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New value from food and industrial wastes – Bioaccumulation of omega-3 fatty acids from an oleaginous microbial biomass paired with a brewery by-product using black soldier fly (*Hermetia illucens*) larvae

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ABSTRACT

Research on bioconversion based on insects is intensifying as it addresses the problem of reducing and reusing food and industrial waste. To reach this goal, we need to find more means of pairing waste to insects. With this goal, brewers' spent grains (BSG) - a food waste of the brewing industry - paired with the oleaginous biomass of the thraustochytrid *Schizochytrium limacinum* cultivated on crude glycerol - a major waste of biodiesel production - were successfully used to grow *Hermetia illucens* larvae. Combining BSG and *S. limacinum* in the diet in an attempt to design the lipid profile of *H. illucens* larvae to contain a higher percentage of omega-3 fatty acids is novel. Insect larvae were grown on three different substrates: i) standard diet for Diptera (SD), ii) BSG, and iii) BSG + 10% *S. limacinum* biomass. The larvae and substrates were analyzed for fatty acid composition and larval growth was measured until 25% of insects reached the prepupal stage. Our data showed that including omega-3-rich *S. limacinum* biomass in the BSG substrate promoted an increase in larval weight compared to larvae fed on SD or BSG substrates. Furthermore, it was possible, albeit in a limited way, to incorporate omega-3 fatty acids, principally docosahexaenoic acid (DHA) from BSG + *S. limacinum* substrate containing 20% of DHA into the larval fat (7% DHA). However, *H. illucens* with this level of DHA may not be suitable if the aim is to get larvae with high omega-3 lipids to feed carnivorous fish.

1. Introduction

As a result of rapid urbanization and population growth, current levels of global waste will have increased by 70 percent in 2050. According to a recent report from the World Bank, 3.4 billion tons of waste will be generated annually within the next 30 years, up from about 2 billion tons in 2016 (Kaza et al., 2018). Industrialized countries are responsible for generating a third of the global waste, despite having only 16 percent of the world's population (Van Huis, 2013).

The volume of waste can be reduced quickest by decreasing economic activity in wealthy countries; however, this strategy will likely not be adopted. Instead, recycling options and reducing food waste could play a major part in helping to remedy the negative impact of waste accumulation. In this view, it is essential to implement robust waste management plans based on circular economy principles, promoting "zero food waste" strategies by which the organic fraction of waste can generate new valuable products and applications.

Insects are taking a leading role in the development of organic waste management strategies by upgrading organic residues to become high-value animal products (Cappellozza et al., 2019). Organic debris is a known target for the larvae of saprophagenic insects. Indeed, the appetite of these insects for food waste is actually now being exploited to bioconvert millions of tons of food waste globally into proteins and lipids for human food and animal feed. In particular, black soldier fly (*Hermetia illucens* L.) is a very efficient and quick bioconverter – offering an excellent means of recycling different kinds of organic substrates (Bonelli et al., 2020; Meneguz et al., 2018). *H. illucens* larvae can feed on a variety of organic residues - for instance, materials of vegetable origin from food processing or agricultural production - and convert them into their own biomass, i.e., a high-value protein resource for feedstuff

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formulation. *H. illucens* larvae can contain more protein – 40% on a dry matter basis – than some alternative sources used in fish feed formulations (Van Huis, 2013; Diener et al., 2011; Ewald et al., 2020). Moreover, soy and fishmeal have traditionally been used to produce protein-rich fish feed, both of them straining the environment to a considerable extent and also being associated with a carbon footprint. Therefore, *H. illucens* larval meal might offer a valid alternative to fish and soybean meal in aquafeeds (Smets et al., 2020).

A wide range of organic substrates have been tested in the rearing of *H. illucens* larvae, such as poultry and fish offal, coffee silverskin, brewery/winery by-products, fruit and vegetable leftovers, and microalgae (Arango Gutièrrez et al., 2014; Cappellozza et al., 2019; Meneguz et al., 2018; Shumo et al., 2019; St-Hilaire et al., 2007; Surendra et al., 2020; Truzzi et al., 2020; Zarantoniello et al., 2020). Although *H. illucens* larvae in general contain 30–35% fat on a dry matter basis, their fatty acid (FA) profile can vary, depending on the rearing substrate. This variation is due to the plasticity of the fat body, an organ involved in lipid storage in the larvae (Pimentel et al., 2017). Such a characteristic could be useful as levels of omega-3 FAs are often low in commercially produced insects and omega-6/omega-3 ratios suboptimal. Indeed, Hoc et al. (2020) reported that *H. illucens* larvae contain high levels of saturated fatty acids (SFAs, lauric acid C12:0, or myristic acid C14:0), but the amount of omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is low. High lipid content and low levels of omega-3 can limit the usefulness of *H. illucens* into fish feed formulations because this would decrease the content of linolenic acid (ALA; C18:3n-3), EPA (C20:5n-3), and DHA (C22:6n-3), which are indispensable for ensuring optimal fish growth and reproduction along with human health benefits derived from consuming fish fillet (El-Dakar et al., 2020).

Owing to the fact that the *H. illucens* FA profile could be related to the dietary fats consumed, several experiments have tried to tailor the FA composition of the larvae through the rearing substrates. When ensiled fresh mussels were present in the rearing substrates, larval biomass displayed high levels of PUFAs (8% of EPA, and 5% of DHA) (Ewald et al., 2020), whereas in other studies, limited EPA and DHA accumulation in *H. illucens* larvae was reported. In particular, larvae reared in seaweed incorporated 1% of EPA (Liland et al., 2017), whereas larvae grown on fish offal contained 2.3–3.1% EPA + DHA of total FAs (St-Hilaire et al., 2007). Ewald et al. (2020) found that the larval FA profile, irrespective of diet composition, mainly consisted of lauric acid C12:0 and other SFAs that are synthesized endogenously by the larvae. Furthermore, the percentage of EPA and DHA tended to decrease with increasing weight *i.e.*, larvae with a higher weight contained a higher percentage of SFAs and a lower percentage of EPA and DHA. Similarly, Knudsen et al. (2021) reported that *H. illucens* larvae can utilise high contents of substrate lipids, but with limited effects on the composition of their biomass and fish oil rich in PUFA supplemented to the substrate had much stronger effects on the *H. illucens* composition of shorter chain FA than on the PUFAs.

In light of the aforementioned considerations, we decided to investigate if thraustochytrids (heterotrophic fungus-like protists), such as *Schizochytrium* spp., might be a valid source of PUFAs to enrich the feeding substrate of *H. illucens* larvae destined for fish feed production. Indeed, El-Dakar et al. (2020) reported DHA bioaccumulation by *H. illucens* larvae fed on the marine protist *Schizochytrium* spp. Within the *Schizochytrium* genus, the species *S. limacinum* is a well-studied thraustochytrid; when growing in a culture medium containing crude glycerol and salt, it has the ability to accumulate lipids for up to 50% of its cell dry matter in a short time and shows a DHA output of 34% of the total lipid concentration (Ethier et al., 2011). The ability of this oleaginous microorganism to use glycerol as a carbon and energy source has been exploited for disposing crude glycerol, which is a major by-product of biodiesel production.

Other potential substrates that can be used in combination with *S. limacinum* for rearing insect larvae can derive from the food and beverage industry. For instance, the brewing industry produces a large amount of organic waste at each stage of the brewing process. For every 1,000 tons of beer produced, between 137 and 173 tons of solid residues can be generated in the form of brewer's spent grains (BSG), spent yeast, and trub (an unwanted precipitation product of the wort boiling process). Some of these by-products contain a significant concentration of organic compounds with a high nutritional value, which places a high biochemical oxygen demand on degradation. Therefore, disposal of these by-products in the environment impacts the ecosystem to a considerable degree.

As already mentioned, about 85 percent of the by-products from the beer brewing process are spent grains, also known as malt bagasse or spent brewery's malt. Breweries around the world produce around 40 million tons of this by-product every year. From a nutritional standpoint, spent grains are rich in fiber (70%), protein (15–26%), lipids (about 10%), vitamins, and minerals (2–5%) (Rachwał et al., 2020). Most brewers have implemented a means of disposing spent grains waste, often being used to feed cattle. However, this industry would like to find new and efficient approaches for turning such waste into an asset and to create new products as part of a circular approach, also with respect to the relationship between food and health (Amoriello and Ciccoritti, 2021).

Accordingly, the aim of the present research was to show whether BSG substrates either alone or enriched with whole-cell *S. limacinum* biomass (cultivated on crude glycerol waste) are optimal for *H. illucens* larval growth and development, and to determine if larvae can incorporate omega-3 FAs through the diet, thus meeting the EPA and DHA requirements of fish feeds.

2. Materials and methods

2.1. S. limacinum fermentation conditions

Heterotrophic cultivation of *S. limacinum* to achieve the highest growth and PUFAs content was performed using crude glycerol in 10 L bioreactors, as described in Terova et al. (2021). In brief, starting cultures for bioreactor inoculation were maintained in a shaker. The exponential phase cultures were then used to inoculate bioreactors to reach a final optical density (OD600) of 0.5. Growth medium for batch fermentations By+ medium (Chi et al., 2007) was modified and adjusted to pH 7 before sterilization, filtered, and added under sterile conditions to the final medium. After 28 h of growth, a crude glycerol supplement was added to the growth medium, as previously reported by Signori et al. (2016), and the fermentation was carried out for 144 h to reach the maximum lipids accumulation in the cells.

After bioreactor fermentation, *S. limacinum* cells were harvested by centrifugation at 7885 × *g* for 20 min and the supernatant was discarded. Then, cells were washed and dry cell weight (DCW) was determined gravimetrically by drying 1 mL of cell culture (Concentrator 5301, Eppendorf AG, Germany). Finally, biomass was frozen in liquid nitrogen and lyophilized (LIO5P, 5Pascal, Milan, Italy).

A large amount of the lyophilized *S. limacinum* biomass (about 2.5 kg) was used in a fish feeding trial as described in Terova et al. (2021), whereas the remainder (500 g), was used in the present study to supplement BSG substrate for insect larval rearing.

2.2. Insect substrates and feeding trials

H. illucens eggs were obtained from a colony established at University of Insubria (Varese, Italy) in 2015. After hatching, neonate larvae were placed in a humid chamber at 27 °C and fed on standard diet for Diptera (Hogsette, 1992) as reported in (Bonelli et al., 2019). After 4 days, batches of 300 larvae were placed into plastic containers (16 × 16 × 9 cm) and fed *ad libitum* with three different diets: i) standard diet for Diptera (SD), ii) BSG, and iii) BSG supplemented with 10% *S. limacinum* (strain SR21). One hundred g of each diet was provided in the rearing

container.

SD was composed of wheat bran (50%), corn meal (30%), and alfalfa meal (20%) mixed in a 1:1 ratio of dry matter/water. BSG were obtained from a local brewery named "A tutto malto", situated in Gallarate, Italy. After the mashing process, spent grains were cooled, vacuum-packed, and frozen at −20 °C until use to avoid excessive fermentation and mould growth. Due to the high moisture content, BSG were provided to the larvae after thawing without adding water.

For preparing the third diet, *S. limacinum* biomass obtained and lyophilized as reported above was appropriately minced and added to the BSG substrate in a 1:9 ratio (10 g *S. limacinum*: 90 g BSG). For the duration of the feeding trials, each substrate was renewed every 2 days until the larvae reached the prepupal stage.

Three independent rearing groups were set up for each diet, and larvae were maintained in the dark at 27 ± 0.5 °C and 70 ± 5% relative humidity. Random samples of larvae (25 to 30 individuals) were weighed every 2 days. For each experimental diet, sampling and larval weight measurement were carried out in triplicate. Weight was recorded until 25% of insects reached the prepupal stage.

For chemical analysis, last instar larvae and the feeding substrates (taken immediately after preparation) were used.

2.3. Chemical analyses

2.3.1. Lipid yield and FA profile of S. limacinum

To determine the lipid content of *S. limacinum* cells, lipids were extracted from 1 g of biomass, based on the method of Bligh and Dyer (1959), as described in Terova et al. (2021). Briefly, after drying in oven, cells were resuspended in HCl and chemically disrupted in a thermostatic bath. Then, lipids were collected by adding n-hexane to the mixture.

2.3.2. Lipid extraction from rearing substrates and the larvae

Total lipid extraction was performed according to the method of Folch et al. (1957). Three gram samples from each substrate and larvae, in triplicate, were thawed and finely ground before lipid extraction. Then, a mixture of chloroform/methanol (2:1 v/v) with 0.01% BHT (butylated hydroxytoluene) was added to each sample before homogenising the sample with an Ultra-Turrax (T25 Digital Ultra-Turrax, IKA®, Germany). After the lipid level was determined gravimetrically, the sample was filtered using anhydrous sodium sulfate and evaporated to dryness under vacuum, at 40 °C.

2.3.3. FA methyl esters

The fatty acid methyl esters (FAME) were prepared using KOH solution (0.5 M/CH_3OH). One hundred mg of crude lipid were mixed with 6 mL of KOH in small sample bottles, heated at 100 °C for 10 min, and then cooled at room temperature. The methylation of FAs was carried out by using catalyst boron trifluoride (BF_3) in each sample and heated at 100 °C for 40 min. The FAME extract was then collected in vials.

The methylated FAs were separated by gas chromatography-mass spectrometry (GC–MS; Thermo-Scientific) equipped with a DB-WAX GC column (30 m × 0.25 mm ID, 0.25 μm film thickness) and identified by comparison to external standards (Supelco 37-component FAME mix, Sigma-Aldrich, Milan, Italy). Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The injection volume was 1 μL. To distinguish each FA, the following thermal conditions were used: 50 °C as initial temperature for 5 min, increased to 200 °C at a rate of 18 °C min^{-1} for 10 min. The last thermal ramp was increased at 248 °C at a rate of 3 °C min^{-1}. The temperature of the injector, the flame ionization detector, and the transfer line was 250 °C. A total run lasted 40 min.

2.4. Statistical analysis

Data were reported as means plus standard deviation. Before statistical analysis, percentage data were transformed using arcsin square root. Shapiro-Wilk's and Levene's test were used to test data for normality and homogeneity of variances, respectively. When the variances were not equal, the Kruskal-Wallis's test was performed, followed by Dunn's *post hoc* test. One-way ANOVA was used to determine differences among groups, followed by Tukey's *post hoc* test. All analyses were performed with Past3 software (Hammer et al., 2001). Principal component analysis (PCA) was carried out on 27 samples and 18 variables (FAs) with software R ("R: A language and environment for statistical computing. R Foundation for Statistical Computing," 2020). Biplot graph was prepared using the "ggplot2" package V (Wickham, 2016).

Table 1
FA profile (% of total FAs) of *S. limacinum* biomass and the rearing substrates.

Fatty acids	% of total Fas			
	S. limacinum Biomass	Substrates SD	BSG	BSG + *S. limacinum*
C10:0	n.d.	n.d	n.d	n.d
C12:0	3.33	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.14
C14:0	6.78	0.38 ± 0.01	0.52 ± 0.03	0.96 ± 0.02
C15:0	4.30	0.33 ± 0.01	0.12 ± 0.02	1.72 ± 0.13
C16:0	56.35	25.44 ± 0.59	31.86 ± 0.51	32.77 ± 0.29
C16:1	n.d.	0.00 ± 0.00	0.00 ± 0.00	1.13 ± 0.00
C17:0	1.11	0.00 ± 0.00	0.11 ± 0.01	1.33 ± 0.03
C17:1	0.13	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.01
C18:0	1.24	2.19 ± 0.04	1.90 ± 0.13	2.49 ± 0.07
C18:1n-9	2.54	22.72 ± 0.49	10.66 ± 0.85	7.71 ± 0.07
C18:2n-6	0.56	44.11 ± 0.10	49.67 ± 0.46	22.82 ± 0.46
C18:3n-3	n.d.	4.23 ± 0.05	4.63 ± 0.14	2.08 ± 0.03
C18:3n-6	n.d.	0.00 ± 0.00	0.00 ± 0.00	0.23 ± 0.01
C20:0	0.13	0.00 ± 0.00	0.00 ± 0.00	0.34 ± 0.04
C20:1n-9	n.d.	0.00 ± 0.00	0.54 ± 0.07	0.24 ± 0.05
C20:4n-6	n.d.	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.04
C20:5n-3	0.38	0.00 ± 0.00	0.00 ± 0.00	0.22 ± 0.02
C22:0	n.d.	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.00
C22:5n-3	n.d.	0.00 ± 0.00	0.00 ± 0.00	4.68 ± 0.17
C22:6n-3	22.82	0.00 ± 0.00	0.00 ± 0.00	19.99 ± 0.61
∑n-3	23.20	4.18 ± 0.07^{B}	4.63 ± 0.17^{B}	26.96 ± 0.44 ^{A}
∑n-6	0.59	44.21 ± 0.14 ^{Ab}	49.67 ± 0.56 ^{Aa}	23.29 ± 0.49 ^{Bab}
∑n-3/∑n-6	39.32	0.09 ± 0.00^{B}	0.09 ± 0.00^{B}	1.16 ± 0.04 ^{A}
∑SFA	73.24	28.38 ± 0.78^{C}	34.50 ± 0.46^{B}	40.53 ± 0.15 ^{A}
∑MUFA	2.67	23.21 ± 0.69 ^{A}	11.20 ± 1.02^{B}	9.21 ± 0.11^{B}
∑PUFA	23.79	48.39 ± 0.07 ^{Bb}	54.30 ± 0.69 ^{Aab}	50.26 ± 0.05 ^{Ba}

SD = standard diet; BSG = 100% brewer's spent grains; BSG + *S. limacinum* = BSG supplemented with 10% of *S. limacinum*. Data are reported as mean ± standard deviation (n = 3). Different capital and small letters indicate statistically significant differences at $p < 0.01$ and $p < 0.05$, respectively. n.d. = not detectable.

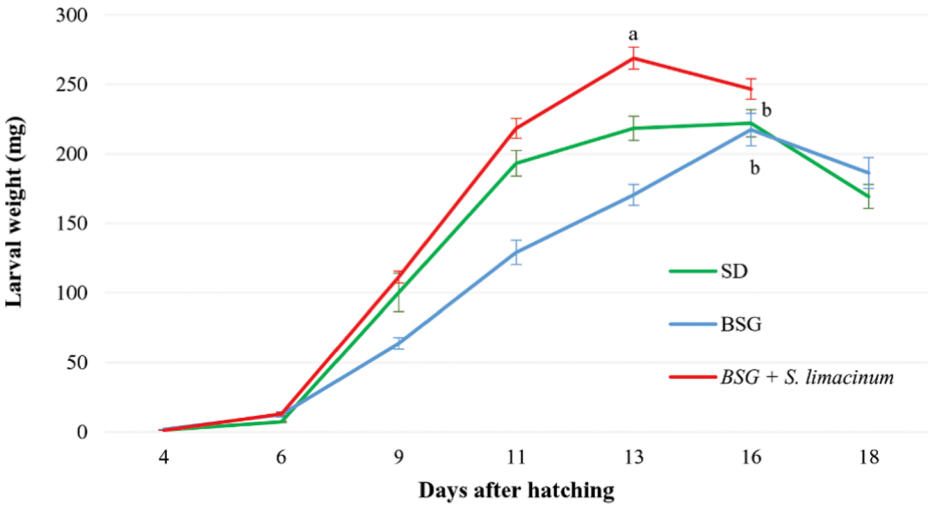

Fig. 1. Weight of *H. illucens* larvae reared on three different experimental substrates. Green, blue and red curves represent the growth of larvae fed on standard diet (SD), 100% brewer's spent grains (BSG) diet, and BSG + 10% of *S. limacinum* diet, respectively. Data are reported as mean ± standard deviation (n = 3). Different letters indicate statistically significant differences between the maximum weights reached by larvae reared on different substrates at $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Dry matter (DM) and lipid content of diets and larval biomass reported in percentage.

	Diets		Larvae	
	DM %	Lipids (% dry weight)	DM %	Lipids (%dry weight)
SD	43.59 ± 0.02^{B}	1.67 ± 0.72^{B}	28.56 ± 0.01^{a}	19.08 ± 1.92 ^{a}
BSG	31.18 ± 0.04 ^{A}	1.33 ± 0.02^{B}	33.45 ± 0.03^{b}	24.85 ± 1.61^{b}
BSG + *S. limacinum*	38.57 ± 0.02^{B}	11.48 ± 0.46^{A}	29.50 ± 0.01^{ab}	18.78 ± 0.46 ^{a}

SD = standard diet; BSG = 100% brewer's spent grains; BSG + *S. limacinum* = BSG supplemented with 10% of *S. limacinum*. Data are reported as mean ± standard deviation (n = 3). Different capital and small letters indicate statistically significant differences at $p < 0.01$ and $p < 0.05$, respectively.

3. Results

3.1. Thraustochytrid S. limacinum culture

After fermentation, a dry cell weight of 8.0 ± 1.1 g/L was obtained with a biomass yield of 0.1 ± 0.09 $g/g_{glycerol}$ (10% w/w). The amount of lipids of the produced biomass was 4.8 ± 0.5 g/L, with a yield of 0.6 ± 0.1 $g/g_{biomass}$ (60% w/w). Table 1 shows the lipid profile of *S. limacinum* obtained from the fermentation. DHA constituted 23% of the total lipids in the biomass, being the second most represented after palmitic acid (56%). Therefore, the DHA titre was 1.1 ± 0.3 g/L with a yield of 0.13 ± 0.03 $g/g_{biomass}$ (13% w/w).

3.2. H. illucens larval growth

At the end of the feeding trial (13–16 days after hatching), performance of the *H. illucens* larvae reared on the three experimental substrates was evaluated. At 13 days from hatching, the final weight reached by the larvae fed upon BSG substrate supplemented with 10% *S. limacinum* was significantly higher than in larvae reared on SD and BSG substrates (Fig. 1). Moreover, larvae reared on BSG + *S. limacinum* substrate reached the prepupal stage earlier than larvae grown on the other two substrates. In particular, insects reared on BSG + *S. limacinum* substrate only needed 13 days to complete their larval stage *versus* 16 days needed for larvae reared on SD and BSG substrates.

3.3. FA content and composition

The dry matter and lipid content of all rearing substrates (diets) and larvae are reported in Table 2. With regard to the dry matter content, SD and BSG + *S. limacinum* substrates showed no differences (44% and 39%, respectively), but they were significantly differed from the DM content of BSG substrate (31%, $p < 0.01$). The DM content of larvae reared on BSG substrate (33 %) was significantly higher than in larvae reared on SD (29%), whereas DM of larvae reared on BSG + *S. limacinum* was similar to the other two dietary groups.

Lipid content was significantly higher in the BSG substrate enriched with 10% of *S. limacinum* (12 %, $p < 0.01$) than in the other two substrates (SD, 2 %; BSG, 1 %), whereas larvae reared on BSG substrate had the highest lipid content compared to those grown on the other two substrates ($p < 0.05$).

The FA profiles of the substrates are shown in Table 1; the content of omega 3, omega 6, and omega-3/omega-6 ratio of each feeding substrate and a comparison of the main FA classes are depicted in Fig. 2.

SFA class representation was greater in the BSG substrate supplemented with the thraustochytrid (41%) than in the other two substrates, whereas the MUFA class (23%) was 2-fold higher in the SD than in the other two substrates. The highest PUFA class content was found in BSG substrate ($p < 0.01$). At a significance level of $p < 0.05$, the comparison between SD and the BSG + 10% of *S. limacinum* was statistically significant for the PUFA class content.

The BSG substrate supplemented with 10% *S. limacinum* showed significantly higher levels of omega-3 (27%; $p < 0.01$) than did the SD (4%; $p < 0.01$) and BSG (5%; $p < 0.01$) substrates (Fig. 2). In contrast, omega-6 was well represented in SD (44%) and BSG (50%), whereas the diet with *S. limacinum* showed low levels of this unsaturated FA class (23%; $p < 0.01$). At a statistically significant level of $p < 0.05$, the omega-6 content was different between BSG and SD substrates. Consequently, the omega-3/omega-6 proved to be significantly higher in the feeding substrate enriched with 10% of *S. limacinum* (1%; $p < 0.01$).

The FA profile of *H. illucens* larvae reared on different substrates is shown in Table 3 and Fig. 3. Levels of saturated FAs, such as C10:0, C12:0, and C14:0, were significantly higher ($p < 0.01$) in larvae fed on SD than in the other two dietary groups. Another FA found to be significantly higher (10%) in the SD larval group than in the BSG and BSG + *S. limacinum* groups was C18:1n-9.

The FA profiles of the larvae showed that including 10% *S. limacinum* in the substrate led to a significant increase in n-3 FAs in comparison to the other two groups ($p < 0.01$). The level of n-6 FAs was similar in larvae reared on BSG and BSG + *S. limacinum* substrates (17% and 18%, respectively), being significantly higher than in larvae fed a SD diet ($p <$

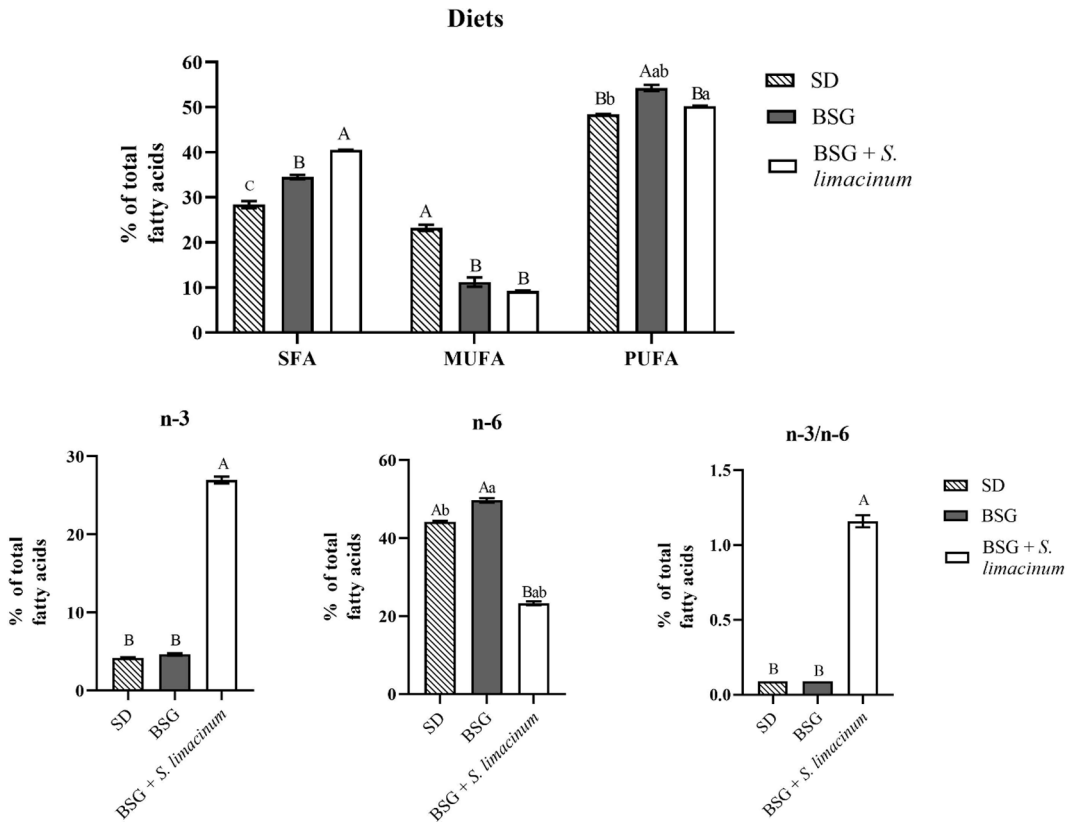

Fig. 2. Comparison of FA classes among the rearing substrates. SD = standard diet; BSG = 100% brewer's spent grains; BSG + *S. limacinum* = BSG supplemented with 10% of *S. limacinum*. Data are reported as mean ± standard deviation (n = 3). Different capital and small letters indicate statistically significant differences at $p < 0.01$ and $p < 0.05$, respectively.

0.01). Within the omega-3 class of FA, C20:5n-3, C22:5n-3, and C22:6n-3 were exclusively detected in the larvae reared on BSG + *S. limacinum* diet.

The omega-3/omega-6 ratio was significantly higher ($p = 0.0012$) in larvae fed on BSG + *S. limacinum* diet (Table 3; Fig. 3) and the same difference was also detected between the substrates (Table 1; Fig. 2).

The SFA class was significantly higher in larvae grown on SD ($p < 0.001$) than in the other dietary groups (Table 3; Fig. 3). The highest content of the MUFAs ($p < 0.01$) was recorded in larvae fed on SD diet as compared to the other feeding groups, whereas at a significance level of 0.05, larvae fed on BSG + *S. limacinum* showed a higher retention of MUFAs than larvae fed on BSG alone.

With regard to PUFAs, the dietary inclusion of thraustochytrids led to a significant increase in this class of FAs in the BSG + *S. limacinum* larvae group ($p < 0.001$) with respect to the other larval groups.

3.4. Principal component analysis

Focusing on the main FAs to explain the maximum amount of variability, a principal component analysis (PCA) of the lipid profiles of *H. illucens* larvae was performed. The first and second principal components (i.e., PC1 and PC2) accounted for 86% and 9% of the data variability, respectively. On the basis of the PC1 values, the bidimensional plot revealed that larvae fed on the same substrate clustered together (Fig. 4A). Indeed, *H. illucens* larvae were assigned to three separate clusters that corresponded to the three substrates. Larvae fed on BSG + *S. limacinum* (red ellipse) were exclusively concentrated in the left half-plane of the biplot (PC1 values < 0), whereas larvae fed on SD (green ellipse) and BSG (blue ellipse) diets were clustered in the right half-plane of the biplot (PC1 values < 0). PC1 clearly discriminated the larvae reared in the substrate supplemented with *S. limacinum*.

Fig. 4B shows the variables, *i.e.*, the FAs. DHA (C22:6n-3) negatively correlated to lauric acid C12:0 and these two FAs only justified the differences between the dietary groups. Finally, as shown in Fig. 3C, the *H. illucens* larvae fed on BSG supplemented with *S. limacinum* were characterized by the highest amount of DHA.

4. Discussion

Circular economy is a new approach based on looping systems (take, make, use, reuse, remake, recycle, and make again) for developing ecologically sound processes with the goal of a "zero waste economy". This new concept is based on the responsible use of resources to promote sustainable development, focusing on positive society-wide benefits. In order to prevent resource depletion and to counter environmental dilemma, it is essential to take a circular economy approach and use waste as a raw material for developing new products and applications (Boyd et al., 2020).

In the present work, we tested the use of a thraustochytrid biomass cultivated on crude glycerol as an omega-3 source for rearing insect larvae destined for animal feed production. In particular, *S. limacinum* biomass was used in combination with BSG, which is a by-product of beer production. A rate of 10% of *S. limacinum* was chosen to supplement the BSG substrate based on the study by Truzzi et al. (2020), which reported that including more than 10% did not provide nutritional benefits to *H. illucens* larvae. Indeed, in that study, growth performance of *H. illucens* larvae was compromised due to increased humidity of the feeding substrate caused by the scarce solubility of *S. limacinum* biomass (Truzzi et al., 2020). The same findings were also reported by Liland et al. (2017).

The FA composition analyses of the three substrates used in our study showed that including 10 % *S. limacinum* to BSG increased levels of DHA and EPA, in comparison to the other two substrates (SD and BSG). This was also the only option, since DHA and EPA was not detected in SD and

Table 3
FA profile (% of total FAs) of *H. illucens* larvae reared on different feeding substrates.

Fatty acids	Larvae			ANOVA *p-value*	Kruskal Wallis *p-value*
	SD	BSG	BSG + *S. limacinum*		
C10:0	0.81 ± 0.09^{A}	0.70 ± 0.02^{B}	0.75 ± 0.06^{AB}	0.007	
C12:0	44.79 ± 3.12^{Aa}	40.47 ± 1.16^{Ba}	28.21 ± 1.39^{Bb}		<0.001
C14:0	8.50 ± 1.03^{A}	7.22 ± 0.66^{B}	5.19 ± 0.40^{B}		<0.001
C15:0	0.86 ± 0.10^{Ab}	0.25 ± 0.03^{Bab}	1.06 ± 0.06^{Aba}		<0.001
C16:0	16.13 ± 1.26^{B}	19.11 ± 2.34^{A}	19.10 ± 0.44^{A}	0.0002	
C16:1	2.94 ± 0.47	2.46 ± 0.51	2.78 ± 0.13		0.08
C17:0	0.60 ± 0.11^{AB}	0.32 ± 0.05^{B}	1.12 ± 0.06^{A}	0.02	
C17:1	0.68 ± 0.15^{Aa}	0.00^{Ba}	0.26 ± 0.03^{ABb}		<0.001
C18:0	2.00 ± 0.60^{B}	1.80 ± 0.13^{B}	2.85 ± 0.29^{A}	0.001	
C18:1n-9	10.24 ± 0.52^{Aa}	8.61 ± 0.50^{Ba}	9.56 ± 0.19^{ABb}		<0.001
C18:2n-6	11.49 ± 0.55^{Bb}	17.46 ± 0.75^{Aab}	16.90 ± 0.96^{ABa}		<0.001
C18:3n-3	1.29 ± 0.10^{B}	1.95 ± 0.17^{A}	1.73 ± 0.13^{A}		<0.001
C18:3n-6	0.00^{B}	0.00^{B}	0.25 ± 0.04^{A}		<0.001
C20:4n-6	0.00^{B}	0.00^{B}	0.76 ± 0.07^{A}		<0.001
C20:5n-3	0.00^{B}	0.00^{B}	2.61 ± 0.28^{A}		<0.001
C22:5n-3	0.00^{B}	0.00^{B}	0.89 ± 0.02^{A}		<0.001
C22:6n-3	0.00^{B}	0.00^{B}	6.76 ± 0.66^{A}		<0.001
∑n-3	1.29 ± 0.10^{Bb}	1.95 ± 0.17^{Ba}	12.00 ± 1.04^{Aab}	<0.001	
∑n-6	11.49 ± 0.55^{B}	17.46 ± 0.10^{A}	17.91 ± 0.92^{A}		<0.001
∑n-3/ ∑n-6	0.11 ± 0.01^{B}	0.11 ± 0.10^{B}	0.67 ± 0.07^{A}	0.0012	
∑SFA	73.68 ± 1.43^{A}	69.89 ± 1.39^{B}	58.28 ± 1.24^{C}	<0.001	
∑MUFA	13.85 ± 0.85^{Aab}	11.08 ± 0.70^{Bb}	12.61 ± 0.32^{Ba}		<0.001
∑PUFA	12.78 ± 0.56^{C}	19.41 ± 0.91^{B}	29.91 ± 1.39^{A}	<0.001	

Data represent mean ± standard deviation (replicates for each group = 3; aliquots per replicates = 3). SD = standard diet; BSG = 100% brewer's spent grains; BSG + *S. limacinum* = BSG supplemented with 10% of *S. limacinum*. Different capital and small letters within row show significant differences among diets at $p < 0.01$ and $p < 0.05$. Statistical differences were determined by one-way ANOVA with Tukey's comparison test and by Kruskal-Wallis with Dunn's test.

BSG substrates. The same result was reported by Truzzi et al. (2020), too. However, unlike Truzzi et al. (2020), who found a decrease in SFA levels due to the omega-3 supplementation in their substrate (coffee-waste silverskin + *S. limacinum*), SFA levels in our experiment were higher in the BSG + 10% *S. limacinum* group. We suggest that the different characteristics of coffee silverskin used by Truzzi et al., (2020) and the BSG used here might account for such difference in SFA levels.

With regard to the BSG feeding media, the FA profile highlighted the predominance of PUFA (54%), followed in a decreasing order by SFA (35 %) and MUFA (11%). These percentages are consistent with those reported by Almeida et al. (2017). In agreement with those authors, linoleic acid (C18:2n-6) was the most abundant FA in our study, followed by palmitic (C18:0) and oleic acid (C18:1n-9). Furthermore, the lipid content of BSG proved to be extremely low (1.3%) and not significantly different from the SD diet. In line with our data, Lordan et al. (2020) found a low lipid content in BSG (1%) that was similar to the malted grain (0.7). Those authors explained these data with the process of barley germination, during which hydrolysis of the triglycerides, which are then metabolized, reduces the barley lipid content by 30% (Anness, 1984).

The largest lipid fraction (58–79% of total lipids) of *H. illucens* larvae in our study was represented by SFA. Here, the medium-chained FA - lauric acid (C12:0) - constituted about 40% of the lipids. This result is in agreement with earlier reports (Barroso et al., 2017; Ewald et al., 2020; Liland et al., 2017; Surendra et al., 2016). Lauric acid is considered a promising nutraceutical as it provides protection against microbial infection, promoting gut health in reared animals (Matsue et al., 2019; Sogari et al., 2019). In fish, dietary lauric acid leads to reduced feed intake as it is quickly oxidized rather than being stored in the liver (Romano et al., 2021), and in mammals, it is considered as a promising candidate against obesity. In a recent study, the anti- inflammatory action of lauric acid was reported in the intestine of fish fed with insect meal, thus mitigating the inflammation caused by insect chitin (Truzzi et al., 2020).

In our study, differently from insect larvae, the feeding substrates were devoid of or contained very low levels of lauric acid (0–0.7%). This difference in the content of lauric acid strongly indicates that *H. illucens* larvae can synthesize this FA by themselves. Other studies (Ewald et al., 2020; Spranghers et al., 2017), too, have reported that, irrespective of the diet, *H. illucens* larval fat consisted mainly of lauric acid, which was endogenously produced by the larvae. Moreover, Spranghers et al. (2017) suggested that the presence of nonstructural carbohydrates (or cell content fractions of plant material) in the feeding media promoted the deposition of lauric acid in the prepupae. This would explain why *H. illucens* larvae grown on SD (about 70 % carbohydrates) diet in our experiment contained the highest level of lauric acid. However, the level of lauric acid accumulated by larvae reared on BSG substrate was similar to that found in larvae grown on SD. Although the BSG substrate is notably rich in structural or cell wall carbohydrates, such as cellulose, it also contains starch (nonfiber carbohydrate), which favour lipid biosynthesis in the larvae. On the other hand, the presence of lignocellulose in BSG might have affected the duration of larval development in our study as reported by Jucker et al. (2019), too.

The dietary inclusion of 10% *S. limacinum* reduced larval synthesis of lauric acid compared to the other two dietary groups and, in general, led to a decrease in SFAs, favouring other FA classes, such as PUFA. This is in line with earlier findings by Truzzi et al. (2020). PCA, too, confirmed the negative correlation existing between DHA (C22:6n-3) and lauric acid (C12:0) in *H. illucens* larvae.

Interestingly, omega-3 (mostly DHA) accumulated in *H. illucens* larvae grown on BSG + *S. limacinum* and were detected in the substrate, too. In contrast, the other two larval feeding groups lacked omega-3 FAs. These data indicate that larvae did not synthesise omega-3, but these were incorporated (mostly DHA) directly from the substrate. PCA levels, too, highlighted that the larval group fed on thraustochytrids was strongly distinct from the other two dietary groups. This clear clustering between groups was feasible due to the presence of omega-3 (mostly DHA) in the BSG + *S. limacinum* feeding substrate. In agreement with our results, Ewald et al. (2020) found the highest percentage of omega-3 in larvae reared on substrates with 10% mussel inclusion, but higher percentages of mussels in the diet decreased omega-3 accumulation in the larvae and negatively affected their growth performance. In Surendra et al. (2016), *H. illucens* prepupae reared on food waste had a low PUFA content (13%), similar to that found in our larvae grown on SD diet. Such low concentration of PUFAs combined with a high concentration of medium chain SFAs, made the prepupa-derived oil an ideal substrate for producing high quality biodiesel. Hence, insect-based bioconversion of organic waste has significant potential in generating high value products for different applications (i.e., raw material for biodiesel production or protein-rich insect meal for animal feed applications) with simultaneous waste valorization.

Thraustochytrid supplementation of the BSG substrate affected larval growth, leading to a reduction in the development time. This

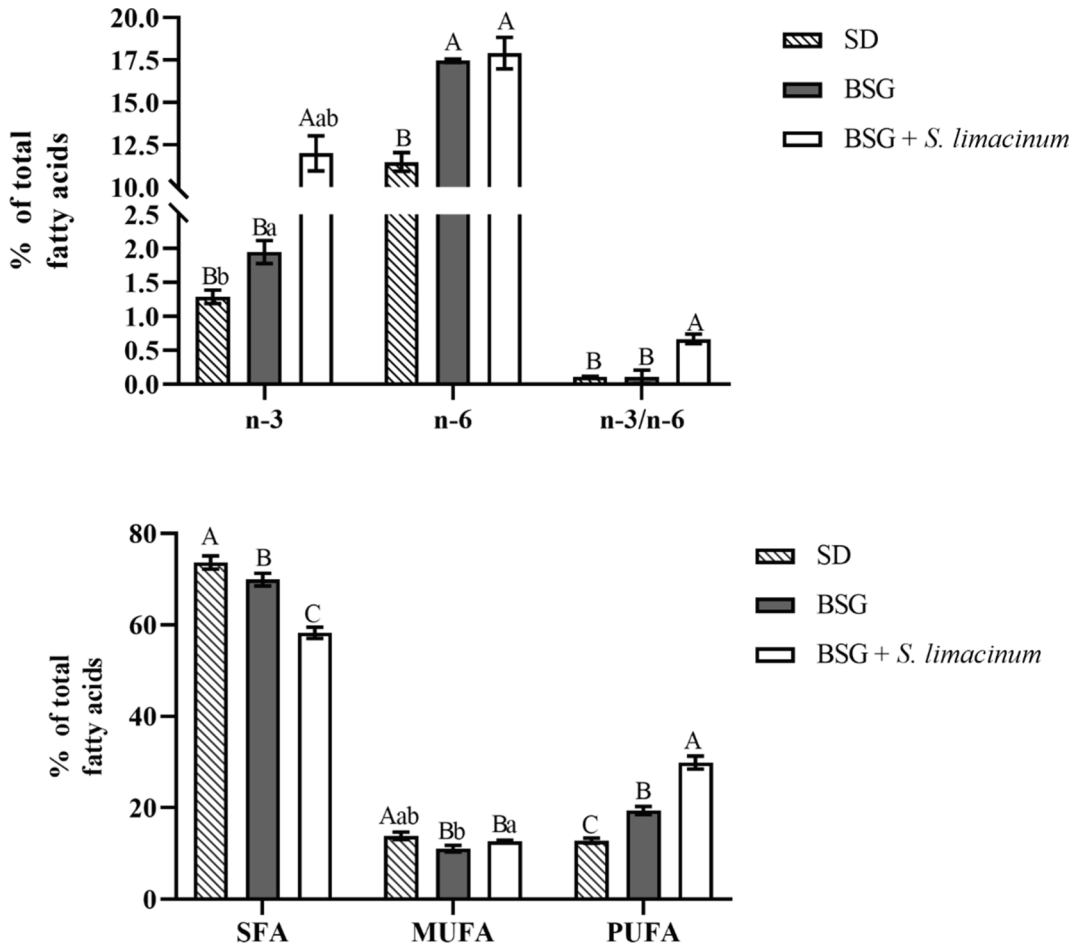

Fig. 3. Comparison of FA classes among the *H. illucens* larvae groups. SD = standard diet; BSG = 100% brewer's spent grains; BSG + *S. limacinum* = BSG supplemented with 10% of *S. limacinum*. Data are reported as mean ± standard deviation (n = 3). Different capital and small letters indicate statistically significant differences at $p < 0.01$ and $p < 0.05$, respectively.

result contradicts El-Dakar et al., (2020), who reported no difference between *H. illucens* larvae grown on 10 % *Schizochytrium* production waste and the control group fed on wheat bran. Nor did those authors find any differences in the larval biomass weight between groups fed on either 10% or 20% *Schizochytrium* waste and the control group. In contrast, the inclusion of 10% *Schizochytrium* in our study promoted a significant increase in larval biomass weight in comparison to larvae fed on SD and BSG.

Notably, optimal development and fat body content are in large part synonymous with the ability of insect larvae to make use of the substrate, and unbalanced dietary fat and protein levels are detrimental for larval growth (Bruno et al., 2019; Spranghers et al., 2017). In our experiment, *H. illucens* larvae fed on BSG + 10% *S. limacinum* performed significantly better than the other two feeding groups and this was certainly related to their well-balanced substrate. Moreover, the developmental time of *H. illucens* fed on BSG or SD diets was similar, albeit a little longer than in larvae fed on BSG + thraustochytrid substrate. Thus, the potential of BSG as a substrate for *H. illucens* rearing is high. Moreover, this substrate has been recently included in the list of a wide range of food waste that can be used to feed this and other insect species processed into fish feed (European Commission, 2017).

5. Conclusions

This research tested in a frame of circular economy insect-based bioconversion as a viable method for turning large quantities of food processing and industrial waste streams into products with high added value. BSG - a food waste of the brewing industry - paired with the biomass of an oleaginous microorganism (*S. limacinum*) cultivated on crude glycerol - a major waste product of industrial biodiesel production - were successfully used to grow insect (*H. illucens*) larvae.

Our data showed that including *S. limacinum*, which is rich in omega-3, in the BSG substrate promoted a significant increase in larval biomass weight in comparison to larvae fed on SD and BSG substrates. Furthermore, it was possible, albeit with some limits, to incorporate omega-3 FAs (mostly DHA) from BSG + *S. limacinum* substrate containing 20% of DHA into the larval fat (7% DHA). However, *H. illucens* with this level of DHA may not be suitable if the aim is to get larvae with high omega-3 lipids to feed carnivorous fish.

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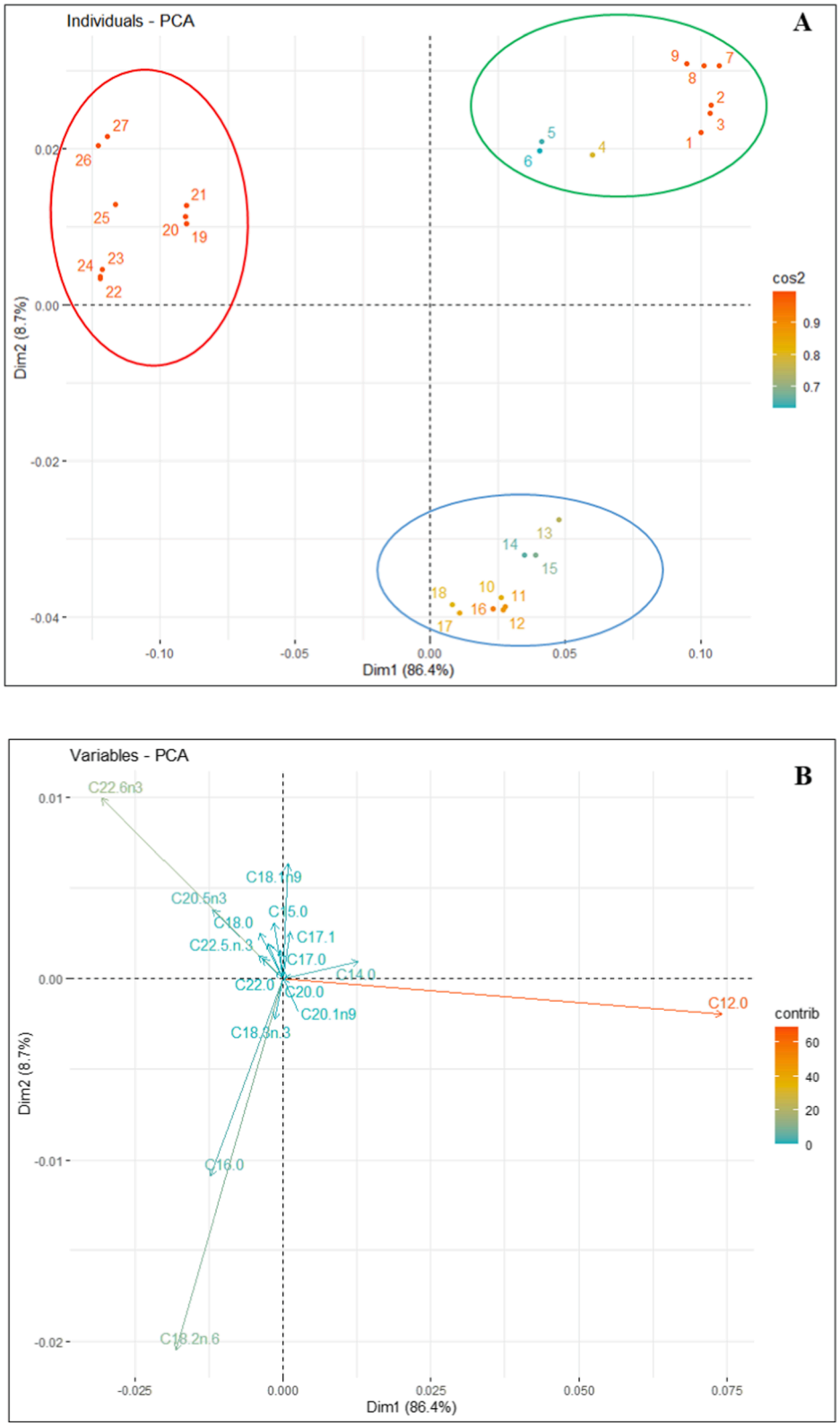

Fig. 4. Principal Component Analysis biplots (A, B, C) showing differences in the lipid profile of *H. illucens* larvae fed on the three diets. The first biplot (A) represents the lipid profile of individual larvae. The second biplot (B) shows the variables represented by FAs. Each coordinate represents a replicate of H. illucens larvae (27 samples). In the biplot B, the color scale and the length of each vector are related to the contribution to the total variance: the longer/darker is the line, the higher is the variance. The third biplot (C) shows the variables separated in the three dietary groups. Different colors are used for each dietary group of larvae (green ellipse: larvae fed on SD diet; blue ellipse: larvae fed on 100% BSG; red ellipse: larvae fed with BSG enriched with 10% of *S. limacinum*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

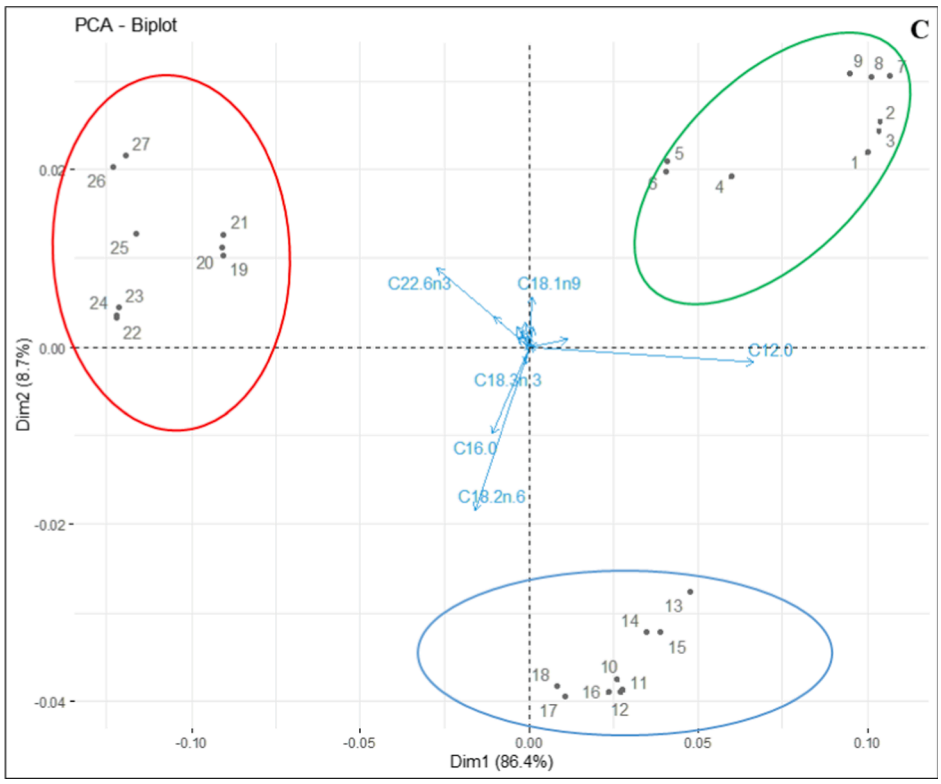

Fig. 4. (*continued*).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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