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# *Hermetia illucens* (Diptera: Stratiomydae) larvae and prepupae: Biomass production, fatty acid profile and expression of key genes involved in lipid metabolism

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ABSTRACT

The Black Soldier Fly (BSF) *Hermetia illucens* provides a promising strategy in the waste valorisation process and a sustainable alternative source of valuable nutrients, including lipids for food and feed. In the present study, the differences in growth performances and nutritional values of BSF V instar larvae and prepupae reared on vegetable waste were analyzed and compared focusing on fat content. V instar larvae showed higher capacity to bioconvert the substrate into biomass than prepupae. The nutritional composition and the fatty acid profiles were dependent on the developmental stage. The expression levels of *acetyl-CoA carboxylase* (*acc*), *fatty acid synthase* (*fas*), *lipase* (*lip*) and *acyl-CoA dehydrogenase* (*acd*) genes involved in the lipid metabolism pathway and herein characterized for the first time, were evaluated in order to understand the molecular basis underlying the observed differences in fatty acid profiles. Our results suggest that the different fatty acid profiles of BSF V instar larvae and prepupae may be related to the modulation of the lipid metabolism-related genes expression during larval development. Our study highlights substantial differences between *H. illucens* V instar larvae and prepupae giving important features regarding the opportunity to modulate the preferable fatty acid profile to meet the industrial requirements.

## 1. Introduction

In recent years, many authors showed that several insects species, especially during their larval development, have a high content in fats, proteins and other nutritive components besides fibres (Fao and van Huis, 2013). Although the nutritional values are highly variable among the different species of both edible (Kouřimská and Adámková, 2016) and feeder insects, it has been established that insect species are characterized by high fat content (Barroso et al., 2014; Ramos-Elorduy, 1997), therefore opening fascinating conceivable outcomes for several applications, such as food and feed yet additionally for other industrial purposes, as biodiesel production (Surendra et al., 2016).

Among insects studied for animal feed, the Black Soldier Fly (BSF) *Hermetia illucens* is one of the richest in lipids (Ramos-Bueno et al., 2016), for up to 45% (Ushakova et al., 2016) making it a natural resource of biologically active hydrophobic substances; moreover, its interest is also due to its high efficiency as bio-converter of organic waste during larval development (Salomone et al., 2017); for these reasons, BSF is considered one of the most promising species for mass production.

Several insect species have been considered as alternative source of nutrients in feed for aquaculture and recently the European Commission (Regulation 2017/893/EC) allowed the use of insects, including *Hermetia illucens*, in feed formulation. Substitution of fish meal

*Abbreviations:* BSF, Black Soldier Fly; BSFL, Black Soldier Fly Larvae; CP, crude protein; CF, crude fat; ADF, acid detergent fibers; NDF, neutral detergent fibers; GR, Growth Rate; $ECD_F$, Efficiency of conversion of digested feed; FCR, Feed Conversion Ratio; BR, Bioconversion Ratio; SR, Substrate Reduction (SR); FA, fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; *acc*, *acetyl-CoA carboxylase*; *fas*, *fatty acid synthase*; *lip*, *lipase*; *acd*, *acyl-CoA dehydrogenase*; *16s rRNA*, *16s ribosomal RNA*; *18s rRNA*, *18s ribosomal RNA*; *ef1-α*, *elongation factor*

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with BSF meal has been evaluated in diverse fish species diets (Kroeckel et al., 2012; Lock et al., 2016; Muin et al., 2017) both as aquafeed ingredient (Cardinaletti et al., 2019; Zarantoniello et al., 2018) or as single feed (Vargas et al., 2018); results showed the maintenance of growth performance and health parameters thus demonstrating that BSF meal could be a potential solution to the current use of fish meal in aquaculture.

Several authors applied different growing substrate to BSF larvae quantifying their proximate composition (Arango Gutiérrez et al., 2004; Li et al., 2011; Manzano-Agugliaro et al., 2012; Oonincx et al., 2015; Rachmawati et al., 2017; Sheppard et al., 1994; St-Hilaire et al., 2007; Veldkamp and Bosch, 2015; Zheng et al., 2012). To date, it is well accepted that the nutritional composition of BSF depends of the quality and quantity of food ingested (Gobbi et al., 2013; Nguyen et al., 2015; Pimentel et al., 2017), the fat content resulting the most variable (from 7 to 39% dry matter) (Barragan-Fonseca et al., 2017). In the lipid extracts of *H. illucens*, fatty acids have been reported as the most conspicuous component with a large proportion of lauric acid characterized by an important antibacterial and antiviral activity. The myristic, palmitic and stearic saturated as well as hexadecenoic and octadecenoic unsaturated acids have also been found; on the contrary, very low concentrations of branched acids and polyunsaturated fatty acids has been found (Oonincx et al., 2015; Ushakova et al., 2016).

Noteworthy, important changes in proximate composition throughout the larval development may occur, but little information has been published on this relevant feature of *H. illucens* physiology. Early studies on the nutritional composition of BSF reported a similar content of crude fat in BSF last instar larvae and prepupae reared on an undefined substrate (Veldkamp and Bosch, 2015); Rachmawati et al. (2017) found a reduction of protein content with age together with an increasing of dry matter content in the later instars. Only recently, Liu et al. (2017) explored the fluctuations in the nutrient content during whole life cycle showing marked variations in the nutritional composition depending on the developmental stage of BSF reared on chicken feed but further investigations are needed to better clarify this important aspect.

Despite the recognized importance of the biochemical composition of BSF larval biomass for future applications in industrial purposes including feed sector, to date the molecular mechanisms defining different metabolic phenotypes are far to be elucidated. Besides, the lipid metabolism pathway in insects has been generally investigated in relation to diapause (Arrese et al., 2010; Canavoso et al., 2001; Gondim et al., 2018; Reynolds et al., 2012; Sim and Denlinger, 2009). At present, the information about lipid metabolism pathway in *H. illucens* is still very limited as well as reports on molecular studies concerning the genes involved in lipid metabolism (Pimentel et al., 2017; Zhu et al., 2019). In previous studies concerning BSF larvae as a candidate species to be used for animal feed, prepupa was the stage most frequently considered for analysis of nutritional composition (Liu et al., 2017; Surendra et al., 2016). However, no scientific reports established that this is the most suitable stage to be used as feed.

Huyben et al. (2019) evaluated the differences in gut microbiota of rainbow trout (*Oncorhynchus mykiss*) fed with *H. illucens* larvae or prepupae demonstrating that the gut bacteria composition in this species is also influenced by the developmental stage of the insect used as meal. These data suggest that larval and prepupal life stages could have different biochemical and nutritional properties that deserve to be deepened before using *H. illucens* for different industrial purposes. To the best of our knowledge, there are no published data comparing differences in nutritional profiles together with the analysis of fat metabolism pathway between the larval and prepupal stages in BSF reared on vegetable waste. In this study, we cloned and characterized, for the first time, four key genes associated with lipid metabolism in insects (Sim and Denlinger, 2009): two of them are implicated in fatty acid biosynthesis in insects, including *acetyl-CoA carboxylase* (acc) and *fatty acid synthase* (*fas*) - whose enzymes catalyze the synthesis of malonyl-CoA from acetyl-CoA and the sequential addition of two-carbon units to an acetyl-CoA primer to make the fatty acid; the other two play a role in lipid degradation, namely *lipase* (*lip*) and *acyl-CoA dehydrogenase* (*acd*) - whose enzymes hydrolyze triglycerides to yield glycerol and free fatty acids, and shorten the acetyl groups of fatty acids, subsequently producing ATPs by β-oxidation of acetyl-CoA, respectively. Their gene expression was evaluated in two different developmental stages, V instar larvae and prepupae, in order to give insights into the molecular mechanisms underlying the potential differences in fat composition during BSF larval development.

The present study was aimed to (*i*) evaluate the BSF V instar larvae and prepupae potential for producing valuable biomass during the bioconversion process, (ii) analyze their nutritional composition focusing on the fatty acid composition and (*iii*) investigate the expression of key genes involved in lipid metabolism in the selected developmental stages of *Hermetia illucens* reared on vegetable waste. Such information could shed light on lipid metabolism of both developmental stages highlighting any differences that may be taken into consideration when selecting the most suitable BSF stage for future applications of the larval biomass in industrial purposes, as animal feed and pharmaceutical sectors or biodiesel production.

## 2. Materials and methods

### 2.1. Rearing substrate and larval feeding experiment

The organic waste used for feeding larvae was composed of a mix of vegetable and fruit wastes (40% pears, 45% banana, 5% tomatoes, 10% various leafy green vegetables). The substrate was ground with a 3 mm die meat mincer (Fama Industrie, Italy) before being used to feed the larvae. A sample of substrate was dried and placed under vacuum for chemical analysis.

Specimens of *Hermetia illucens* were bred and collected from an established colony (www.progettohermetia.it) (Italy). After hatching, BSF larvae were put on a mix of flour (35%) and water (65%) for 5 days. Then the larvae were distributed in six plastic boxes (27 cm x 17 cm x 5 cm), kept at a temperature of 27/32 °C, humidity > 70% and fed every other day until the end of the feeding phase using a uniform feeding rate of 75 mg larva$^{-1}$ day$^{-1}$. The developmental larval stages were recognized following published criteria (Kim et al., 2010) and V instar larvae were collected from three replicates tanks. In the other three replicates tanks, instead, the larvae were allowed to grow until they had changed (in colour) from cream to black/brown prepupae (Tomberlin et al., 2009). The V instar larvae and prepupae samples were collected, weighed (fresh weight) and set at 4 °C to allow quiescence to be reached. Samples were either stored at −80 °C for biomolecular analyses or killed by freezing, dried at 70 °C until a constant weight and ground for chemical analysis.

### 2.2. Vegetable waste bioconversion and biomass production efficiency

The performance of vegetable waste bioconversion by BSF V instar larvae and prepupae was evaluated to estimate the biomass production perspective. The growth performance and the bioconversion indices of BSF V instar larvae and prepupae were evaluated by applying the equations below, as described previously (Banks et al., 2014; Diener et al., 2009; Leong et al., 2016; Meneguz et al., 2018; Mohd-Noor et al., 2017; Nyakeri et al., 2017). The value of the weight gained was used to calculate the Growth Rate (**GR**) (Eq. 1). The efficiency of the BSF to consume and therefore reduce organic matter content in the fed substrates was determined by calculation of Substrate Reduction **(SR)** (Eq. 2); the feed conversion into increased body mass efficiency was evaluated by the Feed Conversion Ratio (**FCR**) (Eq. 3) and the Bioconversion Ratio (**BR**) (Eq. 4). Moreover, the efficiency of conversion of digested feed ($\mathbf{ECD_F}$) (Eq. 5) was calculated:

$$(GR) = \frac{[\text{final body weight–initial body weight}]\ (g)}{\text{trial duration(d)}} \quad (1)$$

$$(SR\%) = \frac{[\text{total feed added–residue feed}](g)}{\text{total feed added(g)}} \times 100 \quad (2)$$

$$(FCR) = \frac{\text{feed added(g)}}{\text{total biomass(g)}} \quad (3)$$

$$(BR\%) = \frac{\text{total biomass(g)}}{\text{total feed added(g)}} \times 100 \quad (4)$$

$$(ECD_F) = \frac{[\text{final larvae dry weight–initial larval dry weight}](g)}{[\text{total dry feed offered–dry residue remained}](g)} \quad (5)$$

The all parameters were calculated in grams on a dry matter basis.

### 2.3. Chemical analysis

Dried substrate, V instar larvae and prepupae samples were analyzed for dry matter (DM), ash, crude protein (CP), crude fat (CF), acid detergent fibers (ADF), neutral detergent fibers (NDF) and fatty acids. The chitin content in the V instar larvae and prepupae samples was estimated by calculating the acid detergent fibers (ADF) and subtracting to this value the amino acids (by weight) contained in the ADF residue (%) as described by Finke (2013). Moreover, the protein values were corrected subtracting the chitin nitrogen from the total nitrogen as described by Spranghers et al. (2017).

#### 2.3.1. Proximate composition of V instar larvae, prepupae and substrate

Dry matter, ash and protein content were determined according to Chemists (1995). Total fat content was determined gravimetrically following the method of Bligh and Dyer (1959). ADF and NDF were determined by the method of Van Soest et al. (1991). All the chemical analyses were carried out in triplicate. The results were expressed as % (w/w) and reported as mean values ± standard error of the mean.

#### 2.3.2. Characterization of the fatty acid profile in V instar larvae, prepupae and substrate

Pooled samples of dried and ground larvae, prepupae and rearing substrate (40 g from each pool) were subjected to fatty acid analysis by gas chromatography based on Raes et al. (2001) via an analytical service laboratory ISO 9001:2015 certified.

### 2.4. Identification and characterization of lipid metabolism genes

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/pathway.html) was used to select the genes associated with the lipid metabolism pathway in *Musca domestica* and *Drosophila melanogaster*. Sequences coding for *acetyl-CoA carboxylase* (*acc*), *fatty acid synthase* (*fas*), *lipase* (*lip*) and *acyl-CoA dehydrogenase* (*acd*) were used to retrieve homologous sequences from flies. Degenerate primers for cloning (Table S1) of the respective homologues from *H. illucens* were designed within the conserved regions of each gene based on the selected sequences from Diptera species reported in Table S2.

Total RNA samples were extracted with TRIzol Reagent (Invitrogen) and used for reverse transcription with QuantiTect Reverse Transcription Kit (Qiagen) after removal of contaminating genomic DNA. RNA quality and quantity were verified as described elsewhere (Giannetto et al., 2017). cDNA samples were obtained from 1 µg of total RNA and amplified by PCR using recombinant Taq DNA polymerase (Invitrogen). PCR products were purified from agarose gel using E.Z.N.A Gel Extraction kit (Omega Bio-tek), cloned and sequenced as detailed by Lazado et al. (2014).

Identities of sequences were confirmed by performing BLASTN searches against the NCBI database (https://blast.ncbi.nlm.nih.gov). Identified sequences of putative lipid metabolism genes from *Hermetia illucens* were used to design gene-specific qPCR primer sets (Table S1).

### 2.5. Sequence analysis

The obtained sequences of *H. illucens acc*, *fas*, *lip* and *acd* genes (designated as *Hiacc*, *Hifas*, *Hilip* and *Hiacd*) were submitted to GenBank. The amino acid sequences of these genes were deduced using the ExPASy Translate tool (https://web.expasy.org/translate) and the open reading frames were identified by ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder). Conserved Domain Search Service (CD Search) of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to predict the protein functional domains. Clustal W algorithm was used for multiple sequence alignment. Evolutionary analyses were conducted in MEGA X using the Neighbor-Joining method with 1000 bootstrap replications and Poisson correction model (Kumar et al., 2018).

### 2.6. Quantitative gene expression

Transcript levels of the herein cloned putative genes related to fat metabolism (*acc*, *fas*, *lip* and *acd*) were determined by quantitative RT-PCR (qPCR) using 1:20 diluted cDNA samples, QuantiTect SYBR® Green PCR Kit (Qiagen) and Rotor-Gene Q 2 plex Hrm thermocycler. The PCR efficiency was evaluated for each gene by a five-point standard curve; for each reaction, replicates, controls and confirmation of specificity were performed as detailed by Giannetto et al. (2013). The *16 s ribosomal RNA* (*16 s rRNA*), *18 s ribosomal RNA* (*18 s rRNA*) and *elongation factor* (*ef1-α*) were used as reference genes. The normalization factor was calculated by GeNorm Software and this value was used to correct the raw data of target genes (Nagasawa et al., 2012). Sequences of qPCR primers are reported in Table S1.

### 2.7. Statistical analysis

The SPSS software 16.0 (SPSS Inc.) was used to perform the analysis of variance followed by Student-Newman-Keuls post hoc tests in order to assess statistically significant differences in the growth performance parameters, proximate composition and expression levels of lipid metabolism-related genes between the two developmental stages. Significance levels were set at $p < 0.05$.

## 3. Results

### 3.1. Substrate bioconversion into biomass of BSF V instar larvae and prepupae

The growth rate in prepupae samples had a value of 0.002; when this parameter was calculated on BSF V instar larvae it was 0.04 (Table 1).

The calculated $ECD_F$ values were 0.18 (V instar larvae) and 0.13 (prepupae) indicating that BSF larvae showed a higher efficiency of conversion, i.e. a higher productivity in digesting and metabolizing the feed medium into biomass in respect to prepupae.

Conversely, the FCR showed an opposite trend being lower at the V instar larval stage (9.29) in respect to the prepupal stage (12.5).

The bioconversion ratio calculated on prepupae samples (8%) was lower with respect to V instar larvae samples (10.8%). The SR was 60% for both larvae and prepupae.

### 3.2. Proximate composition

The values of dry matter, ash, NDF, ADF, estimated chitin content and corrected protein content were significantly higher in the prepupae than in the V instar larvae (Table 2). However, larvae showed higher content of crude fat than prepupae. Regarding the crude protein and

**Table 1**
Growth and bioconversion parameters of *H. illucens* V instar larvae and prepupae reared on vegetable waste. Growth Rate (GR), Efficiency of conversion of digested feed ($ECD_F$), Feed Conversion Ratio (FCR), Bioconversion Ratio (BR), Substrate Reduction (SR). Results based as mean ± standard deviation, n = 3, on dry matter basis.

| Developmental stage | GR (g $d^{-1}$) | $ECD_F$ | FCR | BR (%) | SR (%) |
|---|---|---|---|---|---|
| V instar larvae | 0.04 ± 0.02[a] | 0.18 ± 0.04[a] | 9.29 ± 0.11 [a] | 10.8 ± 0.02[a] | 60 ± 0.08[a] |
| Prepupae | 0.002 ± 0.01[b] | 0.13 ± 0.01[b] | 12.5 ± 0.05[b] | 8 ± 0.06[b] | 60 ± 0.09[a] |

Data in the same column not sharing common letters are significantly different ($p < 0.05$).

**Table 2**
Proximate composition of substrate and *H. illucens* V instar larvae and prepupae. Results are expressed as mean ± standard deviation of the mean, n = 3 on dry matter basis.

| Composition (%) | Substrate | V instar larvae | Prepupae |
|---|---|---|---|
| Dry matter | 73.66 ± 0.11 | 85.51 ± 0.12[a] | 89.46 ± 0.06[b] |
| Crude protein (CP) | 9.49 ± 0.88 | 36.70 ± 2.69[a] | 39.88 ± 1.11[a] |
| Ash | 11.99 ± 0.02 | 5.00 ± 0.03[a] | 5.66 ± 0.07[b] |
| Crude fat (CF) | 3.42 ± 0.05 | 32.97 ± 0.16[a] | 30.80 ± 0.20[b] |
| NDF | 48.24 ± 0.27 | 33.40 ± 0.55[a] | 39.14 ± 0.56[b] |
| ADF | 37.92 ± 0.29 | 22.51 ± 0.32[a] | 27.40 ± 0.11[b] |
| Recovery of ADF (as amino acids) | 4.03 ± 0.06 | 14.86 ± 0.17[a] | 16.31 ± 1.39[a] |
| Estimated chitin content | | 19.16 ± 0.32[a] | 22.97 ± 0.45[b] |
| Corrected protein content | | 33.35 ± 0.37[a] | 35.40 ± 0.37[b] |

Within a line, different superscripted alphabets indicate significant differences between means ($p < 0.05$).

recovery of ADF, no statistical differences were observed between the V instar larvae and prepupae (Table 2).

### 3.3. Fatty acid profiles

Saturated fatty acid content was higher in prepupae (84.51 %) in respect to V instar larvae samples (73.48 %). On the contrary, total unsaturated fatty acid content was higher in larvae (26.52 %) than in prepupae (15.49 %) with higher proportion of both monounsaturated (14.95 % *vs* 8.36 %) and polyunsaturated (11.57 % *vs* 7.13%) fatty acids (Table 3; Fig. 1). Trans fatty acids were mainly detected in prepupae samples, being almost absent in larvae. Interestingly, BSF V instar larvae and prepupae were characterized by comparable levels of omega-3 fatty acids, while omega-6 content was higher in larvae than prepupae (2.5 g/100 g vs 0.99 g/100 g).

Fatty acid profiles of BSF V instar larvae and prepupae revealed that the lauric unsaturated acid was the major component in prepupae (61.87%), while its content was dominant (28.10%) in larvae where the major component was the heptadecanoic unsaturated acid (33.62%). The main differences between the two developmental stages counted for the content of lauric acid which was far higher in prepupae than V instar larvae (61.87% vs 28.10%), heptadecanoic acid showing 3.22% in prepupae in respect to 33.62% in larvae, myristic acid (9.07% in prepupae against 3.85% in larvae), linolenic acid (4.75% in prepupae against 10.3% in larvae), pentadecanoic acid with 0.03% vs 0.81% in prepupae and larvae, respectively. The pentadecenoic acid was found exclusively in V instar larvae (0.41%) as well as the eicosenoic acid (7.96%) and undecanoic acid (0.23%) (Table 3).

**Table 3**
Fatty acid composition of substrate, BSF V instar larvae and prepupae. Values are expressed as % p/p of total fatty acids unless otherwise stated, on dry matter basis.

| Fatty acids | Substrate | V instar larvae | Prepupae |
|---|---|---|---|
| Butyric (C4:0) | < 0.01 | < 0.01 | < 0.01 |
| Caproic (C6:0) | < 0.01 | < 0.01 | < 0.01 |
| Caprylic (C8:0) | < 0.01 | < 0.01 | < 0.01 |
| Capric (C10:0) | 0.13 | **0.46** | **1.19** |
| Caproleic (C10:1) | < 0.01 | < 0.01 | < 0.01 |
| Undecanoic (C11:0) | < 0.01 | 0.23 | < 0.01 |
| Lauric (C12:0) | 2.36 | **28.10** | **61.87** |
| Lauroleic (C12:1) | < 0.01 | < 0.01 | < 0.01 |
| Myristic (Cl4:0) | 0.95 | **3.85** | **9.07** |
| Myristoleic (C14:1) | < 0.01 | 0.66 | 0.54 |
| Pentadecanoic (C15:0) | 0.15 | **0.81** | **0.03** |
| Pentadecenoic (C15:1) | < 0.01 | 0.41 | < 0.01 |
| Palmitic (C16:0) | 25.94 | 5.78 | 7.90 |
| Palmitoleic (C16:1) | 0.73 | 1.65 | 2.44 |
| Heptadecanoic (C17:0) | 0.33 | **33.62** | **3.22** |
| Heptadecenoic (C17:1) | < 0.01 | < 0.01 | < 0.01 |
| Stearic (C18:0) | 4.56 | 0.72 | 1.15 |
| Oleic (C18:1) | 8.10 | 4.27 | 5.26 |
| Linoleic (C18:2) | 11.95 | 1.27 | 2.38 |
| Linolenic (C18:3) | 42.93 | **10.30** | **4.75** |
| Arachidic (C20:0) | 0.98 | < 0.01 | < 0.01 |
| Eicosenoic (C20:1) | < 0.01 | **7.96** | < **0.01** |
| Behenic (C22:0) | < 0.01 | < 0.01 | 0.08 |
| Omega-3 | 0.83 g/100 g | 2.88 g/100 g | 2.99 g/100 g |
| Omega-6 | 0.23 g/100 g | **2.5 g/100 g** | **0.99 g/100 g** |
| Trans Fatty Acids | < 0.01 | < **0.01** | **0.12** |
| Saturated Fatty Acids | 36.29 | 73.48 | 84.51 |
| Monounsaturated Fatty Acids | 8.83 | **14.95** | **8.36** |
| Polyunsaturated Fatty Acids | 54.88 | **11.57** | **7.13** |

Main differences are highlighted in bold.

### 3.4. Characterization and phylogenetic analysis of lipid metabolism-related genes

The obtained cDNA sequences of 4 lipid metabolism genes *(Hiacc, Hifas, Hilip* and *Hiacd*) were deposited to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with the following accession numbers: *Hiacc* (MK906816), *Hifas* (MK906817), *Hilip* (MK906818) and *Hiacd* (MK906819).

The partial cDNA sequence of *Hiacc* was 2680 bp long and contained an open reading frame of 891 amino acid residues (Fig. S1). The predicted amino acid sequence of *Hi*ACC exhibited 61% identity to acetyl-CoA carboxylase from *Homo sapiens* (NP_942131.1) and *Mus musculus* (XP_006532016.1), and 62% identity to ACC from *Danio rerio* (XP_017211602.1). The putative HiACC protein was significantly conserved among dipteran insects as blastp search showed that the protein from BSF had 81–82% identity with the homologues from other flies; moreover, it was characterized by the presence of relevant domains featuring peculiar functions that are encountered in the relative homologous proteins from other eukaryotic organisms.

In particular, the HiACC showed to contain the Acetyl-CoA carboxylase_central region (ACC_central superfamily, accession cl24207) domain in the amino acid range 1–105 (E-value 1.27 e-44) and a Carboxyl transferase domain (Accession pfam01039) in the amino acid interval 205–757 (E-value 3.08 e-177), both characterizing motifs of acetyl-CoA carboxylases (Fig. 2A).

The cloned partial *Hifas* cDNA had a length 1074 bp and encoded a polypeptide of 358 amino acids (Fig. S2). The predicted amino acid sequence of *Hi*FAS exhibited 62%, 42%, 41% and 40% identity to fatty acid synthase from *Drosophila melanogaster* (NP_001015405.3), *Homo sapiens* (NP_004095.4), *Mus musculus* (NP_032014.3) and *Danio rerio* (XP_021328256.1), respectively. Multiple sequence alignment of HiFAS with other fatty acid synthases revealed 61–64% identity with

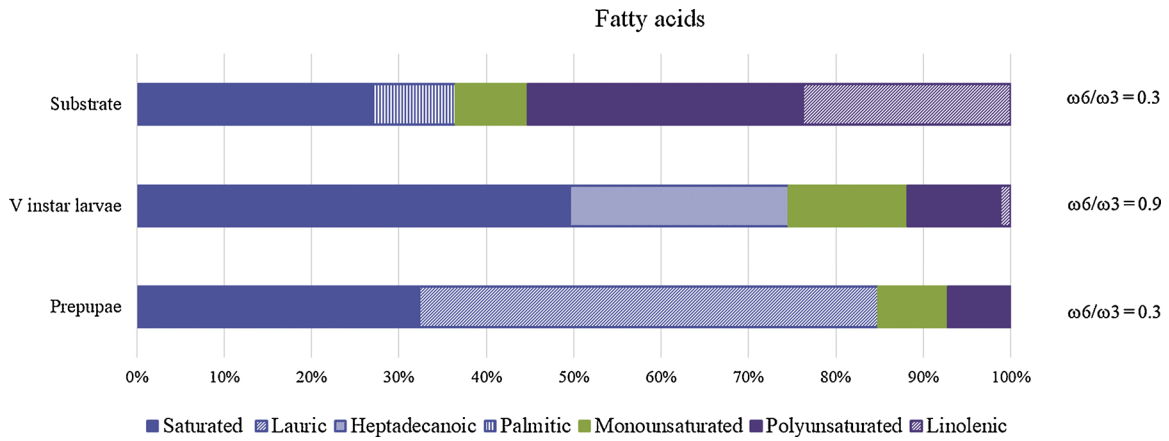


**Fig. 1.** Graphical representation of the major fatty acid components of substrate, *H. illucens* V instar larvae and prepupae. The proportion of lauric, heptadecanoic and palmitic within saturated (blue), monounsaturated (green), and linolenic within polyunsaturated (violet) fatty acids are depicted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

homologous proteins from other flies. Conserved domain search revealed two known functional regions, namely the Short-chain dehydrogenases/reductases (SDR) superfamily domain (accession cl25409) in the amino acid interval 4–174 (E-value 5.02 e-53) and the Phosphopantetheine attachment site (PKS_PP, accession smart00823) in the amino acid interval 186–236 (E-value 3.08 e-06). Moreover, the SDR domain contained all the four residues that compose the active site on the conserved domain KR_1_FAS_SDR_x; specifically, the residues KSYN that compose this conserved feature have been mapped to the query sequence at position 56, 81, 95 and 99, respectively (Fig. 2B).

Partial fragment of *Hilip* (1086 bp) gene involved in lipolysis was isolated and characterized, too. The 1086 bp cDNA sequence encoded for HiLIP consisting of 362 amino acid residues (Fig. S3) and shared 49% amino acid identity with the lipase of *Drosophila melanogaster* (NP_477331.1) and 41% with human gastric triacylglycerol lipase (NP_004181.1). Prediction of conserved domain indicated that the deduced HiLIP protein sequence encoded for a peculiar feature of lipases that is the PLN02872 super family domain (Accession cl28691, residues 1–357, E-value 9.51 e-50) and contained the specific Abhydro_lipase domain (Accession pfam04083) from residue 3 to 60 (E-value 9.99e-24) (Fig. 2C).

Full-length *acd* cDNA gene sequence was obtained from *H. illucens*. *Hiacd* contained the complete open reading frame of 1245 bp encoding 415 amino acid polypeptides (Fig. S4) with a predictive molecular mass of about 45 kDa and a theoretical isoelectric point (pI) of 8.02. Sequence analysis of the HiACD amino acid sequences indicated that it shared 76–78 % identity with homologues from other flies, 63%, 61% and 56% with acyl-CoA dehydrogenases from *Homo sapiens* (NP_001600.1), *Mus musculus* (NP_080102.1) and *Danio rerio* (XP_021336130.1), respectively. Moreover, the HiACD contained the specific conserved domain SCAD_SBCAD in the amino acid interval 40–411 (accession cd01158, E-value 0e + 00) that is characterized by the 8 (of 8) residues composing the FAD binding site, by the 8 (of 8) residues composing the substrate binding pocket and by the 32 (of 32) residues composing the homotetramer interface. The multi-domain CaiA was predicted to be contained in the HiACD, from residue 38 to 413 (Accession COG1960, E-value 4.54e-123); it is constituted by three different domains characterized by specific secondary structures, namely the Acyl-CoA_dh_N domain (all-alpha domain), the Acyl-CoA_dh_M domain (β-barrel fold) and the Acyl-CoA_dh_1 domain (all-alpha, four helical up-and-down bundle) (Fig. 2D).

The proteins from *Hermetia illucens* herein identified showed a high degree of primary structure similarity with their relative homologous proteins from other flies; besides, the finding of functional domains in all the putative proteins is indicative of conserved functions throughout evolution suggesting that HiACC, HiFAS, HiLIP and HiACD are really involved in BSF lipid metabolism.

Evolutionary analyses of the deduced amino acid sequences from *Hermetia illucens* were conducted after blastp search of public databases with putative HiACC, HiFAS, HiLIP and HiACD as a query (Table S2). Phylogenetic tree reconstruction with the retrieved protein sequences of ACC (Fig. 3A), FAS (Fig. 3B), LIP (Fig. 3C) and ACD (Fig. 3D) showed that all the BSF proteins grouped with dipteran orthologs. All the vertebrate homologs grouped together in a different branch of the phylogenetic tree.

### 3.5. Gene expression

All the analyzed genes (acc, fas, lip and acd) showed transcriptional differences depending on the different phase of the life cycle (Fig. 4).

In particular, all transcripts were significantly down-regulated in prepupae ($p < 0.05$) with acc and fas genes involved in the fatty acid biosynthesis showing 2.59-fold and 1.91-fold lower transcript levels as compared to V instar larvae. Besides, the genes implicated in lipid degradation herein identified showed decreased transcript levels in prepupae with value 3.10-fold (lip) and 1.79-fold (acd) lower than V instar larvae.

Overall, the expression profiles of the four genes implicated in lipid metabolism suggest they influence the lipid content in the different BSF developmental stages.

## 4. Discussion

In this study we compared two different developmental stages of *H. illucens* in terms of efficiency to convert vegetable waste into valuable biomass and in terms of nutritional composition focusing on lipid metabolism. We showed that V instar larvae and prepupae exhibit peculiar characteristics to consider for using BSF as alternative lipid source.

Bioconversion ratio indicates the efficiency of consumption of a substrate. In contrast, the FCR indicates the proportion of digested food that is assimilated and therefore ends up as biomass; the lower the value, the higher the efficiency of conversion of substrate to biomass. Indeed, high FCR values indicate a digestible but poor nutrient substrate that is for the most part excreted. Our data from the bioconversion process, showed that BSF V instar larvae had lower FCR and correspondingly higher BR than prepupae. This implicates that the substrate was not only effectively consumed but also highly assimilated into biomass with BSF showing better performance at the larval stage than prepupal stage. Moreover, our results showed that BSF V instar larvae had higher efficiency of conversion of digested feed values compared to prepupae proving that overall, with the same quantity and quality of substrate, BSF V instar larvae (BSFL) had higher capacity to

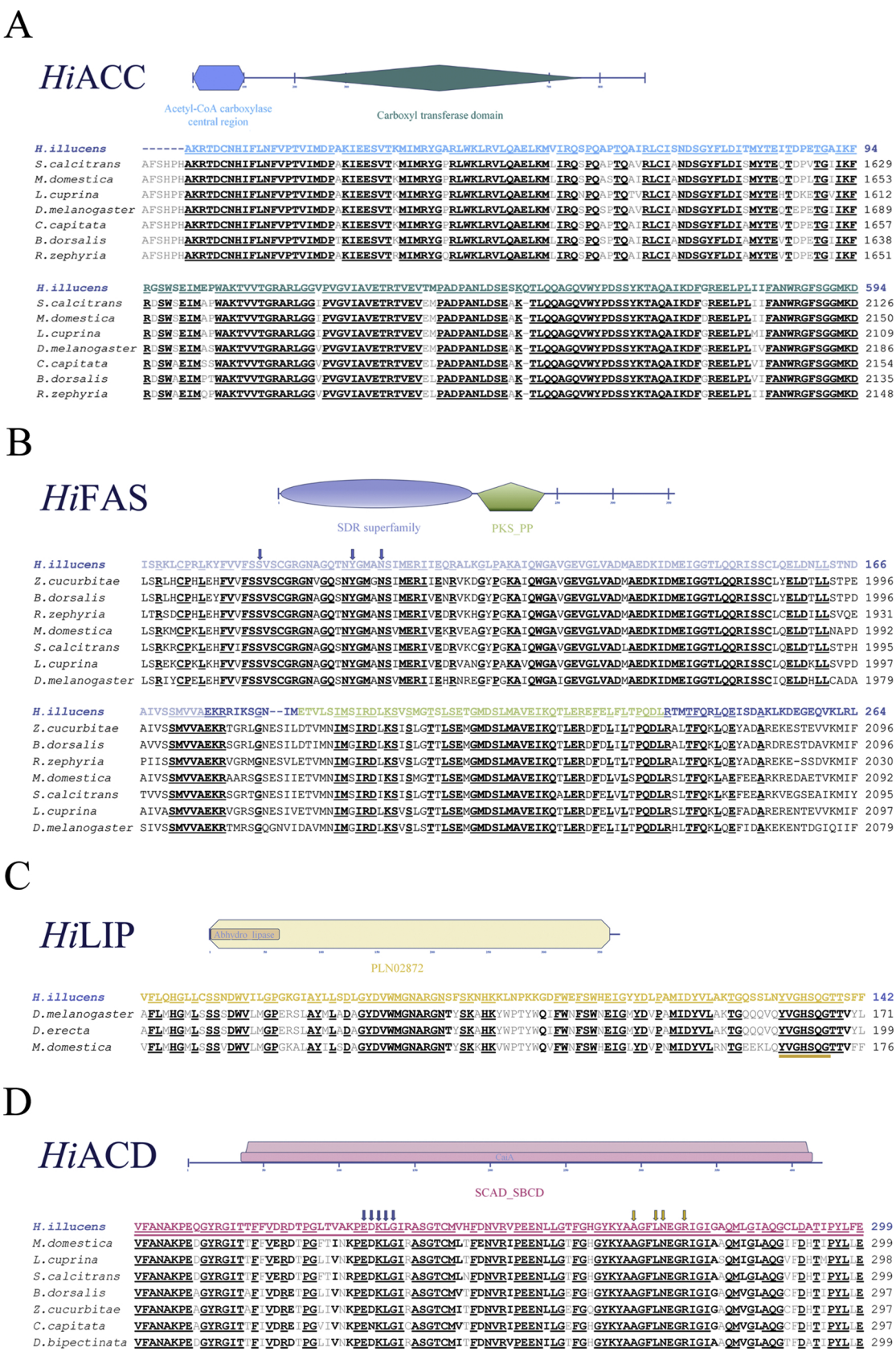


**Fig. 2.** Relevant conserved regions of *H. illucens* proteins involved in lipid metabolism. Schematic diagram of functional domains deduced from the cloned genes and alignment of relevant conserved regions within the identified motifs are represented. (A) Acetyl-CoA carboxylase (*Hi*ACC) protein sequence with conserved functional domains acetyl-CoA carboxylase, central region (in blue) and carboxyl transferase (in green); (B) fatty acid synthase (*Hi*FAS) protein sequence with conserved functional domains SDR superfamily (in light blue) and PKS_PP (in green); (C) lipase (*Hi*LIP) protein sequence with conserved functional domains PLN02872 and Abhydro_lipase (in orange); (D) acyl-CoA dehydrogenase (*Hi*ACD) protein sequence with conserved functional domains SCAD_SBCD (in pink) and CaiA (in pink-white). Sequences details are given in the supplementary material (Figs. S1–S4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

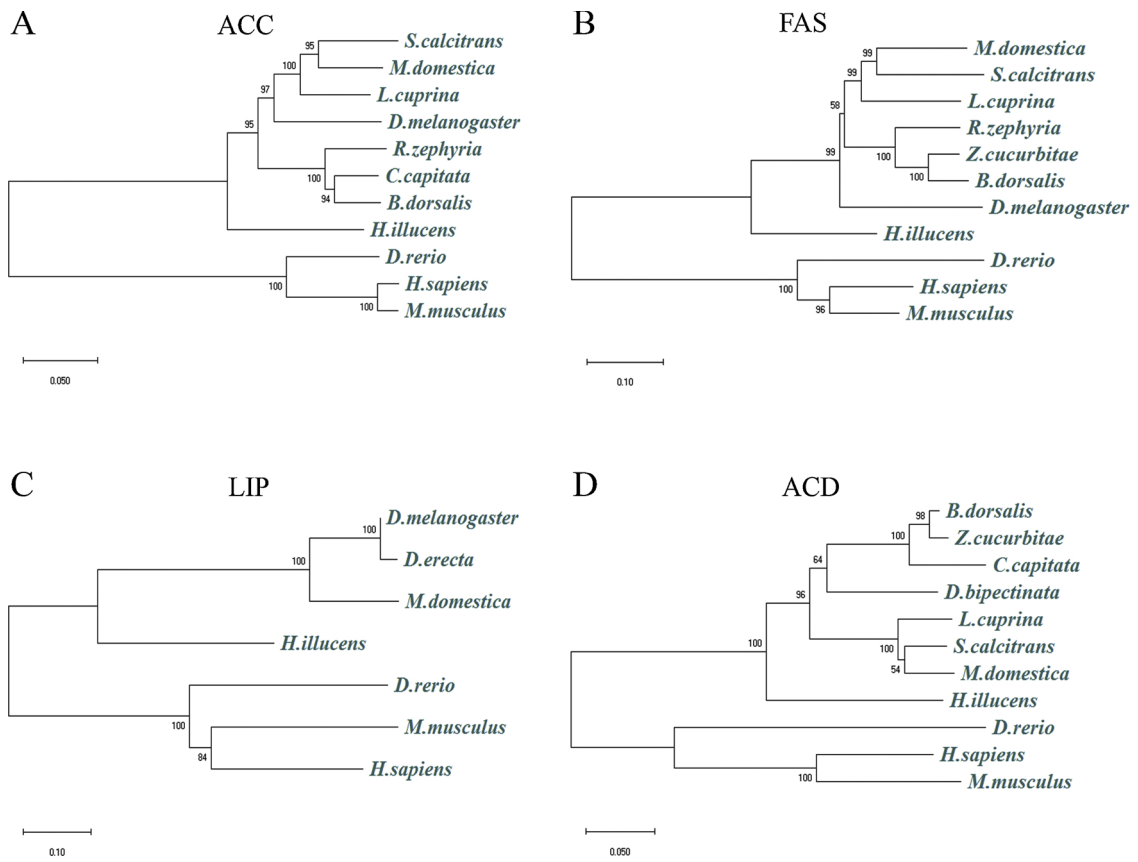


**Fig. 3.** Phylogenetic analysis of *H. illucens* proteins with the relative orthologs from different species. (A) ACC, (B) FAS, (C) LIP and (D) ACD. The trees were constructed using the Neighbor-Joining method; bootstrap values (1000 replicates) are reported next to branches. GenBank accession numbers of the sequences used in these analyses are listed in Table S2.

bioconvert the substrate into biomass than prepupae.

Literature from the last decade have recognized the bioconversion by *H. illucens* as a promising and eco-friendly biotechnology to efficiently reduce a wide variety of organic wastes into valuable insect biomass with potential benefits for animal feed and biodiesel production (Surendra et al., 2016). Several studies reported that BSFL development as well as biological traits of adult flies are strongly dependent on the quality and quantity of rearing substrate; as a consequence, the protein and fat composition of BSF larvae is influenced by the food supplied to larvae (Wang and Shelomi, 2017). Although different studies pointed BSFL as a valuable source of proteins and lipids, a great variation in their amounts have emerged because of different experimental designs (growth substrate, feeding rate, developmental stage, methods to extract protein and lipids, chitin content evaluation). Remarkable, BSF V instar larvae and prepupae were characterized by higher nutrient content than substrate confirming the role of *H. illucens* as an ideal player in the waste valorisation process. The BSF proximate composition found in our study is comparable to previously published results on the same species grown on vegetable and fruit wastes (Meneguz et al., 2018); starting from 9.49% protein provided in the substrate, BSF contained 36.70% protein at the larval stage and 39.88% at the prepupal stage. The same tendency was observed when the protein content was corrected by chitin values, where prepupal stage showed values significantly higher than the larval stage, indicating that the protein contained in chitin can lead to an over estimation of the protein fraction (Diener et al., 2009). Therefore, an estimation of chitin appears to be needful when the proximate composition of insects is analyzed to use the larval/prepupal biomass in feed field as the chitin content, even at low levels has been reported to negatively influence nutrient digestibility in some fish species (Kroeckel et al., 2012; Olsen et al., 2006). Nevertheless, chitin from insect can provide an alternative source for chitosan, a sugar with several scientific and commercial applications for health care, agriculture, as well as cosmetic and textile industries. On the contrary, starting from 3.42% lipid in the substrate, BSF contained 32.97% lipid at the larval stage and 30.80% at the prepupal stage showing an opposite trend in respect to protein content measured in the two developmental stages. Our data on the lipid characterization of BSF V instar larvae and prepupae provided further information on the biochemical features of *H. illucens* developmental stages and suggest remarkable information on the potential of BSF in artificial breeding for the feed sector. Among fatty acids (FA), the polyunsaturated (PUFA) was the predominant category of the rearing substrate used as feed in this experiment; nevertheless, BSF fatty acid profiles were dominated by saturated fatty acids (SFA), as already demonstrated by other authors (Meneguz et al., 2018). In particular, our results showed that larval and prepupal stages are characterized by specific proportions of both saturated and unsaturated fatty acids. While the omega-3 fatty acid content was comparable between both development stages, the quantity of omega-6 was greater in V instar larvae. Paul et al. (2017) calculated the ω6/ω3 ratio in different insect species showing a great range of variability: values were very high for *Tenebrio molitor* (204), *Acheta domesticus* (37.4) and *Conocephalus discolour* (25.08), or very low for *Chorthippus parallelus* (0.33). Taking into account that the optimal ratio of omega-6 to omega-3 is estimated to be near 1:1 for a balanced feeding (Barroso et al., 2017), BSF at the larval stage showed the best potential dietary benefits. Noteworthy, since the omega-3 and omega-6 contents in both V instar larvae and prepupae were remarkable higher than that provided in the substrate, it could be inferred that BSF is able to synthesize valuable fatty acids.

Both V instar larvae and prepupae contained high proportions of lauric, palmitic and oleic acids, fatty acids known to be present in *H. illucens* (Surendra et al., 2016), but their contents were substantially

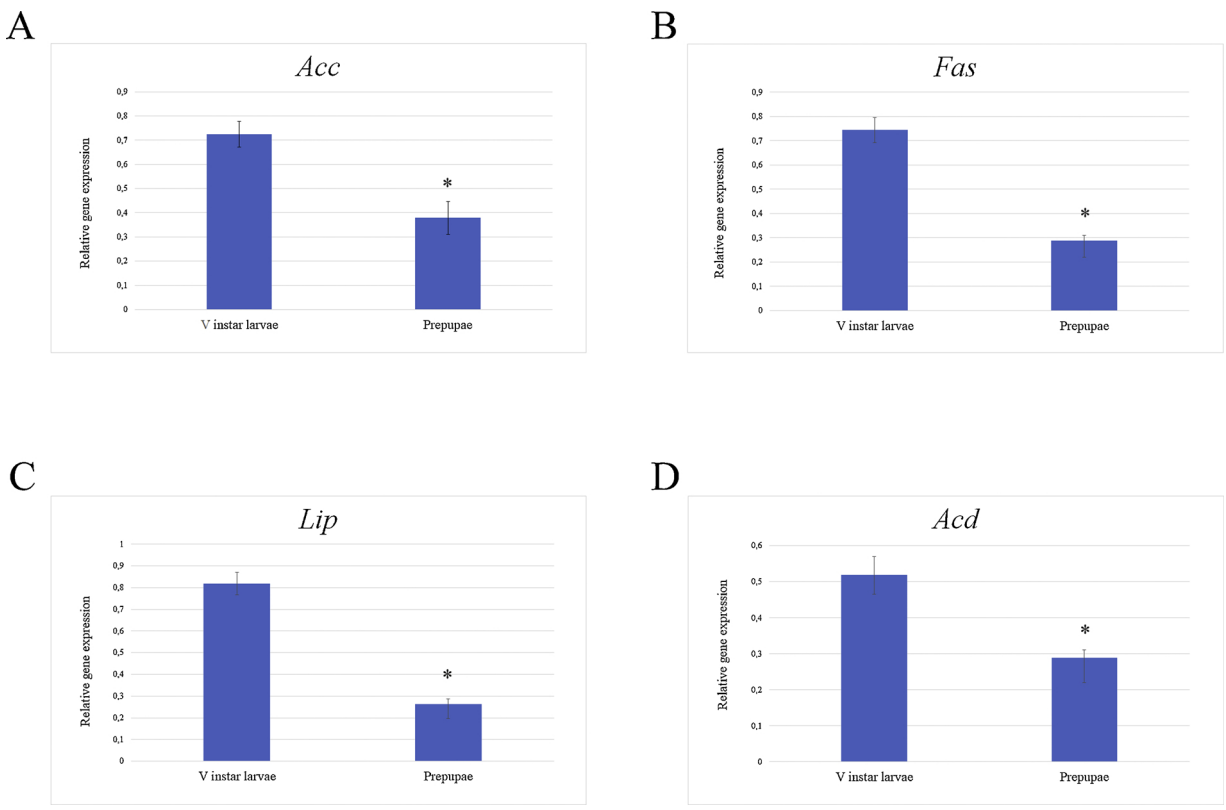


**Fig. 4.** mRNA expression levels of lipid metabolism-related genes in *H. illucens*. Relative gene expression of (A) *Hiacc*, (B) *Hifas*, (C) *Hilip* and (D) *Hiacd* were evaluated by qPCR in V instar larvae and prepupae. Results are presented as mean ± SD (n = 6). Asterisks (*) denote significant differences between the selected developmental stages ($p < 0.05$).

different between V instar larvae and prepupae samples. Lauric acid content in prepupae resulted twice higher than in V instar larvae; beside its use as a nutritional supplement, lauric acid is known for its anti-microbial activity (Kim and Rhee, 2016) through cellular membrane lysis and for its anti-inflammatory properties (Zarantoniello et al., 2019) that could mitigate the effects of chitin in fish fed with BSF meal. These aspects make BSF a leading actor both in animal feed and pharmacological fields. It is a common opinion that the fatty acid content in the diet affect the BSF fatty acid profile; Sealey et al. (2011) indeed, found that BSF can incorporate some omega-3 fatty acids when these occur in their diet; our data are in agreement with this assumption showing that providing C18-3 linolenic acid in the diet, BSF incorporates it; nevertheless, we found that it is incorporated much more at the larval stage than prepupa. On the contrary, Oonincx et al. (2015) indicated limited possibilities to adapt BSF fatty acid profile showing that higher dietary fat content is reflected in a larger proportion of fatty acids being metabolized to lauric acid. Therefore, although diet has been suggested to exert strong influences on FA profiles, there are other factors such as fatty acid biosynthesis that should also be taken into account to explain overall FA composition (Stanley-Samuelson et al., 1988).

We found a surprisingly high value of C17 heptadecanoic acid being alone the 33% of the total fatty acids (it is second to lauric acid) in V instar larvae against the 3% in the prepupae. Also, the C15 pentadecanoic acid resulted more abundant in V instar larvae than in prepupae samples. C15 and C17 are odd chain saturated fatty acids (OCS-FAs) that have been found in relation with reduced risk for developing pathologies and play a role in membrane functionality increasing their fluidity in a similar manner as PUFA in order to meet the homeostatic range requirements of membrane functionality (Jenkins et al., 2015).

Trans fatty acids were detected in prepupae samples only. It is known that the increase in trans fatty acids is detrimental to health because they interfere with the desaturation and elongation of both omega-6 and omega-3 fatty acids.

Besides the diet, species and environmental conditions, the stage of life can also influence the fatty acid profile of insects (Guil-Guerrero et al., 2018). They can biosynthesize and accumulate different fatty acids at various stages of life, depending upon the utility of fatty acid in the body (Liu et al., 2017; Zhu et al., 2019). In our study, even though BSF V instar larvae and prepupae were fed with the same diet, i.e. vegetable waste, the two developmental stages exhibited a different fatty acid composition indicating that fatty acid profiles in *Hermetia illucens* change during the life cycle, too.

Despite the great body of data on these physiological and biochemical traits, most of the previous studies on BSF as feed reported the nutrition composition analysis limited to prepupae (Spranghers et al., 2017), but scientific evidences on improved performance of this BSF developmental stage in respect to others are scarce. Liu and colleagues (2017) focused on the nutritional composition during whole life cycle of *H. illucens* grown on commercial broiler chicken feed providing preliminary supporting information on the metabolic biology of this excellent feed source in the animal and aquaculture feed industry. However, the molecular mechanisms determining the metabolic variations, during insect development still remain to be clarified.

To date, the knowledge of the genes involved in lipid metabolism in insects is still scarce as most of studies focused on their expression patterns associated to diapause (Alabaster et al., 2011; Reynolds et al., 2012; Sim and Denlinger, 2009). Molecular investigations on lipid metabolism pathway in *Hermetia illucens* have been inadequate till now, especially recognizing the great potential of this insect as a valuable lipid source for diverse industrial purposes and the fascinating opportunity to modulate the fatty acid profiles by manipulating the composition of the rearing substrate.

In the present study, four key genes selected from the KEGG

pathway database and involved in the lipid metabolism in insects, namely *acetyl-CoA carboxylase, fatty acid synthase*, *lipase* and *acyl-CoA dehydrogenase*, were characterized in the Black Soldier Fly (BSF) *Hermetia illucens* for the first time and designated *Hiacc*, *Hifas*, *Hiacd* and *Hilip*, respectively. Sequences and phylogenetic analyses revealed that the obtained cDNAs and their deduced proteins showed a high similarity with their relative homologues from other flies. The multiple sequence alignment of the deduced proteins showed their molecular structures were highly conserved, which probably denotes similar function in different species. Indeed, this was further supported by the identification of specific protein functional domains, namely the Acyl-CoA carboxylase and the Carboxyl transferase domains in *Hi*ACC; the SDR superfamily and the PKS_PP domains in the *Hi*FAS; the PLN02872 superfamily and the Abhydro_lipase domains in the *Hi*LIP; the SCAD_SBCD and the CaiA domains in the *Hi*ACD. All the predicted domains constitute peculiar motifs characterizing the relative protein families suggesting conserved function for the protein herein identified.

The observed differences in mRNA expression patterns between the V instar larvae and prepupae suggest that the investigated genes are implicated in lipid metabolism during BSF larval development. Specifically, the overexpression of *fas* and *acc* in V instar larvae might have a role in the rate-limiting steps of fatty acid biosynthesis thus participating in accumulating nutrient and energy reserves to be used during metamorphosis and for further development. This process is especially important in holometabolous insects that rely on these stored reserves to support growth, life and reproduction (Arrese and Soulages, 2010). At the stage of prepupa, *fas* and *acc* transcript levels were decreased, instead: the amount of nutrient stored during the larval feeding stage is probably adequate to further support BSF development. This is consistent with previous studies reporting lipid metabolism (primarily fatty acid metabolism) genes differentially expressed during different developmental stages in insects (Harker et al., 2013; Zhu et al., 2019). Recently, a fat accumulation model in developing BSF identified the genes associated with FA biosynthesis (*acc* and *fas*) as upregulated in early stages and suggested that BSF accumulate fat synthesizing short-chain fatty acids (mainly lauric acid), thus providing a foundation for metabolic engineering (Zhu et al., 2019). Besides, the differential expression patterns of *lip* and *acd* in larval and prepupal stages may account to mobilize lipids stores to support energy requirements associated to non-feeding periods and sustain development, as reported in *Gryllus bimaculatus* (Anand and Lorenz, 2008) and *Manduca sexta* (Arrese et al., 2010).

Our data on gene expression modulation during larval and prepupal stages strongly suggest their involvement in the accumulation of lipid stores that might serve for the developmental growth, energy requirements for the metamorphosis and the reproduction of the adult. These findings also give important knowledge to understand the molecular basis of the process determining different metabolic phenotypes showing peculiar nutritional content in BSF V instar larvae and prepupae reared on vegetable waste.

Overall, our results show remarkable differences in biomass production, nutritional values and chemical characteristics between V instar larvae and prepupae, including the protein, lipid and chitin content, the proportion of total polyunsaturated fatty acids with peculiar or dominant fatty acids (such as lauric, heptadecanoic, linolenic, eicosenoic), as well as the omega-6 to omega-3 ratio. Also, our data strongly suggest that these differences in the fatty acid profile might be determined by lipid metabolism-related genes differentially expressed in the two investigated developmental stages.

The bioconversion by BSF represents a new agri-food biotechnology and an environmentally friendly solution oriented towards waste valorization instead of a waste prevention strategy in a circular economy context. In this process *H. illucens* during the larval development represents a valuable bioreactor for the waste management transforming negative value substances into bioactive products with promising applications. The proteins and lipids obtained via *H. illucens* constitute an alternative resource and our study demonstrate that the different nutritional values of larvae and prepupae could be exploited *ad hoc* to formulate species-specific diets for fulfilling the nutritional needs of the different fish species in aquaculture field as well as selecting preferable fatty acid profiles in order to meet the alimentary and industrial requirements. As such, the BSF mass production opens innovative scenarios on the potential of BSF as a sustainable alternative source of lipids, proteins and bioactive substances for relevant purposes including but not limited to the feed sector, pharmaceutical applications and biodiesel production.

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## Declaration of Competing Interest

None declared.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2019.10.015.

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