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

Short running title: Lipid profile and growth of *Hermetia illucens* reared on food by-products

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Abstract

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

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

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

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

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

INTRODUCTION

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

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

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

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

Currently, these by-products are only partially utilized as animal feed, for composting, or biogas production. An investigation into alternative uses of these by-products is becoming urgent and it ranges from the extraction of high-value compounds, such as lipids for biodiesel, or polyphenols for their antioxidant activity, or other substances of high nutritional value before choosing to use by-products as a substrate for biogas production.[7–13] Finally, spent coffee ground and brewery by-products are conveniently utilized for the cultivation of edible mushrooms.[14,15]

A very interesting solution to the problem is the bioconversion of by-products into valuable organic fractions, such as proteins, fat, and chitin carried out by scavenger insects, such as the 'black soldier fly' (BSF) *Hermetia illucens* (Linnaeus, 1758) (Diptera, Stratiomyidae). In the last few years, this species has been used in different studies for waste bioconversion.[16] Indeed, BSF larvae are extremely voracious as well as highly suitable to being fed different organic wet substrates (with a wide range of pH and moisture), including by-products originating from the food industry, agricultural and livestock processes, municipal garden waste and household food scraps.[17–25]

The content and the profile of BSF biomass, as well as the performance of larval growth, can vary to some extent depending on the different rearing substrates. In particular, the lipid profile is largely affected by the larval stage and the chemical composition of the rearing substrates.[2,26,27] According to the type and content of the main constituents, various applications of whole larvae can be hypothesized. However, the limitations imposed by the

EU legislation about origin and kind of bio-waste authorized for insect rearing is fostering research into larvae processing in order to isolate and purify their main constituents. For these reasons, the present study aims at assessing the lipid content and fatty acids composition of BSF prepupae reared on different food by-products to outline its prospective applications.

EXPERIMENTAL

Laboratory colony

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

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

Collection of substrates

The alfalfa meal used in the control diet came from a pet store, while the wheat bran and the cornmeal were sourced at a local grocery store. The substrates tested in the experiments were collected from local companies and grouped into two categories: (i) industrial by-products, and (ii) by-products from retailers. The former consisted in brewery spent grains obtained from a local craft brewery Modena, Emilia-Romagna, Italy; cow's milk whey collected from the consortium of Parmigiano-Reggiano cheese (Reggio Emilia, Emilia-Romagna, Italy); grape stalks (*Vitis vinifera*) obtained from a local winery (Reggio Emilia, Emilia-Romagna, Italy); tomato peels and seeds (*Solanum lycopersicum*) collected from local companies (Reggio Emilia and Parma, Emilia-Romagna, Italy).

The latter consisted of bread dough, prepared by mixing and kneading 250 g of wheat flour (*Triticum aestivum*), 150 g of water, 0.5 g of brewer's yeast, 5 g of olive oil, and 5 g of sodium chloride; fish scraps of European bass (*Dicentrarchus labrax*), including heads, fins, fishbones, and offal, which came from a local fish shop (Reggio Emilia, Emilia-Romagna, Italy) and were later cut into small pieces; spent coffee ground (*Coffea* spp.) which was collected from a local vending company (Reggio Emilia, Emilia-Romagna, Italy).

All substrates were stored at -20 °C to avoid external contamination (microorganisms, mites, and insects) before their use.

Chemicals

All the reagents and solvents were of AR grade and were purchased from authorized suppliers. Butylated hydroxyanisole (BHA), chloroform, hexane, hydrochloric acid, methanol, potassium hydroxide (KOH), sodium chloride (NaCl), and anhydrous sodium sulfate (an. Na_2SO_4) were purchased from WVR Srl (Milan, Italy). Pure standard fatty acids (FAs) were purchased from Carlo Erba (Milan, Italy). Saline solution was prepared at a 12.5 g kg^{-1} (w/v) of NaCl in deionized water. Undecanoic acid methyl ester, used as internal standard, was purchased from Fluka (Milan, Italy) and prepared at 10 g kg^{-1} concentration (w/v) in chloroform-methanol 2:1. Deionized water was obtained through an Elix 3^{UV} purification system (Merck-Millipore, Milan, Italy).

Experimental trials

The experimental substrates were administered at the beginning of the experiment (200 g) and were placed into glass containers together with 100 BSF larvae (5-7 days old), for each replicate. Unlike all other substrates, cow's milk whey was prepared by mixing the "Gainesville House Fly" dry diet with 60% cow's milk whey.

For each substrate, including the standard diet considered as a control, three replicates were performed inside climatic chambers under the same conditions described for laboratory colony, and the entomological checks were performed 3 times per week until the larvae reached the prepupal stage and were ready to be manually collected. Afterward, prepupae were gently washed with tap water to remove any residue of the substrate, dried with absorbent paper, counted and weighed. Finally, prepupae were killed by freezing at -20 °C and stored until analysis could be carried out.[31]

Fat extraction and determination of total lipid content

Fat extraction was performed through the Folch method[32] as adjusted and described in detail by Montevecchi et al. (2019) on previously frozen prepupae.[33]

The total lipid content was weighed with an analytical scale (AX224, Sartorius AG, Goettingen, Germany) and expressed as g_{fat} $Kg^{-1}{}_{prepupae}$ fresh weight. Each sample was analyzed rigorously following the same procedure.

Free and bound fatty acids acid-catalyzed esterification and transesterification procedure, gas chromatography-mass spectrometry peak identification, and gas chromatographic profile of fatty acids.

On each sample, free and bound fatty acids (FA) determination was carried out using the method described by Christie.[34] In a glass tube, the prepupal fat was weighed (100 mg) and 1 mL of hydrochloric acid in methanol (50 mL L^{-1}) was added. The tube was sealed and placed at a temperature of 100 °C for 1 h in order to derivatize FA into fatty acids methyl esters (FAME). Afterward, the tube was cooled down and 500 µL of hexane and 500 µL of deionized water were added. The mixture was centrifuged at 1752.4 g for 5 min to facilitate the separation of the organic upper phase.

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

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

GC-MS was used for identification only. Peaks were identified by comparing retention times and mass spectra of pure standards and by comparing the mass spectra with those present in the data system library dedicated to FAs (Famedb23.l and Famedbwax.l; Agilent Technologies).

The FAME fraction (1 µL) of each sample was injected in a gas chromatograph (Focus, Thermo Fisher Scientific, Rodano, MI, Italy), equipped with a split/splitless injector and FID detector to determine the individual FAs. The chromatograms were acquired in the same conditions described for GC-MS using the Chrom-Card software (Thermo Fischer Scientific, Rodano, MI, Italy). The peaks were identified by comparing the retention times of the analytes with those of the pure standards. Quantification was performed using the external standard method in the presence of an internal standard. Each sample was analyzed rigorously following the same procedure.

Statistical analysis

Univariate and multivariate analyses were carried out on the data set. Differences among the lipid composition of the prepupae reared on the different substrates were assessed by analysis of variance (one-way ANOVA) based on two or three replicates for each sample. When a significant effect (at least $p < 0.05$) was detected, comparative analyses were carried out using the post hoc Tukey's multiple comparison test. Principal component analysis (PCA) of the autoscaled values was also carried out. All statistical tests were performed using Statistica version 8.0 software (Stat Soft Inc., Tulsa, OK, USA).

RESULTS

Prepupal growth performance

Parameters of prepupal growth performance obtained with the different substrates are shown in table 1. On the spent coffee ground, no larval growth was recorded and all larvae died within 15 days. Poor growth was also observed on grape stalks, bread dough, and fish scraps, where BSF survival was lower than 34% of the initial population. Development-time parameters were used to observe the achievement of the prepupal stage for both 50% and 95% of the living specimens. Only the BSF larvae grown on brewery by-products, cow's milk whey, and tomato peels and seeds, along with those grown on the control substrate, reached at least 50% of living specimens throughout the experiments. BSF larvae fed with cow's milk whey showed the significantly shortest time of growth ($p \leq 0.001$), followed by those fed with tomato peels and seeds. The percentage of living prepupae at the end of the experiment was similar for these substrates.

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

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

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

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

Total lipid content

The total lipid contents are reported in table 2. The one-way ANOVA showed statistically significant differences among the BSF reared on the different substrates ($p \leq 0.001$). Prepupae fed on brewery by-products, bread dough, and control diet had the highest lipid content (around 130 g kg^{-1} fresh weight), whereas those grown with grape stalks showed the lowest lipid content (about three times lower than the other diets). All the other samples had total lipid content around 110 g kg^{-1} of fresh weight.

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

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

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

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

Lipid profile

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

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

The comparison of the results obtained showed good accordance with some recent studies. $C_{12:0}$ was often the most abundant FA with content higher than 500 mg g^{-1} prepupal fat in BSF reared on the cow's milk whey, as well as in the control. This peculiar $C_{12:0}$ 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 experiment. PC3 (13.48% of the total variance) was characterized by $C_{10:0}$ with a negative sign on this PC (Fig. 1B).

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

DISCUSSION AND CONCLUSIONS

Prepupal growth performance

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

In the presence of tomato peels and seeds, the larval growth was faster than in the control diet or brewery by-products. Moreover, faster growth (95% prepupal achievement in less than 24 days) was observed, although the final mean weight per prepupa was lower than in the experiments that used other vegetables or fruits as growth substrates.[38,39] Tomato peels are mainly composed of polysaccharides, such as pectin, cellulose, and hemicellulose. Furthermore, they also contain bioactive compounds, such as flavonoids, lycopene, and ascorbic acid, which are associated with a high antioxidant activity.[40]

Tomato peels were reduced by BSF larvae to extremely thin pale-orange sheets, thus showing that larvae are able to eat up the residual pulp and to let the outer membrane rather intact. Furthermore, this suggests that the larvae were able to rapidly grow using all the nutrients available and were not negatively affected by the presence of polyphenols. However, specific studies should focus on the effect of these compounds on BSF larval performance.

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

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

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

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

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

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

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

Total lipid content and lipid profile

The lipid content of insects, as well as its composition and FA profile, can vary with diet. In fact, the lipid content and composition are strongly affected by the insect species and by several other factors, such as the diet provided, habitat, temperature and moisture conditions, metamorphic stage.[47] The modulation of the factors allows to target lipid amount and quality according to the planned fat application.

In a recent study, Caligiani and coll.[35] have found in BSF larvae a crude lipid content of 371 g kg^{-1} (dry matter basis) that once converted into a fresh matter basis (340 g kg^{-1} dry matter) was 126 g kg^{-1} fresh weight. This value is in perfect accordance with the fat content found in the present study using brewery by-products, as well as the control treatment. However, all other substrates with the exception of grape stalks yielded a fat content higher than 110 g kg^{-1} (fresh matter basis). A content of fat around 40 g kg^{-1} was found in the literature for BSF larvae reared on grape stalks.[38] In the present study, the fat yield shown by BSF prepupae grown on grape stalks was consistent to the unsatisfactory growth performance associated to this substrate and it is likely caused by the deficiency of crucial nutrients.

In general, the range of BSF larval lipid content reported in the literature ranges from 50 to 130 g kg^{-1} (fresh matter basis), thus representing a good source of energy.[48,49] Moreover, the low cholesterol content, similar structure and functionality as butter, and a sensory acceptance up to 25% replacing of butter, makes this fat suitable for the food industry.[50] However, due to the hindrances imposed by the current legislation, the use of insect fat as a biofuel is one the most profitable alternative to reduce the consumption of fossil fuels. Biodiesel is chemically obtained by reacting lipids with short-chain alcohols (methanol, ethanol, propanol), thus providing individual esters of fatty acid. Biodiesel has good lubricating properties and higher cetane ratings in comparison to the common low-sulfur diesel fuels.[51] In the literature, a cetane number (CN) as high as 64.8 has been reported for BSF fat. This value has also been associated with a fatty acid profile adequate for biodiesel production.[52] In the present study, cetane numbers for the different samples were obtained (table 3) in accordance with Freedman and Bagby (1990).[51] Fat obtained using cow's milk whey achieved the highest CN figure (60), as did the control, while tomato peels and seeds provided the lowest value[52].

Aside from its interesting properties as a fuel, biodiesel also shows other crucial characteristics, which make it more efficient than standard diesel. In particular, it allows to reduce the production and release of environmental contaminants, such as carbon monoxide, heavy metals, sulfur oxides, aliphatic and aromatic hydrocarbons, as well as fine particulate matter.[53,54] Furthermore, the energy balance of the life cycle shows an extremely positive result with an energy consumption of about a quarter compared to the energy produced. Since it derives from renewable vegetable crops and animal products, it implies the zeroing of the CO_2 cycle when biodiesel is burned. No additional CO_2 is emitted because the carbon dioxide in output has already been compensated by that which the plants fixed during their growth. The only source of carbon dioxide in surplus could be provided by the alcohols used in the transesterification process, unless they have a bio-origin, as well. Finally, biodiesel has a lower environmental impact. Its toxicity is lower than its fossil equivalents. In fact, biodiesel degrades completely in 21 days, but even after 2 days in contact with the air, the esters of the fatty acids are no longer detectable. Many microorganisms, can use biodiesel as a source of carbon, thus limiting the problems arising from accidental or chronic fuel losses.[55-59]

As already described, FA profile is significantly affected by the rearing substrate (table 3) and these data are confirmed by literature.[48] As a consequence, a targeted diet can give rise to different types of fat characterized by an optimal composition for a specific purpose: e.g. either production of biodiesel or high PUFA oil for food and feed. On the other hand, a complete diet gives rise to a sort of "standard fat", as does a rearing substrate composed of HO.RE.CA. by-products, in which carbohydrates, lipids, and proteins from different food sources are supplied in large quantities. At the moment, the first results not yet published using HO.RE.CA. by-products are very promising in terms of fat yield and quality. Moreover, it remains to be assessed what percentage of fat the larvae can tolerate in the substrate.

Returning to the present study, the possibility of affecting and modulating the FA composition was strongly confirmed. Noteworthy differences were found out in the ratio between unsaturated (UFAs) and saturated (SFAs) FA sums, thus outlining peculiar profiles for each rearing substrate. Brewery by-products provided a higher concentration of UFAs in comparison with cow's milk whey, likely due to higher polyunsaturated fatty acids (PUFA) content. The UFAs/SFAs increased in fish scraps, mainly because of the higher concentration of the monounsaturated fatty acids (MUFA) content. The bread dough was characterized by the highest MUFA content (around 340 mg g^{-1} prepupal fat). Finally, tomato peels and seeds and grape stalks (despite their nutritional shortage), both showed UFAs/SFAs higher than 1, as well as the highest PUFA content (around 390 mg g^{-1} prepupal fat) along with the lowest $C_{12:0}$ concentration.

The cow's milk whey showed the best performance in terms of larval growth. However, to be considered suitable as a substrate, this by-product needs to be absorbed on a solid support which in the present study was represented by the control diet. Also, in light of the results, the control diet could be effectively replaced by tomato peels and seeds. These latter gave rise to prepupae with a prevalence of unsaturated fatty acids, even though larvae were not able to crush tomato seeds and effectively exploit the substances contained. Follow-up studies should, therefore, consider a preliminary grinding step to help release tomato-seed inner nutrients into the substrate. For all these reasons, the use of ground tomato peels and seeds as solid support for cow's milk whey, and in the same way for *ricotta* whey, may represent an advantageous combination of substrates for BSF rearing, which is worth ascertaining.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Figure captions

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 prepupa; 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, unsaturated fatty acid sum to saturated 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 (%).

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Table 1. Parameters of prepupae growth performance using different substrates (values are means of three replicates ± the standard deviation).

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 × MWP/T ($g_{biomass}$ per day)**
ANOVA (F_{value})	499.7***	1017.4***	100.5***	121.8***	89.3***
Control	28 ± 0 c	32 ± 1 c	99 ± 1 b	0.22 ± 0.01 e	0.67 ± 0.01 a
SUBSTRATES GROUP A†					
Brewery by-products	31 ± 0 d	33 ± 0 c	97 ± 4 b	0.22 ± 0.00 e	0.66 ± 0.01 a
Cow's milk whey	17 ± 0 a	18 ± 1 a	100 ± 0 b	0.19 ± 0.02 d	1.0 ± 0.2 b
Grape stalks	=	=	15 ± 4 a	0.04 ± 0.00 a	=
Tomato peels and seeds	20 ± 2 b	24 ± 1 b	100 ± 0 b	0.14 ± 0.00 c	0.59 ± 0.04 a
SUBSTRATES GROUP B‡					
Bread dough	=	=	34 ± 14 a	0.08 ± 0.00 b	=
Fish scraps	=	=	21 ± 11 a	0.10 ± 0.01 b	=

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 ($P \leq 0.05$)

***: $P \leq 0.001$.

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

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

PLP × MWP/T%P: biomass yield.

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

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

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 ($P \leq 0.05$).

***: $P \leq 0.001$.

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

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 × MLC/T: lipid yield.

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

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**	**MUFA 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
SUBSTRATES GROUP A†																
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
SUBSTRATES GROUP B‡																
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

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 ($P \leq 0.05$).

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

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

SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UFAs/SFAs, 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.

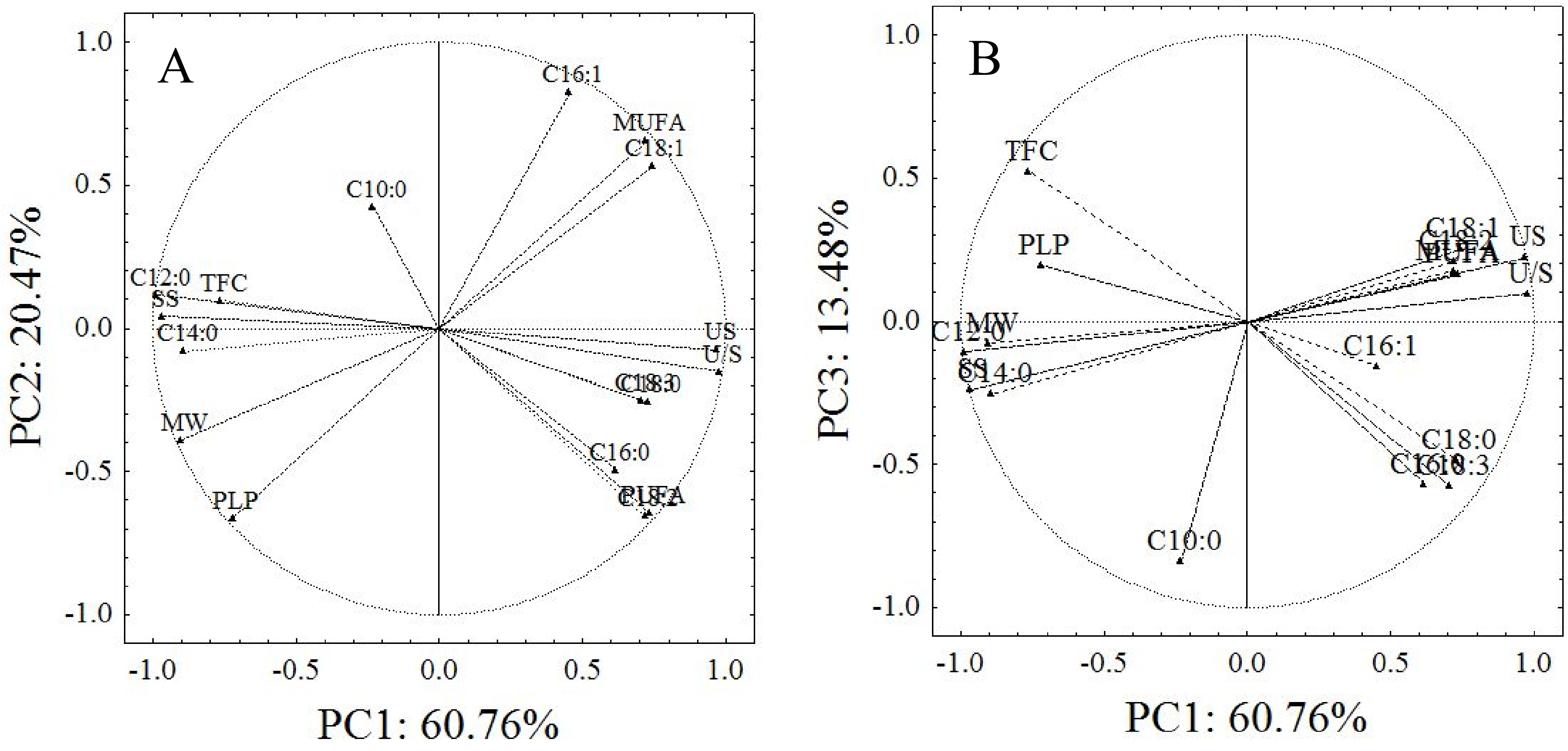

Figure 2. (A) PC1 vs PC2 and (B) PC1 vs PC3 score plots of substrates used for black soldier fly growth with the explained variance (%).