM) and blended on ice for 15 min. The suspension was then centrifuged at 3,000 × *g* for 15 min at 4 °C. The pellet was discarded, and the supernatant was centrifuged at 18,000 × *g* for 30 min at 4 °C. The pellet containing BBM was resuspended in 50-100 µl of homogenisation buffer. Protein concentration in homogenates and membrane suspensions was determined by Coomassie Plus™ Protein Assay Reagent (Thermo Fisher Scientific), with BSA as standard.

APN activity was measured as described in Bonelli *et al.* (2019) using leucine-p-nitroanilide as substrate. One unit (U) of APN activity was defined as the amount of enzyme that releases 1 µmol of p-nitroaniline per min per mg of sample proteins (molar extinction coefficient, ε: 9.9 mM^{-1} cm^{-1}).

All the experiments were conducted at least in triplicate using samples obtained from larvae of at least two independent rearing batches.

Statistical analysis

The effect of the diet on larval maximum weight and on indexes related to larval performance and bioconversion process was analysed using generalised linear models (GLMs) with proper distribution. The '*glm*' and '*dropI*' functions from the stats package were used to perform the analysis and obtain the *P* values. Statistical differences between groups were considered significant at *P* value < 0.05. This statistical analysis was performed in R environment (R version 4.1.0).

The Two-Way ANOVA statistical analysis followed by the Holm-Šídák's post-hoc test has been used in RT-qPCR experiments to assess the statistical significance of the differences in NRQ among the same midgut region from larvae reared on the two different diets. Statistical differences were considered significant at *P* value < 0.05. This statistical analysis was performed with GraphPad Prism (version 9).

Welch Two Sample *t*-test was performed to evaluate differences in the enzymatic activity among the same midgut region from larvae reared on the two different diets. Statistical differences between groups were con-

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TABLE 1 Chemical composition and moisture content of the two experimental rearing substrates. Values (g/100 g of diet) are expressed on "as fed" (calculated on the diet as fed to the larvae -taking into consideration the water content-) and "dry matter" (moisture-free) basis

	s-OFMSW		Modified s-OFMSW	
Component	As fed	Dry matter (%)	As fed	Dry matter (%)
Crude protein	7.9	22.1	3.1	14.7
Crude lipid	2.9	8.0	0.9	4.1
Crude fibre[a]	0.1	0.4	0.4	1.8
Nitrogen-free extract[b]	23.6	66.1	16.2	76.0
Ash	1.2	3.4	0.7	3.4
Hemicellulose[c]	4.1	11.6	2.2	10.3
Cellulose[c]	0.4	1.2	0.6	2.7
Lignin[c]	0.4	1.2	0.4	2.0
Starch	14.8	41.5	7.8	36.5
Glucose and fructose	2.7	7.4	4.6	21.6
Water content	64.3	–	78.7	–

[a]Includes most of cellulose and insoluble lignin.[b]Includes sugars, organic acids, pectins, soluble lignin, hemicellulose, and a small percentage of cellulose. [c]Values calculated from neutral and acid detergent fibre analyses.

sidered significant at P-value < 0.05. This statistical analysis was performed in R environment (R version 4.1.0).

3 Results

Nutrient content of the rearing substrates

The analysis of the nutrient content of s-OFMSW and modified s-OFMSW revealed that the two substrates were considerably different in terms of chemical composition (Table 1). In particular, s-OFMSW had a higher amount of the main macronutrients (i.e. crude protein, crude lipid, and starch) both on "as fed" and "dry matter" basis. Unlike starch, monosaccharides (i.e. glucose and fructose) were more abundant in modified s-OFMSW (Table 1), probably because of the higher fruit content of this substrate (Supplementary Table S1).

Since proteins are essential macronutrients, the amount of crude protein in the rearing substrates is important to estimate their nutritional value and quality. Nevertheless, since the nutritional value of proteins can vary depending on their digestibility and amino acid content (e.g. amount of essential amino acids and ratio between essential and non-essential amino acids), the amino acid profile of s-OFMSW and modified s-OFMSW was analysed (Figure 1). The total amino acid content (Figure 1A, Supplementary Table S3), as well as the content of each amino acid (Figure 1B, Supplementary Table S3), were higher in s-OFMSW compared to modified s-OFMSW, thus reflecting the different content of crude protein in the two rearing substrates. However, in both diets all essential amino acids were present and accounted for 40% of the total amino acid content (Figure 1B, Supplementary Table S3).

The effect of the different nutrient content of the diets (e.g. 2.5:1 for crude protein content, 3:1 for crude lipid content, and 2:1 for starch on "as fed" basis of s-OFMSW *versus* modified s-OFMSW, and lower amount of amino acids in modified s-OFMSW) on BSFL was determined by evaluating their growth performance, the efficiency of the bioconversion process, the chemical composition of the insect biomass, and the digestion capabilities of the insects.

Larval growth performance and bioconversion efficiency

Firstly, the potential impact of the different nutrient content of the rearing substrates on larval growth was addressed. Unexpectedly, the developmental time and maximum weight of the larvae were not affected by the diet, as insects completed the larval stage in 19 days in both cases and their maximum weight on the two substrates was not statistically different (duration of larval stage (day): 19.0 ± 0.6 for s-OFMSW and 18.7 ± 0.3 for modified s-OFMSW, P = 0.9251; maximum weight (mg): 260 ± 8 for s-OFMSW and 270 ± 5 for modified s-OFMSW, P = 0.3709; values obtained from three independent experiments) (Figure 2).

Moreover, relative growth rate (RGR) confirmed that the growth performance of BSFL on the two

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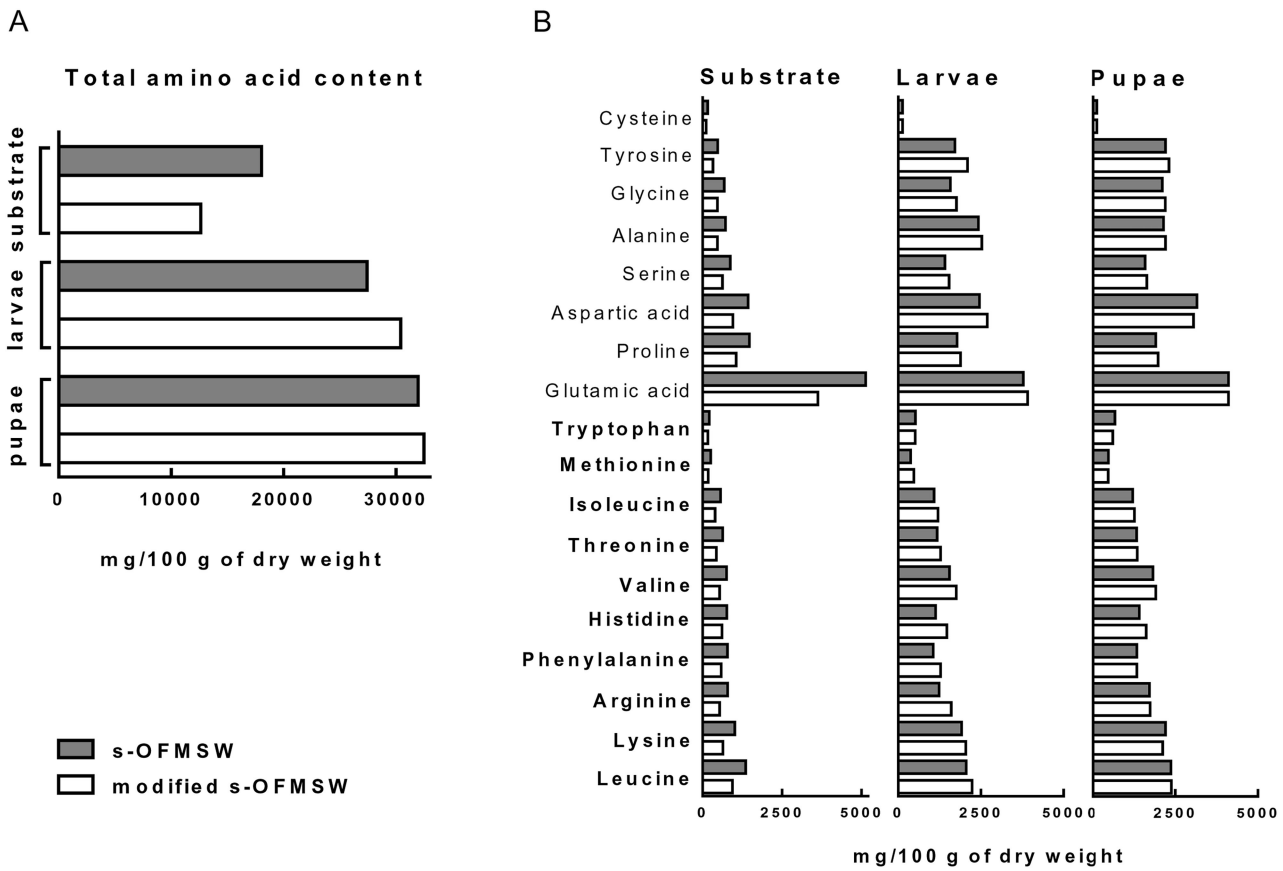

FIGURE 1 Analysis of amino acids profile. Content of total amino acids (A) and of each amino acid (B) expressed as mg/100 g of dry matter in s-OFMSW, modified s-OFMSW, larvae, and pupae. Essential amino acids are indicated in bold. Asparagine and glutamine contents are included respectively in Aspartic acid and Glutamic acid contents.

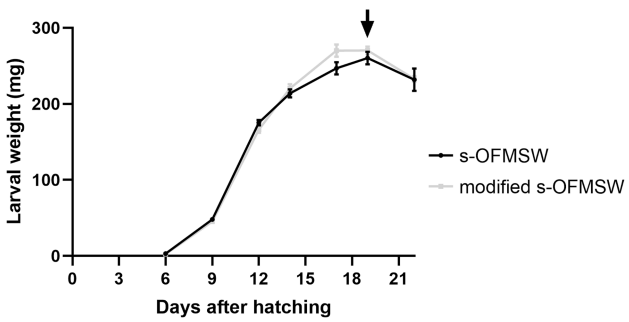

FIGURE 2 Growth curves of BSFL reared on s-OFMSW and modified s-OFMSW. The weight of larvae reared on s-OFMSW (black line) and modified s-OFMSW (grey line) was recorded until 25% of insects reached pupal stage (end of the bioconversion process). The arrow indicates the moment in which the maximum weight was reached. This day was considered the end of the larval stage (arrow). Then, insects entered prepupal stage and stopped feeding.

feeding substrates was not statistically different (P = 0.9323) (Table 2). However, the composition of the substrate influenced the feed conversion efficiency, as indicated by indexes related to the bioconversion process (Table 2). In particular, waste reduction index (WRI) and the related substrate reduction (D) were significantly lower for modified s-OFMSW, although larvae reared on this diet displayed higher efficiency in the conversion of ingested food (ECI) and nitrogen conversion efficiency (NCE). Finally, the survival rate (SR), although different in the two substrates, was higher than 90% for both diets.

Chemical composition of insect biomass

Despite the significant difference in the nutrient content of the two diets, the chemical composition of larvae -and pupae- reared on the two substrates was unexpectedly comparable (Table 3). In particular, the larvae on both diets were characterised by a similar protein and lipid content. The same pattern was observed for pupae (Table 3).

The total amino acid content (expressed as mg/100 g dry weight) was comparable in pupae obtained from both rearing substrates, while larvae reared on modified s-OFMSW showed a slightly higher content of total amino acid and of each amino acid -except tryptophan- compared to their counterparts on s-OFMSW, although the latter diet had a higher amount of amino acids com-

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TABLE 2 Indexes to evaluate the growth performance and the efficiency of the bioconversion process of BSFL reared on s-OFMSW and modified s-OFMSW. All indexes were calculated on dry matter basis. Asterisks indicate statistically significant effect of the diet. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Index	s-OFMSW	Modified s-OFMSW
Relative growth rate (RGR)	0.08 ± 0.0	0.08 ± 0.0
Substrate reduction (D) (%)	83.5 ± 2.8	71.9 ± 1.6*
Waste reduction index (WRI) (%)	6.4 ± 0.2	5.5 ± 0.1*
Efficiency of conversion of ingested food (ECI) (%)	20.8 ± 0.8	26.9 ± 1.2*
Nitrogen conversion efficiency (NCE) (%)	33.9 ± 1.1	56.6 ± 2.2***
Survival rate (SR) (%)	91.1 ± 1.3	99.6 ± 0.1**

TABLE 3 Chemical composition of larval and pupal biomass. Values are expressed as percentage on dry matter

	s-OFMSW		Modified s-OFMSW	
Component	Larvae	Pupae	Larvae	Pupae
Crude protein	36.2	42.3	38.1	40.8
Crude lipid	43.7	40.1	44.9	42.7
Nitrogen-free extract[a]	11.2	7.1	7.5	5.5
Ash	3.7	2.8	3.1	3.6
Chitin	5.2	7.7	6.4	7.4

[a]Includes sugars, organic acids, pectins, soluble lignin, hemicellulose, and a small percentage of cellulose.

pared to modified s-OFMSW (Figure 1, Supplementary Table S3). Moreover, the amount of each amino acid - except cysteine and alanine for s-OFMSW, and cysteine, alanine, and methionine for modified s-OFMSW- and the total amino acid content were higher in pupae than in larvae for both rearing substrates (Figure 1, Supplementary Table S3). Interestingly, the amount of each amino acid in larvae and pupae was always higher than in the feeding substrate, except glutamic acid/glutamine and cysteine in larvae reared on s-OFMSW (Figure 1, Supplementary Table S3).

Despite these slight differences in amino acid composition between larvae and pupae reared on the two substrates, insects displayed a similar chemical profile. This evidence may be explained by the ability of the larvae to modulate midgut functionality, in particular digestive capabilities, to meet their nutritional requirements even when the rearing substrates have a different nutrient content. To address this aspect, the expression and the activity of key digestive enzymes were evaluated in the three morphofunctional distinct regions of the midgut: anterior, middle and posterior (Bonelli *et al.*, 2019).

Gene expression analysis of digestive enzymes

Starting from a *de novo* transcriptome of BSFL midgut and the analysis of differentially expressed genes in the midgut of larvae reared on diets with different nutrient content (Bonelli *et al.*, 2020), transcripts encoding digestive enzymes (i.e. endo- and exopeptidases, amylases, and lipases) were selected to evaluate if and how the different nutrient content of the diets altered their expression profile. In particular, nine transcripts encoding endo- and exopeptidases involved in protein digestion were examined: three transcripts encoding trypsin-like proteins (Try_DN11026, Try_DN13300, and Try_DN11081), four encoding chymotrypsin-like proteins (Chy_DN12383, Chy_DN8352, Chy_DN8552, and Chy_DN9167), and two encoding membrane GPI-anchored APN-like (APN_DN10599 and APN_DN10282). RT-qPCR expression analysis of genes encoding enzymes involved in protein digestion showed that: (1) regardless of the rearing substrate, their expression was recorded mostly in the posterior midgut; (2) a significant number of the selected endopeptidase-like genes changed their expression profile depending on the rearing substrate; and (3) transcripts encoding membrane GPI-anchored APN did not show any significant variation (Figure 3, Supplementary Table S4). These results strongly suggest that protein digestion could be regulated to optimally exploit diets with different protein content.

Regarding carbohydrate and lipid digestion, two transcripts encoding α-amylase-like proteins (α-Am_DN796

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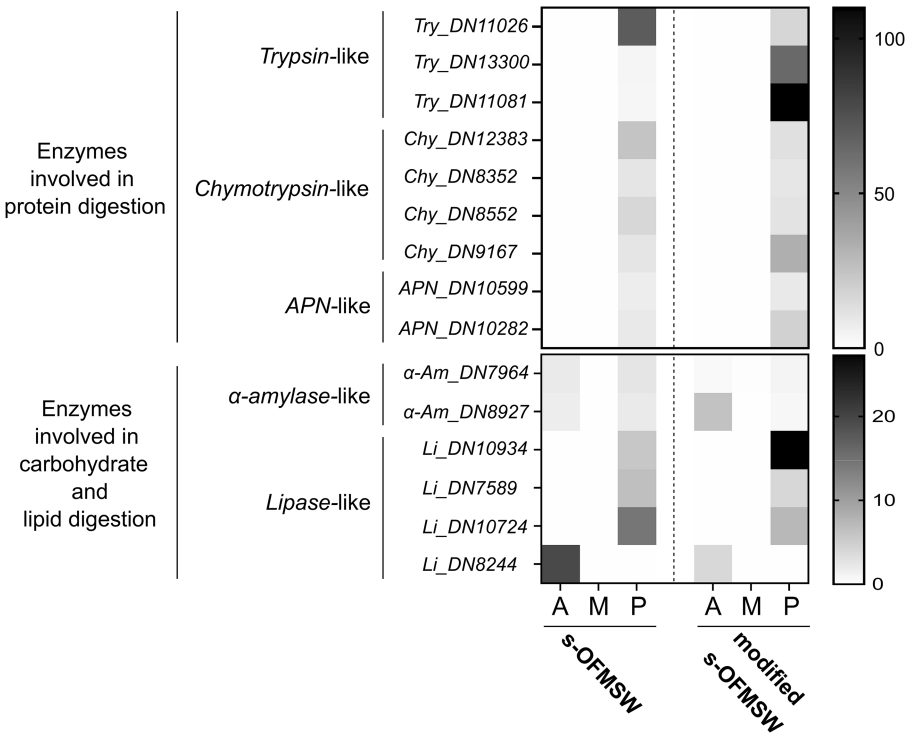

FIGURE 3 Expression profile of BSFL midgut genes involved in the digestion of proteins (trypsin- and chymotrypsin-like, aminopeptidase N (APN)-like), carbohydrates (α-amylase-like), and lipids (lipase-like) of larvae grown on different diets (s-OFMSW and modified s-OFMSW). The expression level of each gene is reported in the heatmap as the mean of Normalised Relative Quantity (NRQ) of respective midgut regions (A = anterior midgut; M = middle midgut; P = posterior midgut). The detailed relative expression values and statistic data for each gene are reported in Supplementary Table S4.

4_i1 and α-Am_DN8927) and four encoding lipase-like proteins (Li_DN10934, Li_DN7589, Li_DN10724, and Li_DN8244) were selected. Regardless of the rearing substrate, the expression levels of all genes were negligible in the middle midgut (Figure 3, Supplementary Table S4). The expression levels of both transcripts encoding α-amylase-like proteins varied in the anterior midgut (Figure 3, Supplementary Table S4). Concerning lipases, only one of the selected transcripts (Li_DN8244) was expressed in the anterior midgut and its expression levels decreased in the modified s-OFMSW (Figure 3, Supplementary Table S4); moreover, regardless of the rearing substrate, this gene was not expressed in the posterior midgut. The other three transcripts encoding lipase-like proteins were expressed only in the posterior midgut of larvae reared on both diets and one of them (i.e. Li_DN10934) showed a different expression pattern in the two conditions (Figure 3, Supplementary Table S4).

The overall results indicated that differences in nutrient composition of the rearing substrate drove significant transcriptional regulation of digestive enzymes (the abundance of eight transcripts out of fifteen was significantly different in at least one midgut tract). This evidence prompted us (1) to investigate whether the activity of digestive enzymes in larvae grown on the two diets were different, and, in this case, (2) to examine whether these differences were in line with the results of gene expression analysis. Indeed, the extent of the hydrolytic activity of an enzyme may not always reflect its transcription level, the timing of the transcriptional regulation may not overlap the variation in enzymatic activity, and, for each hydrolytic enzyme family, transcripts that have not been analysed herein may account for significant changes in the overall luminal activity.

Activity of digestive enzymes in the different midgut regions

Since a significant transcriptional regulation of genes encoding digestive enzymes induced by diet composition was demonstrated (Figure 3), the activity of hydrolytic enzymes involved in protein, carbohydrate, and lipid digestion in larvae reared on the two diets was evaluated.

The total proteolytic activity was almost exclusively present in the midgut juice isolated from the posterior region, regardless of the rearing substrate (Figure 4A), confirming that protein digestion mainly occurs in this district where optimal alkaline pH for serine protease activity is present (Bonelli *et al.*, 2019; Bonelli *et al.*,

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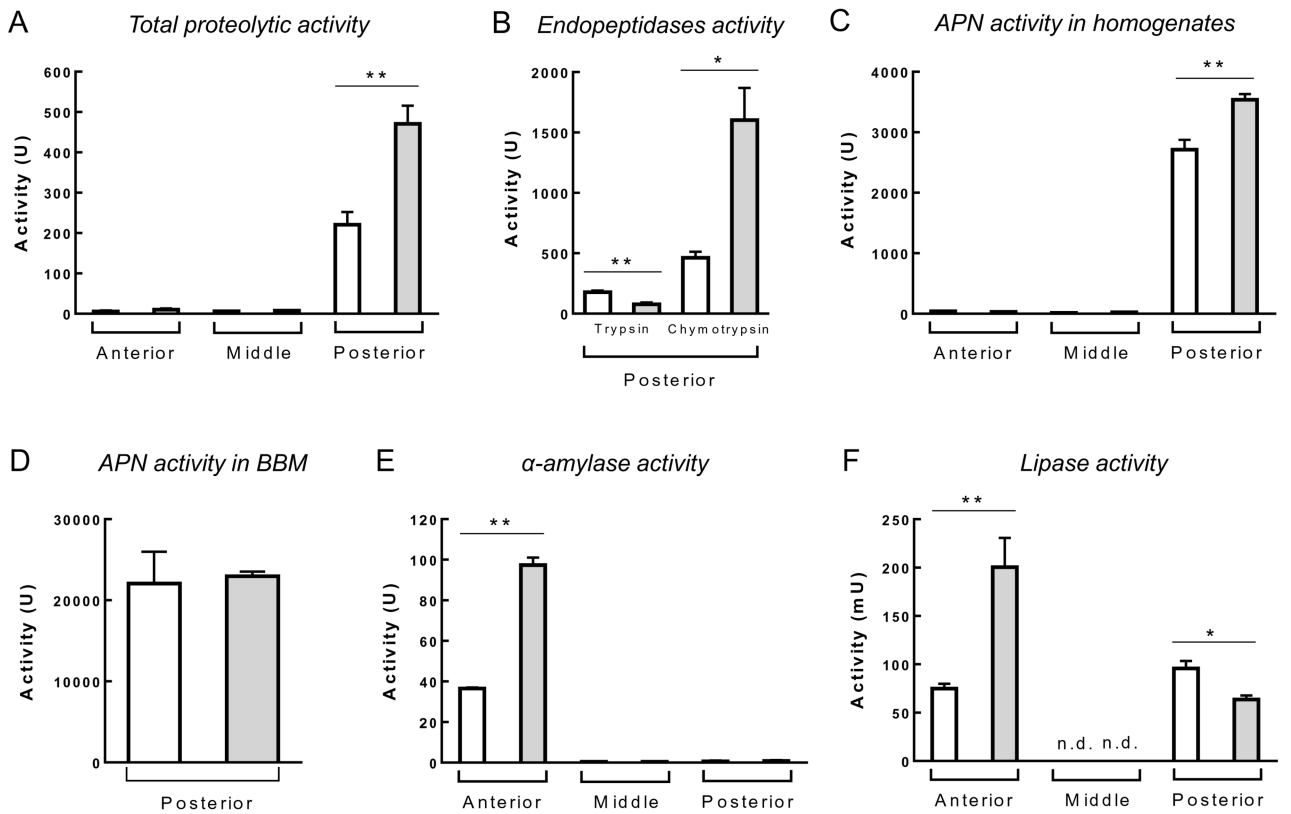

FIGURE 4 Enzymatic activities in the midgut juice (A, B, E, F), midgut tissue homogenate (C), and brush border membranes (BBM) (D) from larvae reared on s-OFMSW (white bars) and modified s-OFMSW (grey bars). Total proteolytic activity in midgut juice from the three midgut regions (A). Trypsin and chymotrypsin (B) activity in midgut juice isolated from the posterior midgut. Aminopeptidase N (APN) activity in the homogenate of the three different midgut regions (C) and in BBM from posterior midgut (D). Alpha-amylase (E) and lipase (F) activity in midgut juice from the three midgut regions. n.d. = non-detectable activity. The values are reported as mean ± SEM of at least 3 experiments. Asterisks indicate statistically significant differences; * $P < 0.05$; ** $P < 0.01$.

2020). Nevertheless, a significantly higher activity was observed in larvae reared on modified s-OFMSW compared to s-OFMSW (Figure 4A), due to a higher activity of chymotrypsin-like proteases (Figure 4B). This evidence demonstrated that the capability of protein digestion in BSFL was strictly dependent on the protein content of the rearing substrate and, in particular, that a positive modulation of the proteolytic activity occurred when the larvae were reared on modified s-OFMSW, the substrate with the lowest content of crude protein (Table 1). These results are in agreement with previous data that showed a positive modulation of the proteolytic activity in BSFL reared on substrates with low protein content, an effect driven by an increase in chymotrypsin-like activity (Bonelli *et al.*, 2020).

To evaluate differences in the final phase of protein digestion, the activity of APN, an exopeptidase responsible for the release of single N-terminal amino acids from peptides, was measured. APN activity was significantly higher in homogenates of the posterior midgut of larvae reared on modified s-OFMSW (Figure 4C), supporting the results on total proteolytic activity (Figure 4A). Nevertheless, APN activity in BBM, to which the enzyme is anchored (Caccia *et al.*, 2019; Terra and Ferreira, 1994), did not show significant differences (Figure 4D). This evidence, supported by molecular data (Figure 3), may be explained by a higher activity of intracellular APN in the posterior region of larvae reared on modified s-OFMSW. Indeed, APNs can be found in vesicles at the base of microvilli, endoplasmic reticulum, and lysosomes, although the function of intracellular APNs is not always related to their enzymatic activity (Danielsen *et al.*, 1995; Hansen *et al.*, 1987; Hansen *et al.*, 1989; Matsui *et al.*, 2006).

Carbohydrate digestion mainly occurred in the anterior midgut as demonstrated by the significant α-amylase activity recorded only in this region on both rearing substrates (Figure 4E). However, a 2.7-fold increase in the activity in the anterior tract of larvae grown on modified s-OFMSW compared to s-OFMSW (Figure 4E) was measured. This increase may depend on the lower

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starch content (on "as fed" basis, Table 1) in modified s-OFMSW compared to s-OFMSW.

Lipase activity was recorded in the anterior and posterior midgut (Figure 4F), regardless of the rearing substrate, with the highest activity observed in the anterior midgut of larvae reared on modified s-OFMSW (Figure 4F).

4 Discussion

BSFL are widely employed as bioconversion agents for various organic wastes and by-products due to their nutritional adaptability, primarily attributed to the morphofunctional plasticity of the midgut and the associated microbiota (Bonelli *et al.*, 2020; Bruno *et al.*, 2019a; De Smet *et al.*, 2018; Eke *et al.*, 2023; Sun *et al.*, 2022; Vandeweyer *et al.*, 2023). The findings of the current study not only confirm this previous evidence, but demonstrate that, regardless of the nutrient content of the OFMSW used as rearing substrate, the fine modulation of BSFL digestive system guarantees comparable and adequate growth performance, efficiency of the bioconversion process, and quality of the insect biomass.

Despite the significant differences in nutrient composition between the two OFMSW utilised in this study, the growth rate and maximum weight of BSFL were similar to those observed with nutritionally balanced diets (Bonelli *et al.*, 2020; Eggink *et al.*, 2022 Jucker *et al.*, 2020; Pliantiangtam *et al.*, 2021). Furthermore, despite slight variations in indexes related to bioconversion efficiency, the chemical composition of BSFL -and pupae- reared on both OFMSW was quantitatively and qualitatively satisfactory, if compared to other organic substrates (Amrul *et al.*, 2022; Surendra *et al.*, 2020). In fact, regardless of the OFMSW and life stage, the amount of key components in the insect biomass, such as crude protein, crude lipid, and chitin (i.e. 39.4, 42.9, and 6.8% on dry matter, respectively; values represent the mean of percentages reported in Table 3), was consistent with, or even exceeded, that obtained through the bioconversion of other organic wastes and by-products (30-50% for crude protein, 20-47% for crude lipid, and 2-6% for chitin, on dry matter) (Amrul *et al.*, 2022; Eggink *et al.*, 2022; Surendra *et al.*, 2020).

Significantly, the percentage of crude protein in larva and pupa samples was consistent with the total amino acid content. Generally, the amount of crude protein is calculated from the total nitrogen content of the biomass by applying a nitrogen-to-protein conversion factor (Kp) of 6.25. However, since in insects the non-protein nitrogen is not negligible due to the presence of chitin, a more appropriate conversion factor (Kp = 5.6) has been empirically determined for insects (Boulos *et al.*, 2020; Janssen *et al.*, 2017). The data presented in this study not only support the accuracy of these determinations, but also demonstrate that a conversion factor of 5.6 is applicable for BSF biomass, irrespective of the developmental stage and rearing substrate.

The protein content of the biomass is a major outcome in insect-mediated bioconversion and, in this regard, BSF is particularly efficient (Anankware *et al.*, 2021; Fowles and Nansen, 2019; Surendra *et al.*, 2020). The amino acid profile of the protein fraction, expressed as percentage of crude protein in the two OFMSW, is comparable. Interestingly, certain amino acids showed an increase in the insect biomass compared to the substrate, indicating the insects' ability to accumulate these important building blocks. In particular, the content of the essential amino acid tryptophan and the non-essential amino acid tyrosine increased by approximately 60% and 130%, respectively, in both larvae and pupae compared to the rearing substrates. These aromatic amino acids play a crucial role in insect growth and development and are precursors of important biological molecules, such as neurotransmitters, vitamins, and melanin which is involved in sclerotization and pigmentation of the cuticle, as well as in immune responses (Marieshwari *et al.*, 2023; Tomberlin *et al.*, 2023). The distinctive spectrum of amino acids in BSFL grown on OFMSW could confer unique characteristics to the biomass, opening avenues for the optimisation of protein-based materials or the development of alternative and novel applications (Qazanfarzadeh and Kumaravel, 2023).

The differences in nutritional composition of the two experimental OFMSW triggered a significant modulation of the larvae' digestive machinery at the transcriptional level. This was accompanied by a change in the activity of crucial enzymes involved in macromolecule digestion. The digestive functions along the midgut appeared to be regionalised as previously observed (Bonelli *et al.*, 2019; Bonelli *et al.*, 2020), with carbohydrate digestion mainly occurring in the anterior midgut, protein digestion mostly in the posterior midgut, and lipid digestion in both the anterior and posterior midgut; the middle midgut was confirmed to be only partially involved in digestive functions. In particular, an increase in total proteolytic (mainly due to chymotrypsin activity), amylolytic, and lipolytic activities was correlated with a lower content of crude protein, starch, and lipid contents in the rearing substrate, respectively, in agree-

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ment with previous studies on BSFL and other insects (Bonelli *et al.*, 2020; Lazarević and Janković-Tomanić, 2015; Lehane *et al.*, 1995). The efficiency of this modulation was reflected in a significantly higher NCE index (i.e. +60%) for larvae reared on the modified s-OFMSW. Indeed, this mechanism allowed the larvae to compensate for the relatively low quantity of proteins in this rearing substrate, as confirmed by the same crude protein amount of insects grown on both diets, despite their different protein content. It is worth noting that differences in the expression levels of genes encoding hydrolytic enzymes did not always align with changes in the corresponding enzymatic activity measured in the lumen of BSFL midgut. This discrepancy may be attributed to the role of midgut microbiota in nutrient digestion (Callegari *et al.*, 2020; Engel and Moran, 2013; Jaffar *et al,*. 2022) and by the not always observed correlation between transcript abundance and relative hydrolytic activity against specific nutrients (Bonelli *et al.*, 2020; Liu *et al.*, 2016; Rocca *et al.*, 2015).

In summary, the results presented here corroborate previous findings on the significant potential of BSFL in the bioconversion of OFMSW (Diener *et al.*, 2011; Ferronato *et al.*, 2023; Papa *et al.*, 2022; Purkayastha and Sarkar, 2022) and demonstrate that the extraordinary plasticity of BSFL digestive system is a key factor for OFMSW bioconversion. This evidence is particularly noteworthy from an applicative perspective as it demonstrates that (1) BSFL can effectively valorise the OFMSW and potential variations in its composition does not influence the quality of the bioconversion process, and (2) the resulting insect biomass contains a substantial percentage of protein, lipids, and chitin, making it suitable for the production of bioplastics, biomaterials, and biodiesel (Ishak and Kamari, 2019; Tettamanti and Bruno, 2024). It must be underlined that the recycling rate of biowaste is not comparable to the considerable growing trend of paper, cardboard, glass, metals, and plastic recycling (European Environment Agency, 2013). Hence, a more focused approach to biowaste collection and recycling is essential, along with the development of new treatment strategies to complement existing methods, such as composting and anaerobic digestion.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.26135329

Conflict of interest

The authors declare no conflict of interest.

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