

Lipid profile and growth of black soldier flies (*Hermetia illucens*, Stratiomyidae) reared on by-products from different food chains

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Complete List of Authors:	<p>Hadj Saadoun, Jasmine; University of Modena and Reggio Emilia, Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA Interdepartmental Centre</p> <p>Montevecchi, Giuseppe; University of Modena and Reggio Emilia, Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA Interdepartmental Centre</p> <p>Zanasi, Luca; University of Modena and Reggio Emilia, Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA Interdepartmental Centre</p> <p>Bortolini, Sara; University of Modena and Reggio Emilia, Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA Interdepartmental Centre</p> <p>Macavei, Laura; University of Modena and Reggio Emilia, Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA Interdepartmental Centre</p> <p>Masino, Francesca; University of Modena and Reggio Emilia, Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA Interdepartmental Centre</p> <p>Maistrello, Lara; Università degli Studi di Modena e Reggio Emilia Dipartimento di Scienze della Vita, Dipartimento di Scienze della Vita</p> <p>Antonelli, Andrea; University of Modena and Reggio Emilia, Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA Interdepartmental Centre</p>
Key Words:	<i>Hermetia illucens</i> , waste valorization, food chain by-products, prepupal fatty acids profile

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10 4 **Short running title:** Lipid profile and growth of *Hermetia illucens* reared on food by-
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17 7 Jasmine Hadj Saadoun,¹ Giuseppe Montevecchi*, Luca Zanasi, Sara Bortolini,² Laura Ioana
18
19 8 Macavei, Francesca Masino, Lara Maistrello, and Andrea Antonelli
20

21 9
22
23
24 10 Department of Life Sciences (Agro-Food Science Area), BIOGEST - SITEIA
25
26 11 Interdepartmental Centre, University of Modena and Reggio Emilia, Piazzale Europa 1,
27
28 12 42124 Reggio Emilia, Italy.
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36
37 16 * Correspondence to: Giuseppe Montevecchi, Department of Life Sciences (Agro-Food
38
39 17 Science Area), BIOGEST - SITEIA Interdepartmental Centre, University of Modena and
40
41 18 Reggio Emilia, Piazzale Europa 1, 42124 Reggio Emilia
42
43
44 19 E-mail: giuseppe.montevecchi@unimore.it
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58 ¹ Department of Food and Drug, University of Parma, Viale Area delle Scienze 49/a, 43124 Parma, Italy

59 ² Faculty of Science and Technology, Free University of Bozen-Bolzano, Piazza Università 5, 39100 Bolzano,
60 Italy

21 Abstract

22 **BACKGROUND:** The total amount of bio-waste produced annually in the EU by the food
23 and beverage chains is estimated at 37 Mtons. The possibility to use insects for the
24 valorization of **by-products** from these value chains may represent a sustainable solution.
25 This study aims at investigating **the by-products obtained from different food chains for the**
26 **rearing of black soldier fly prepupae to evaluate lipid content and profile and outline its**
27 **possible applications.**

28 The substrates used in this experiment were: (i) industrial **by-products (brewery spent grains,**
29 **cow's milk whey, grape stalks, and tomato peels and seeds)** and (ii) **by-products from**
30 **retailers (bread dough, fish scraps, and spent coffee ground).** Fat extracted from prepupae
31 using an adjusted Folch method was utilized for total lipid content and fatty acids profile.

32 **RESULTS:** Best larval performances were obtained from beer (**0.22 g_{weight} per prepupa**),
33 tomato (**0.19 g_{weight} per prepupa**), and cheese (**0.14 g_{weight} per prepupa**) food-chain **by-**
34 **products.** The extremely different composition of the substrate was reflected in the
35 **differentiated lipid profile of black soldier fly prepupae and in a range of ratios between**
36 **unsaturated and saturated fatty acids comprised from 0.37 for cow's milk way to 1.34 for**
37 **tomato peels and seeds.**

38 **CONCLUSION:** The high content and type of lipids, together with the proteins, and chitin
39 extracted from prepupae are high-value bio-based products that could be used in the
40 feed/food industry or for the development of innovative biomaterials, such as biodiesel.
41 These results suggest **that food chain by-products are the best candidate for insect-**
42 **bioconversion purposes.**

43
44 **Keywords:** *Hermetia illucens*; waste valorization; food chain **by-products**; prepupal fatty
45 acids profile

47 INTRODUCTION

48 Waste management is one of the main problems the world population has been facing in
49 modern times.^{1,2} The amount of organic biodegradable waste produced by the EU is
50 estimated at 76-102 Mtons per year of food and gardening waste, included in the solid
51 undifferentiated municipal waste.³ The amount of waste brought about by food and beverage
52 companies reaches 37 Mtons per year and is often considered a net loss.³ This loss may
53 originate from different stages in the food-chain: production scraps of agro-food industry,
54 discards due to commercial or aesthetical reasons or close to an imminent expiration date,
55 and goods unsold by retailers and vending companies.⁴

56 In 2008, the European Union (EU) unequivocally established the order of priority in the
57 waste treatment, the first being waste reuse and the last its landfill disposal.⁵ It later
58 committed itself in a great effort to reduce or reuse bio-waste. In 2015, the European
59 Commission adopted the Circular Economy Action Plan,⁶ which includes measures aimed at
60 stimulating the European transition towards a circular economy and fostering sustainable
61 economic growth. In particular, all Member States are required to take specific measures to
62 cope with food waste.

63 Emilia-Romagna is one of the most important regions in Italy and Europe for agri-food
64 production, therefore the amount of bio-waste accumulated by the food-chain companies is
65 huge. The main production activities in the Emilia-Romagna region include tomato
66 processing, winemaking and, dairy productions. These food chains cause the accumulation of
67 large amounts of vegetal (tomato peels and seeds, and grape pomaces, seeds, and stalks) and
68 animal (cow's milk whey and *ricotta* whey) **by-products**. **Since** these huge amounts of
69 seasonal crops are concentrated **in most cases** in the very short harvesting time, they are one
70 of the major challenges to be faced.

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3 71 In recent times, other types of **by-products** have been causing a lot of concern. In particular,
4
5 72 brewery **by-products** from the thriving of craft breweries; bread dough and pre-cooked and
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7 73 other semi-finished bakery products which are distributed at shopping centers where the last
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9 74 cooking phase is carried out; spent coffee grounds from vending machines; and animal
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11 75 carcasses from fish and butcher's shops.

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14 76 Currently, these **by-products** are only partially utilized as animal feed, for composting, or
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16 77 biogas production. An investigation into alternative uses of these **by-products** is becoming
17
18 78 urgent and it ranges from the extraction of high-value compounds, such as lipids for
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20 79 biodiesel, or polyphenols for their antioxidant activity, or other substances of high nutritional
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22 80 value before choosing to use **by-products** as a substrate for biogas production.⁷⁻¹³ Finally,
23
24 81 spent coffee ground and brewery **by-products** are conveniently utilized for the cultivation of
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26 82 edible mushrooms.^{14,15}

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29 83 A very interesting solution to the problem is the bioconversion of **by-products** into valuable
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31 84 organic fractions, such as proteins, fat, and chitin carried out by scavenger insects, such as the
32
33 85 'black soldier fly' (BSF) *Hermetia illucens* (Linnaeus, 1758) (Diptera, Stratiomyidae). In the
34
35 86 last few years, this species has been used in different studies for waste bioconversion.¹⁶
36
37 87 Indeed, BSF larvae are extremely voracious as well as highly suitable to being fed different
38
39 88 organic wet substrates (with a wide range of pH and moisture), including **by-products**
40
41 89 originating from the food industry, agricultural and livestock processes, municipal garden
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43 90 waste and household food scraps.¹⁷⁻²⁵

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46 91 **The content and the profile of BSF biomass, as well as the performance of larval growth, can**
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48 92 **vary to some extent depending on the different rearing substrates. In particular, the lipid**
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50 93 **profile is largely affected by the larval stage and the chemical composition of the rearing**
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52 94 **substrates.**^{2,26,27} **According to the type and content of the main constituents, various**
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54 95 **applications of whole larvae can be hypothesized. However, the limitations imposed by the**
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3 96 EU legislation about origin and kind of bio-waste authorized for insect rearing is fostering
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5 97 research into larvae processing in order to isolate and purify their main constituents. For these
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7 98 reasons, the present study aims at assessing the lipid content and fatty acids composition of
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9 99 BSF prepupae reared on different food by-products to outline its prospective applications.
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101 **EXPERIMENTAL**

102 **Laboratory colony**

103 The BSF larvae used for all the experiments came from the mother colony which is kept in
104 the laboratory of Applied Entomology, Technopole of Reggio Emilia (Italy) that has in turn
105 been established from prepupae collected in composters located in the provinces of Modena
106 and Cuneo (Northern Italy). Both larvae and adults were kept in climatic chambers under
107 controlled conditions at 27 ± 0.5 °C, 60-70% relative humidity and 16:8 h light:dark
108 photoperiod.

109 About 400-500 larvae for each glass container ($21 \times 13 \times 8$ cm, L×W×H) were reared on
110 “Gainesville House Fly” diet (50% wheat bran, 30% alfalfa meal and 20% cornmeal) mixed
111 with 60% water.^{18,28,29} The larvae were fed with fresh substrate three times per week. After
112 reaching the prepupal stage, the individuals were manually collected and placed into
113 cylindrical containers for emergence. Subsequently, the newly emerged flies were transferred
114 into cages (BugDorm-4 Insect Rearing Cage, $32.5 \times 32.5 \times 32.5$ cm, L×W×H, NHBS Ltd,
115 Totnes, UK). The adults were provided with a small plastic cap filled with cotton soaked with
116 sucrose. As oviposition site, a patented 3D-printed device,³⁰ developed in our laboratory, was
117 used. The eggs were manually collected three times per week and placed directly on the
118 rearing substrate, inside the glass containers described above.

119

120 **Collection of substrates**

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3 121 The alfalfa meal used in the control diet came from a pet store, while the wheat bran and the
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5 122 cornmeal were sourced at a local grocery store. The substrates tested in the experiments were
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7 123 collected from local companies and grouped into two categories: (i) industrial **by-products**,
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9 124 and (ii) **by-products** from retailers. The former consisted in brewery spent grains obtained
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11 125 from a local craft brewery Modena, Emilia-Romagna, Italy; cow's milk whey collected from
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13 126 the consortium of Parmigiano-Reggiano cheese (Reggio Emilia, Emilia-Romagna, Italy);
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15 127 grape stalks (*Vitis vinifera*) obtained from a local winery (Reggio Emilia, Emilia-Romagna,
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17 128 Italy); tomato peels and seeds (*Solanum lycopersicum*) collected from local companies
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19 129 (Reggio Emilia and Parma, Emilia-Romagna, Italy).
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24 130 The latter consisted of bread dough, prepared by mixing and kneading 250 g of wheat flour
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26 131 (*Triticum aestivum*), 150 g of water, 0.5 g of brewer's yeast, 5 g of olive oil, and 5 g of
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28 132 sodium chloride; **fish scraps of European bass (*Dicentrarchus labrax*), including heads, fins,**
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30 133 **fishbones, and offal**, which came from a local fish shop (Reggio Emilia, Emilia-Romagna,
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32 134 Italy) and were later cut into small pieces; spent coffee ground (*Coffea* spp.) which was
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34 135 collected from a local vending company (Reggio Emilia, Emilia-Romagna, Italy).
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37 136 All substrates were stored at -20 °C to avoid external contamination (microorganisms, mites,
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39 137 and insects) before their use.
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44 139 **Chemicals**

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47 140 All the reagents and solvents were of AR grade and were purchased from authorized
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49 141 suppliers. Butylated hydroxyanisole (BHA), chloroform, hexane, hydrochloric acid,
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51 142 methanol, potassium hydroxide (KOH), sodium chloride (NaCl), and anhydrous sodium
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53 143 sulfate (an. Na₂SO₄) were purchased from WVR Srl (Milan, Italy). Pure standard fatty acids
54
55 144 (FAs) were purchased from Carlo Erba (Milan, Italy). Saline solution was prepared at a
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57 145 12.5 g kg⁻¹ (w/v) of NaCl in deionized water. Undecanoic acid methyl ester, used as internal
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3 146 standard, was purchased from Fluka (Milan, Italy) and prepared at 10 g kg⁻¹ concentration
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5 147 (w/v) in chloroform-methanol 2:1. Deionized water was obtained through an Elix 3^{UV}
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8 148 purification system (Merck-Millipore, Milan, Italy).
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11 12 150 **Experimental trials**

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15 151 The experimental substrates were administered at the beginning of the experiment (200 g)
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17 152 and were placed into glass containers together with 100 BSF larvae (5-7 days old), for each
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19 153 replicate. Unlike all other substrates, cow's milk whey was prepared by mixing the
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21 154 "Gainesville House Fly" dry diet with 60% cow's milk whey.
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24 155 For each substrate, including the standard diet considered as a control, three replicates were
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26 156 performed inside climatic chambers under the same conditions described for laboratory
27
28 157 colony, and the entomological checks were performed 3 times per week until the larvae
29
30 158 reached the prepupal stage and were ready to be manually collected. Afterward, prepupae
31
32 159 were gently washed with tap water to remove any residue of the substrate, dried with
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34 160 absorbent paper, counted and weighed. Finally, prepupae were killed by freezing at -20 °C
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36 161 and stored until analysis could be carried out.³¹
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41 42 163 **Fat extraction and determination of total lipid content**

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44 164 Fat extraction was performed through the Folch method³² as adjusted and described in detail
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46 165 by Montevecchi et al. (2019) on previously frozen prepupae.³³
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48
49 166 The total lipid content was weighed with an analytical scale (AX224, Sartorius AG,
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51 167 Goettingen, Germany) and expressed as $g_{fat} Kg^{-1}_{prepupae}$ fresh weight. Each sample was
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53 168 analyzed rigorously following the same procedure.
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3 170 **Free and bound fatty acids acid-catalyzed esterification and transesterification**
4
5 171 **procedure, gas chromatography-mass spectrometry peak identification, and gas**
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8 172 **chromatographic profile of fatty acids.**

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10 173 On each sample, free and bound fatty acids (FA) determination was carried out using the
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12 174 method described by Christie.³⁴ In a glass tube, the prepupal fat was weighed (100 mg) and
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14 175 1 mL of hydrochloric acid in methanol (50 mL L⁻¹) was added. The tube was sealed and
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16
17 176 placed at a temperature of 100 °C for 1 h in order to derivatize FA into fatty acids methyl
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19 177 esters (FAME). Afterward, the tube was cooled down and 500 µL of hexane and 500 µL of
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21 178 deionized water were added. The mixture was centrifuged at 1752.4 g for 5 min to facilitate
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23 179 the separation of the organic upper phase.

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26 180 The FAME fraction (1 µL) of three samples (control) was withdrawn from the upper phase of
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28 181 the tube and injected into a gas chromatograph (GC) HP 6890 series instrument (Hewlett-
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30 182 Packard, Waldbronn, Germany) coupled with a mass spectrometer (MS) detector (HP 5973,
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33 183 Hewlett-Packard Waldbronn, Germany), equipped with a capillary column (Mega-10, 100%
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35 184 cyanopropyl polysiloxane, Mega snc, Legnano, MI, Italy) 50 m, having an internal diameter
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37 185 of 0.25 mm and film thickness of 0.20 µm.

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40 186 The injection was performed through a split/splitless injection port in split mode at 245 °C
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42 187 (split ratio 50:1). The carrier gas was ultrapure helium (with a constant flow rate of 1.5 mL
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44 188 min⁻¹). The temperature of the GC oven was set at 110 °C, held for 1 min and then increased
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46 189 at 10 °C min⁻¹ up to 230 °C and finally held for 2 min (15 min in total). The molecular
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48 190 fragmentation was obtained by electron ionization (EI). The data were obtained in full-scan
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50 191 mode and the mass to charge ratio (*m/z*) was recorded between 33 and 400 at 70 eV.

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53 192 GC-MS was used for identification only. Peaks were identified by comparing retention times
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55
56 193 and mass spectra of pure standards and by comparing the mass spectra with those present in

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3 194 the data system library dedicated to FAs (Famedb23.1 and Famedbwax.1; Agilent
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5 195 Technologies).

6
7
8 196 The FAME fraction (1 μ L) of each sample was injected in a gas chromatograph (Focus,
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10 197 Thermo Fisher Scientific, Rodano, MI, Italy), equipped with a split/splitless injector and FID
11
12 198 detector to determine the individual FAs. The chromatograms were acquired in the same
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14 199 conditions described for GC-MS using the Chrom-Card software (Thermo Fischer Scientific,
15
16 200 Rodano, MI, Italy). The peaks were identified by comparing the retention times of the
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18 201 analytes with those of the pure standards. Quantification was performed using the external
19
20 202 standard method in the presence of an internal standard. Each sample was analyzed
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22 203 rigorously following the same procedure.
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27 28 205 **Statistical analysis**

29
30 206 Univariate and multivariate analyses were carried out on the data set. Differences among the
31
32 207 lipid composition of the prepupae reared on the different substrates were assessed by analysis
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34 208 of variance (one-way ANOVA) based on two or three replicates for each sample. When a
35
36 209 significant effect (at least $p < 0.05$) was detected, comparative analyses were carried out
37
38 210 using the post hoc Tukey's multiple comparison test. Principal component analysis (PCA) of
39
40 211 the autoscaled values was also carried out. All statistical tests were performed using Statistica
41
42 212 version 8.0 software (Stat Soft Inc., Tulsa, OK, USA).
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47 48 49 214 **RESULTS**

50 51 215 **Prepupal growth performance**

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53 216 Parameters of prepupal growth performance obtained with the different substrates are shown
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55 217 in table 1. On the spent coffee ground, no larval growth was recorded and all larvae died
56
57 218 within 15 days. Poor growth was also observed on grape stalks, bread dough, and fish scraps,
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60

219 where BSF survival was lower than 34% of the initial population. Development-time
220 parameters were used to observe the achievement of the prepupal stage for both 50% and
221 95% of the living specimens. Only the BSF larvae grown on brewery **by-products**, cow's
222 milk whey, and tomato peels and seeds, along with those grown on the control substrate,
223 reached at least 50% of living specimens throughout the experiments. BSF larvae fed with
224 cow's milk whey showed the significantly shortest time of growth ($p \leq 0.001$), followed by
225 those fed with tomato peels and seeds. The percentage of living prepupae at the end of the
226 experiment was similar for these substrates.

227 The mean weight per prepupa varied significantly according to the rearing substrate
228 ($p \leq 0.001$). When BSF larvae were fed on grape stalks, bread dough, and fish scraps, the
229 prepupal mean weight showed the lowest values, whereas the highest weights were detected
230 when fed on the control diet and cow's milk whey, followed by brewery **by-products** and
231 tomato **by-products**. Finally, the biomass yield was calculated using the following formula:

$$\text{Biomass yield} = \frac{\text{PLP} \times \text{MWP}}{\text{T}}$$

233 where PLP is the percentage of living prepupae at the end of the experiment (%); MWP is
234 mean weight per prepupa; T is the time to achieve 95% of living prepupae.

235 The biomass yield varied significantly among the BSF reared on the different substrates
236 ($p \leq 0.001$). Highest yield values were obtained with cow's milk whey, while brewery **by-**
237 **products**, tomato peels and seeds, and the control diet constituted a single statistical group
238 (around two/third of whey).

239

240 **Total lipid content**

241 The total lipid contents are reported in table 2. The one-way ANOVA showed statistically
242 significant differences among the BSF reared on the different substrates ($p \leq 0.001$).
243 Prepupae fed on brewery **by-products**, bread dough, and control diet had the highest lipid

244 content (around 130 g kg⁻¹ fresh weight), whereas those grown with grape stalks showed the
245 lowest lipid content (about three times lower than the other diets). All the other samples had
246 total lipid content around 110 g kg⁻¹ of fresh weight.

247 The mean lipid content per prepupa was calculated using the total lipid content and the mean
248 weight per prepupa. Successively, the mean lipid content per prepupa was used to calculate
249 the lipid yield, using the following formula:

$$250 \quad \text{Lipid yield} = \frac{\text{PLP} \times \text{MLC}}{\text{T}}$$

251 where PLP is the percentage of living prepupae at the end of the experiment (%); MLC is
252 mean lipid content per prepupa; T is the time to achieve 95% of living prepupae.

253 The lipid yield varied significantly among BSF larvae reared on the different substrates
254 ($p \leq 0.001$). BSF grown in presence of cow's milk whey showed the highest lipid yield
255 values, followed by those fed on brewery by-products and control diet.

257 **Lipid profile**

258 The results of the analysis of the lipid profile are shown in table 3. The whole data set was
259 subjected to one-way ANOVA. Statistical differences were found for each FA, as well as for
260 the other indexes considered, with $p \leq 0.001$, except C_{10:0} that showed a $p \leq 0.01$.

261 Prepupae reared on cow's milk whey and the control showed a 1:3 unsaturated fatty acid sum
262 to saturated fatty acids sum ratio (UFAs/SFAs), while brewery by-products provided a
263 UFAs/SFAs around 1:2. Bread dough, grape stalks, and tomato peels and seeds showed
264 UFAs/SFAs higher than 1.

265 The comparison of the results obtained showed good accordance with some recent studies.
266 C_{12:0} was often the most abundant FA with content higher than 500 mg g⁻¹ prepupal fat in
267 BSF reared on the cow's milk whey, as well as in the control. This peculiar C_{12:0}
268 concentration has been already plentifully reported in the literature.^{2,35,36} The concentrations

of C_{14:0} were positively correlated with those of C_{12:0} ($r = 0.90$; $p < 0.001$), while C_{16:0} was negatively correlated with C_{12:0} ($r = -0.57$; $p < 0.05$). Similar behavior was described by Meneguz and coll.² In addition, C_{12:0} was negatively correlated with C_{18:0} ($r = -0.66$; $p < 0.05$), C_{18:1} ($r = -0.67$; $p < 0.01$), C_{18:2} ($r = -0.80$; $p < 0.001$), and C_{18:3} ($r = -0.57$; $p < 0.05$). Good accordance was observed with the results described by Meneguz and coll. on the brewery by-products, with a high increase of the PUFA fraction.²

Principal component analysis

A principal component analysis (PCA) was carried out on the autoscaled values to explore the parameters with figures for all the samples and to evaluate the relationship among the variables and the overall distribution of the samples on the score plot. The first three principal components (PCs), all with eigenvalues > 1.0 , explained 94.72% of the total variance. All factors with eigenvalues < 1.0 were discarded according to Kaiser's criterion.³⁷

The main SFA (C_{12:0}), along with C_{14:0}, SFA sum, and total lipid content weighed on PC1 (60.76% of the total variance) with a negative sign (Fig. 1A) and were grouped together, thus showing a high positive correlation among them. The mean weight and the percentage of living prepupae at the end of the experiment were characterized by a high negative weight on the PC1, as well as a rather negative value also on the PC2. PUFA (C_{18:2} and C_{18:3}), as well as UFA sum, UFAs/SFAs, and two saturated FAs (C_{16:0} and C_{18:0}) weighed on the PC1 with positive sign, and finally the main MUFA (C_{16:1} and C_{18:1}), along with their sum, mainly weighed on the PC2 (20.47% of the total variance). A negative correlation was observed between each of the parameters related to SFA, notably C_{12:0}, SFA sum, and total lipid content and each of the parameters related to UFA, notably UFA sum and UFAs/SFAs. A less obvious negative correlation was highlighted between parameters related to MUFA, notably MUFA sum and C_{18:1}, and the percentage of living prepupae at the end of the

294 experiment. PC3 (13.48% of the total variance) was characterized by C_{10:0} with a negative
295 sign on this PC (Fig. 1B).

296 The cow's milk whey lay close to the control in the center-left part of the score plot (Fig.
297 2A), due to their high concentrations of C_{12:0}, C_{14:0}, SFA sum, and total lipid content.
298 Brewery **by-products** diverged along the PC2 for the high values of mean weight and
299 percentage of living prepupae at the end of the experiment. The grape stalks treatment was
300 located in the opposite part of the plot due to its high UFA content. Fish scraps and bread
301 dough were set in the positive quadrant of the PC1, along the PC2 and were mainly
302 characterized by a high concentration of MUFA, while tomato peels and seeds were isolated
303 in the negative quadrant of the PC1 for their high concentrations of C_{18:2} and PUFA sum.

304

305 DISCUSSION AND CONCLUSIONS

306 **Prepupal growth performance**

307 BSF larval development followed different trends according to the composition of each
308 substrate. As for substrates of the first category (industrial **by-products**), the best larval
309 performance in terms of shorter development time was recorded using cow's milk whey
310 (instead of water in the control diet) and tomato peels and seeds. Indeed, cow's milk whey
311 contains a wide range of nutrients, such as lactose, lactic acid, proteins, fats, and mineral
312 salts, which are crucial for faster larval development. Although this by-product of the dairy
313 industry is normally used to make another dairy product, *ricotta* cheese, the present study
314 showed that it might find a possible convenient use to reduce growth times of larvae in a BSF
315 industrial farming system when combined with an appropriate solid standard diet.

316 In the presence of tomato peels and seeds, the larval growth was faster than in the control diet
317 or brewery **by-products**. Moreover, faster growth (95% prepupal achievement in less than 24
318 days) was observed, although the final mean weight per prepupa was lower than in the

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3 319 experiments that used other vegetables or fruits as growth substrates.^{38,39} Tomato peels are
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5 320 mainly composed of polysaccharides, such as pectin, cellulose, and hemicellulose.
6
7 321 Furthermore, they also contain bioactive compounds, such as flavonoids, lycopene, and
8
9 322 ascorbic acid, which are associated with a high antioxidant activity.⁴⁰

10
11
12 323 Tomato peels were reduced by BSF larvae to extremely thin pale-orange sheets, thus showing
13
14 324 that larvae are able to eat up the residual pulp and to let the outer membrane rather intact.
15
16 325 Furthermore, this suggests that the larvae were able to rapidly grow using all the nutrients
17
18 326 available and were not negatively affected by the presence of polyphenols. However, specific
19
20 327 studies should focus on the effect of these compounds on BSF larval performance.

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22
23 328 Tomato seeds contain essential amino acids, minerals (iron, magnesium, zinc, and copper),
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25 329 and fatty acids, notably oleic acid.⁴¹ However, the seeds were not intentionally crushed before
26
27 330 use. As a consequence, it was observed that the seeds were left intact, thus suggesting that
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29 331 larvae were not able to perforate the outer tegument and gain access to inner nutrients.

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32 332 Brewer spent grains are the most abundant **by-products** generated from the brewing process.
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34 333 They represent the insoluble part of the barley grains that are still rich in proteins (20-30%),
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36 334 fibers (30-50%), lipids (4-10%), and ash (3.5-4.5%).⁴² The main components of the fibers are
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38 335 arabinoxylans and cellulose.⁴³ This kind of substrate did not provide development
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40 336 performance as fast as that obtained with milk whey and tomato. However, they are
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42 337 particularly rich in proteins and, as a matter of fact, the percentage of living prepupae at the
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44 338 end of the experiment was close to 100%. The mean weight per prepupa, as well as its mean
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46 339 lipid content, presented the highest values, thus showing that this substrate provided a
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48 340 balanced nutrient composition for BSF larval growth.

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51 341 In the present study, BSF prepupae reared on brewers' spent grains after 18 days reached an
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53 342 average weight of 0.22 g. These data are consistent with those reported by Meneguz and
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55 343 coll.² who found a maximum larval weight of 0.12 g, after 8 days.

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3 344 Grape stalks effectively proved to be one of the most inadequate substrates as they are almost
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5 345 devoid of nutrients. They are indeed composed of fibers, such as cellulose (36%), lignin
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7 346 (34%), and hemicellulose (24%).⁴⁴ The rest is represented by polyphenols and salts, and other
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10 347 minor, worthless substances. BSF prepupae reached an average weight of 0.04 g, after 37
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12 348 days. Meneguz and coll. recorded a maximum larval weight of 0.15 g, after 26 days.²⁵ This
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14 349 apparent inconsistency in growth performance with said study is very likely due to the
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16 350 different composition of the substrate. Indeed, Meneguz and coll. used whole winery **by-**
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18 351 **products**, including grape seeds, pulp, peels, stalks, and leaves. Grape peels are similar to
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20 352 tomato peels, which gave good results. For this reason, Meneguz and coll.'s results are not
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22 353 comparable with the sole stalks used in the present study.²

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26 354 Among the substrates belonging to the second category (retailer **by-products**), spent coffee
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28 355 ground showed that it was not fit for purpose at all since all larvae died within 15 days. The
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30 356 high content of indigestible fibers and toxic alkaloids, mainly caffeine, as well as the products
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32 357 of Maillard reaction that brings about the permanent modification of sugars and proteins,
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34 358 were probably the causes that made this substrate totally unfavorable to BSF larval growth.⁴⁵

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37 359 Larvae reared on fish scraps and bread dough showed low growth performance in terms of
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39 360 mean weight and prepupal survival. Fish scraps have a very high content of proteins and
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41 361 polyunsaturated fatty acids but they are poor in carbohydrates and probably this is the reason
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43 362 for the poor performance. Likewise, Nguyen and coll. (2013)⁴⁶ reported that a fish offal
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45 363 protein-carbohydrate percentage of 90.9:1.0 was associated with a poor survival percentage
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47 364 of 47%. On the contrary, a protein-carbohydrate balanced diet (21:21 as a percentage in
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49 365 weight in the diet)²⁰ led to faster development rate and higher larval survival. A protein-
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51 366 carbohydrate percentage in weight in the diet of 35:7 led to a development time of around 45
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54 367 days, with a survival percentage of 46%. These authors confirmed the importance of an
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56 368 adequate moisture rate in the substrate, being 70% of relative humidity the best condition for
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3 369 growth performance. In general, an unbalanced diet and fast dehydration of fish scraps may
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5 370 probably be an unfavorable environment for BSF larval development.

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8 371 As for bread dough, the larval growth was mainly hindered by the texture of the substrate. In
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10 372 fact, the dough outer layer quickly dried up thus forming a thick hard surface, whereas the
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12 373 internal core had a very sticky texture that firmly trapped most of the larvae constraining their
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14 374 movements and, as a consequence, they died.

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18 19 376 **Total lipid content and lipid profile**

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21 377 The lipid content of insects, as well as its composition and FA profile, can vary with diet. In
22
23 378 fact, the lipid content and composition are strongly affected by the insect species and by
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25 379 several other factors, such as the diet provided, habitat, temperature and moisture conditions,
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27 380 metamorphic stage.⁴⁷ The modulation of the factors allows to target lipid amount and quality
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29 381 according to the planned fat application.

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33 382 In a recent study, Caligiani and coll.³⁵ have found in BSF larvae a crude lipid content of
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35 383 **371 g kg⁻¹** (dry matter basis) that once converted into a fresh matter basis (**340 g kg⁻¹** dry
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37 384 matter) was **126 g kg⁻¹** fresh weight. This value is in perfect accordance with the fat content
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39 385 found in the present study using brewery **by-products**, as well as the control treatment.
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41 386 However, all other substrates with the exception of grape stalks yielded a fat content higher
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43 387 than **110 g kg⁻¹** (fresh matter basis). A content of fat around **40 g kg⁻¹** was found in the
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45 388 literature for BSF larvae reared on grape stalks.³⁸ In the present study, the fat yield shown by
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47 389 BSF prepupae grown on grape stalks was consistent to the unsatisfactory growth performance
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49 390 associated to this substrate and it is likely caused by the deficiency of crucial nutrients.

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53 391 In general, the range of BSF larval lipid content reported in the literature ranges from **50 to**
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55 392 **130 g kg⁻¹** (fresh matter basis), thus representing a good source of energy.^{48,49} **Moreover, the**
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57 393 **low cholesterol content, similar structure and functionality as butter, and a sensory**

394 acceptance up to 25% replacing of butter, makes this fat suitable for the food industry.⁵⁰

395 However, due to the hindrances imposed by the current legislation, the use of insect fat as a
396 biofuel is one the most profitable alternative to reduce the consumption of fossil fuels.

397 Biodiesel is chemically obtained by reacting lipids with short-chain alcohols (methanol,
398 ethanol, propanol), thus providing individual esters of fatty acid. Biodiesel has good
399 lubricating properties and higher cetane ratings in comparison to the common low-sulfur
400 diesel fuels.⁵¹ In the literature, a cetane number (CN) as high as 64.8 has been reported for
401 BSF fat. This value has also been associated with a fatty acid profile adequate for biodiesel
402 production.⁵² In the present study, cetane numbers for the different samples were obtained
403 (table 3) in accordance with Freedman and Bagby (1990).⁵¹ Fat obtained using cow's milk
404 whey achieved the highest CN figure (60), as did the control, while tomato peels and seeds
405 provided the lowest value⁵².

406 Aside from its interesting properties as a fuel, biodiesel also shows other crucial
407 characteristics, which make it more efficient than standard diesel. In particular, it allows to
408 reduce the production and release of environmental contaminants, such as carbon monoxide,
409 heavy metals, sulfur oxides, aliphatic and aromatic hydrocarbons, as well as fine particulate
410 matter.^{53,54} Furthermore, the energy balance of the life cycle shows an extremely positive
411 result with an energy consumption of about a quarter compared to the energy produced. Since
412 it derives from renewable vegetable crops and animal products, it implies the zeroing of the
413 CO₂ cycle when biodiesel is burned. No additional CO₂ is emitted because the carbon dioxide
414 in output has already been compensated by that which the plants fixed during their growth.
415 The only source of carbon dioxide in surplus could be provided by the alcohols used in the
416 transesterification process, unless they have a bio-origin, as well. Finally, biodiesel has a
417 lower environmental impact. Its toxicity is lower than its fossil equivalents. In fact, biodiesel
418 degrades completely in 21 days, but even after 2 days in contact with the air, the esters of the

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3 419 fatty acids are no longer detectable. Many microorganisms, can use biodiesel as a source of
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5 420 carbon, thus limiting the problems arising from accidental or chronic fuel losses.⁵⁵⁻⁵⁹
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8 421 As already described, FA profile is significantly affected by the rearing substrate (table 3)
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10 422 and these data are confirmed by literature.⁴⁸ As a consequence, a targeted diet can give rise to
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12 423 different types of fat characterized by an optimal composition for a specific purpose: e.g.
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14 424 either production of biodiesel or high PUFA oil for food and feed. On the other hand, a
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16 425 complete diet gives rise to a sort of “standard fat”, as does a rearing substrate composed of
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18 426 HO.RE.CA. by-products, in which carbohydrates, lipids, and proteins from different food
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20 427 sources are supplied in large quantities. At the moment, the first results not yet published
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22 428 using HO.RE.CA. by-products are very promising in terms of fat yield and quality.
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24 429 Moreover, it remains to be assessed what percentage of fat the larvae can tolerate in the
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26 430 substrate.
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31 431 Returning to the present study, the possibility of affecting and modulating the FA
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33 432 composition was strongly confirmed. Noteworthy differences were found out in the ratio
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35 433 between unsaturated (UFAs) and saturated (SFAs) FA sums, thus outlining peculiar profiles
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37 434 for each rearing substrate. Brewery by-products provided a higher concentration of UFAs in
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39 435 comparison with cow’s milk whey, likely due to higher polyunsaturated fatty acids (PUFA)
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41 436 content. The UFAs/SFAs increased in fish scraps, mainly because of the higher concentration
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43 437 of the monounsaturated fatty acids (MUFA) content. The bread dough was characterized by
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45 438 the highest MUFA content (around 340 mg g⁻¹ prepupal fat). Finally, tomato peels and seeds
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47 439 and grape stalks (despite their nutritional shortage), both showed UFAs/SFAs higher than 1,
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49 440 as well as the highest PUFA content (around 390 mg g⁻¹ prepupal fat) along with the lowest
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51 441 C_{12:0} concentration.
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56 442 The cow’s milk whey showed the best performance in terms of larval growth. However, to be
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58 443 considered suitable as a substrate, this by-product needs to be absorbed on a solid support
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3 444 which in the present study was represented by the control diet. Also, in light of the results, the
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5 445 control diet could be effectively replaced by tomato peels and seeds. These latter gave rise to
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7 446 prepupae with a prevalence of unsaturated fatty acids, even though larvae were not able to
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9 447 crush tomato seeds and effectively exploit the substances contained. Follow-up studies
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11 448 should, therefore, consider a preliminary grinding step to help release tomato-seed inner
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13 449 nutrients into the substrate. For all these reasons, the use of ground tomato peels and seeds as
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15 450 solid support for cow's milk whey, and in the same way for *ricotta* whey, may represent an
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17 451 advantageous combination of substrates for BSF rearing, which is worth ascertaining.
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33
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36 459 *mediante insetti per l'ottenimento di biomateriali per l'agricoltura*).
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41 42 461 **Conflict of Interest Statement**

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44 462 The authors declare that there is no conflict of interest regarding the publication of this
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46 463 article.
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3 465 **Figure captions**
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8 467 **Figure 1.** (A) PC1 vs PC2 and (B) PC1 vs PC3 loading plots of main parameters related to
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10 468 the analyses of substrates used for black soldier fly growth with the explained variance (%).
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12 469 MW: mean lipid content per prepupa; PLP: percentage of living prepupae at the end of the
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14 470 experiment; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SS,
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16 471 saturated fatty acids; TFC, total fat content; US, unsaturated fatty acids; U/S, unsaturated
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18 472 fatty acid sum to saturated fatty acids sum ratio.
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24 474 **Figure 2.** (A) PC1 vs PC2 and (B) PC1 vs PC3 score plots of substrates used for black
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26 475 soldier fly growth with the explained variance (%).
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652 **Table 1.** Parameters of prepupae growth performance using different substrates (values are means of three replicates \pm the standard deviation).

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Substrate	Achievement of 50% living prepupae (day)	Achievement of 95% living prepupae (day; T)	Percentage of living prepupae at the end of the experiment (%; PLP)	Mean weight per prepupa (g; MWP)	Biomass yield PLP \times MWP/T (g _{biomass} per day)
ANOVA (F_{value})	499.7***	1017.4***	100.5***	121.8***	89.3***
Control	28 \pm 0 c	32 \pm 1 c	99 \pm 1 b	0.22 \pm 0.01 e	0.67 \pm 0.01 a
<i>SUBSTRATES</i>					
<i>GROUP A†</i>					
Brewery by-products	31 \pm 0 d	33 \pm 0 c	97 \pm 4 b	0.22 \pm 0.00 e	0.66 \pm 0.01 a
Cow's milk whey	17 \pm 0 a	18 \pm 1 a	100 \pm 0 b	0.19 \pm 0.02 d	1.0 \pm 0.2 b
Grape stalks	=	=	15 \pm 4 a	0.04 \pm 0.00 a	=
Tomato peels and seeds	20 \pm 2 b	24 \pm 1 b	100 \pm 0 b	0.14 \pm 0.00 c	0.59 \pm 0.04 a
<i>SUBSTRATES</i>					
<i>GROUP B‡</i>					
Bread dough	=	=	34 \pm 14 a	0.08 \pm 0.00 b	=
Fish scraps	=	=	21 \pm 11 a	0.10 \pm 0.01 b	=

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655 Results of one-way ANOVA and Tukey's test are reported as F_{value} and letters (for statistically significant F_{value}), respectively. Different letters
656 identify samples that are significantly different ($P \leq 0.05$)657 ***: $P \leq 0.001$.658 †: industrial **by-products**; ‡: retailer **by-products**.

659 T: time to achieve 95% of living prepupae; PLP: percentage of living prepupae at the end of the experiment; MWP: mean weight per prepupa.

660 PLP \times MWP/T%P: biomass yield.

662 **Table 2.** Total lipid content. Values (g kg^{-1} prepupal fresh weight) are means of three repetitions \pm the standard deviation

663

Substrate	Total lipid content \pm S.D. (g kg^{-1} F.W.)	Mean lipid content per prepupa (g; MLC)	Lipid yield PLP \times MLC/T (g lipid per day)
ANOVA (F_{value})	183.5***	183.9***	201.9***
Control	128 \pm 11 cd	0.028 \pm 0.001 e	0.086 \pm 0.002 b
<i>SUBSTRATES GROUP A</i> †			
Brewery by-products	128 \pm 3 d	0.028 \pm 0.000 e	0.083 \pm 0.003 ab
Cow's milk whey	110 \pm 10 b	0.020 \pm 0.003 d	0.11 \pm 0.02 c
Grape stalks	43 \pm 2 a	0.002 \pm 0.000 a	=
Tomato peels and seeds	115 \pm 7 bc	0.016 \pm 0.000 c	0.067 \pm 0.002 a
<i>SUBSTRATES GROUP B</i> ‡			
Bread dough	132 \pm 1 d	0.010 \pm 0.001 b	=
Fish scraps	111 \pm 10 b	0.012 \pm 0.002 b	=

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665 Results of one-way ANOVA and Tukey's test are reported as F_{value} and letters (for statistically significant F_{value}), respectively. Different letters identify samples that are significantly different ($P \leq 0.05$).

666 ***: $P \leq 0.001$.

667 †: industrial by-products; ‡: retailer by-products.

668 F.W. = fresh weight; MLC: mean lipid content per prepupa; PLP: percentage of living prepupae at the end of the experiment; T: time to achieve 95% of living prepupae; PLP \times MLC/T: lipid yield.

670

672 **Table 3.** FA profile of the sample set. Values (mg g^{-1} prepupal fat) are means of three repetitions \pm the standard deviation.

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Substrate	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:0}	SFA sum	UFA sum	MUF A sum	PUFA sum	UFAs/SFAs	Cetane number
ANOVA (F_{values})	12.6 **	146.6 ***	158.2 ***	47.3 ***	288.1 ***	157.5 ***	138.3 ***	428.9 ***	75.4 ***	407.5 ***	214.6 ***	214.5 ***	237.5 ***	364.0 ***	103.1 ***	
Control	15 ^b ± 1	557 ^e ± 14	76 ^d ± 4	83 ^a ± 3	20.5 ^a ± 0.4	9 ^{ab} ± 1	111.3 ^a ± 0.2	110 ^a ± 3	13.2 ^b ± 0.7	5.5 ^c ± 0.2	745 ^e ± 6	255 ^a ± 6	131.8 ^a ± 0.8	123 ^a ± 5	0.34 ^a ± 0.01	60
<i>SUBSTRATE S GROUP A†</i>																
Barley beer (brewery by-product)	14.0 ^b ± 0.4	485 ^{cd} ± 12	56 ^c ± 1	107.1 ^b ± 0.4	19.3 ^a ± 0.9	10.8 ^{ab} ± 0.8	82 ^a ± 3	205 ^c ± 2	21 ^c ± 1	0.00 ^a ± 0.00	673 ^d ± 9	327 ^b ± 9	101 ^a ± 6	226 ^c ± 4	0.49 ^{ab} ± 0.02	57
Cow's milk whey	13.1 ^b ± 0.9	534 ^{de} ± 17	78.0 ^d ± 0.7	88 ^a ± 2	19.6 ^a ± 0.0	16.6 ^c ± 0.3	114 ^a ± 5	129 ^a ± 8	8 ^a ± 1	0.00 ^a ± 0.00	730 ^{de} ± 19	271 ^{ab} ± 19	133 ^a ± 7	137 ^a ± 13	0.37 ^a ± 0.04	60
Grape stalks	13.9 ^b ± 0.6	170 ^a ± 11	29.2 ^a ± 0.2	128 ^c ± 4	49 ^c ± 3	33.0 ^d ± 0.0	229 ^c ± 9	318 ^d ± 9	29.5 ^d ± 0.8	0.00 ^a ± 0.00	374 ^a ± 23	626 ^e ± 23	278 ^c ± 9	348 ^d ± 14	1.68 ^d ± 0.17	51
Tomato peels and seeds	7.4 ^a ± 0.2	280 ^b ± 8	32 ^{ab} ± 1	89.5 ^a ± 0.1	15.6 ^a ± 0.8	12.3 ^b ± 0.5	165 ^b ± 5	374 ^e ± 3	13.4 ^b ± 0.2	4.2 ^b ± 0.3	425 ^{ab} ± 12	568 ^d ± 13	181 ^b ± 9	389 ^e ± 4	1.34 ^c ± 0.07	51
<i>SUBSTRATE S GROUP B‡</i>																
Bread dough	11.4 ^{ab} ± 0.0	334 ^b ± 9	39.8 ^b ± 0.3	84 ^a ± 1	41 ^b ± 1	10.7 ^{ab} ± 0.4	300 ^d ± 12	169 ^b ± 3	11.0 ^{ab} ± 0.8	0.00 ^a ± 0.00	479 ^b ± 10	521 ^d ± 10	341 ^d ± 15	180 ^b ± 5	1.09 ^c ± 0.04	55
Fish scraps	14.8 ^b ± 0.8	439 ^c ± 9	38.4 ^b ± 0.0	77 ^a ± 1	71.6 ^d ± 0.2	8.3 ^a ± 0.8	205 ^c ± 5	105 ^a ± 3	13.0 ^b ± 0.7	6.5 ^d ± 0.2	584 ^c ± 14	395 ^c ± 10	276 ^c ± 8	118 ^a ± 5	0.68 ^b ± 0.04	59

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675 Results of one-way ANOVA and Tukey's test are reported as F_{value} and letters (for statistically significant F_{value}), respectively. Different letters
676 identify samples that are significantly different ($P \leq 0.05$).

677 **: $P \leq 0.01$; ***: $P \leq 0.001$.

678 †: industrial **by-products**; ‡: retailer **by-products**.

679 SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UFAs/SFAs,
680 unsaturated fatty acid sum to saturated fatty acids sum ratio.

Figure 1. (A) PC1 vs PC2 and (B) PC1 vs PC3 loading plots of main parameters related to the analyses of substrates used for black soldier fly growth with the explained variance (%).

MW: mean lipid content per prepupae; PLP: percentage of living prepupae at the end of the experiment; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SS, saturated fatty acids; TFC, total fat content; US, unsaturated fatty acids; U/S, saturated fatty acid sum to unsaturated fatty acids sum ratio.

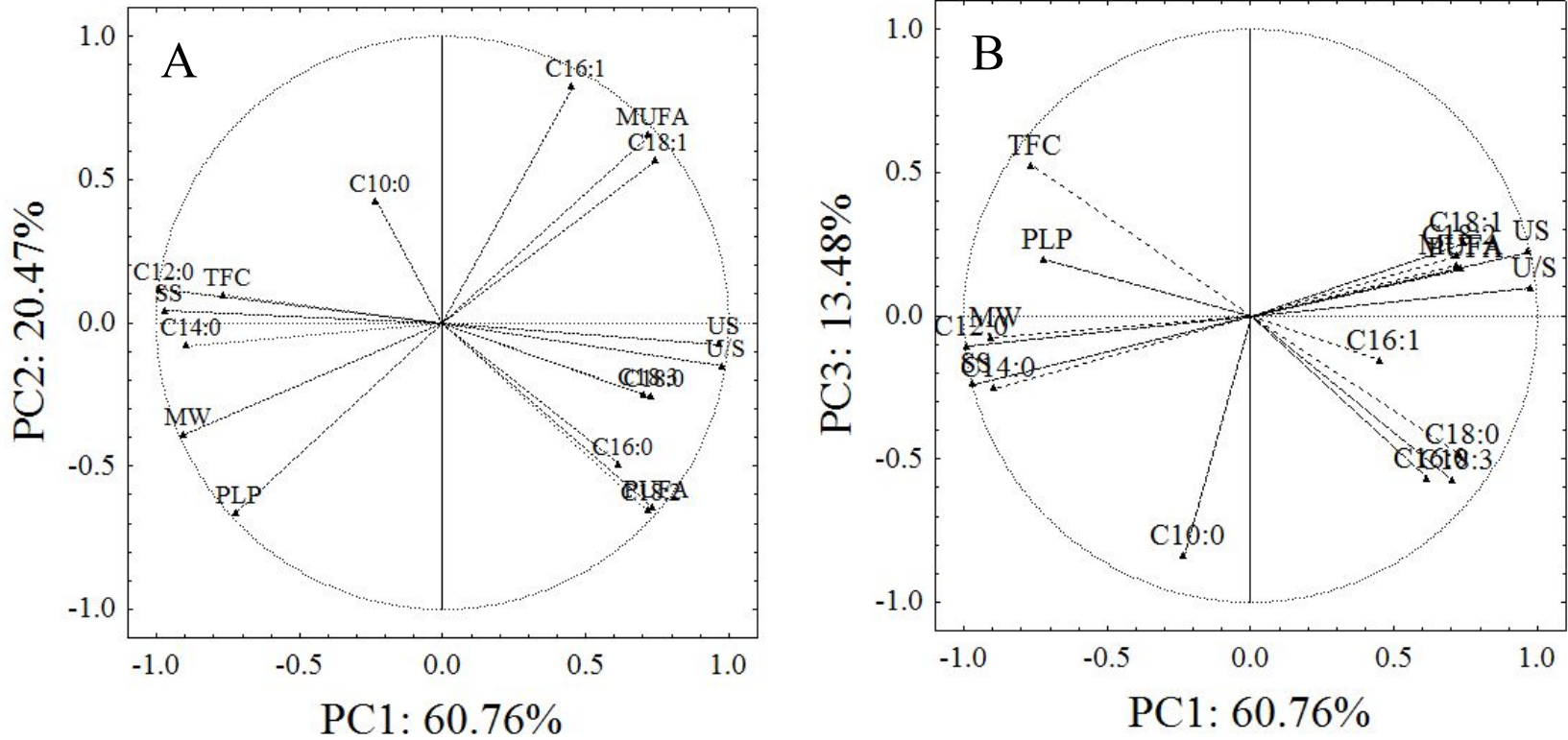


Figure 2. (A) PC1 vs PC2 and (B) PC1 vs PC3 score plots of substrates used for black soldier fly growth with the explained variance (%).

