Food Research International 196 (2024) 115151

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Improved nutritional and antioxidant properties of black soldier fly larvae reared on spent coffee grounds and blood meal by-products

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ARTICLE INFO

Keywords:
Spent coffee grounds
Blood meal
Black soldier fly larvae
Co-digestion
In vitro digestion
In vitro fermentation

ABSTRACT

Black Soldier Fly larvae (BSFL) are a promising and sustainable alternative to obtain proteins. Due to their high growth rate and ability to use different substrates as feeding stocks, BSFL can be also used to valorize food waste. Thus, the aim of this research was to unravel the potential use of Spent Coffee Grounds (SCG) and blood meal alone or mixed as feedstocks for BSFL and the nutritional changes for BSFL meal, especially after simulated human *in vitro* digestion and fermentation. Chicken feed was used as a control. Chicken feed showed the highest BSFL growth ($P < 0.05$) compared with blood meal and the mix made of blood meal and SCG; the latter caused the lowest growth. The meal obtained from BSFL fed with blood meal had the highest protein content, as well as the highest levels of short chain fatty acids (SCFAs) produced after *in vitro* fermentation by the human gut microbiota. On the other hand, the meal from larvae fed with SCG showed higher antioxidant capacity than the others in the DPPH, FRAP and ABTS assays. The digestibility of macronutrients, release of antioxidant capacity and production of SCFAs of the BSFL meal were improved when using these substrates, compared to chicken feed.

1. Introduction

Nowadays, agricultural and food waste is a major problem as it brings with it negative environmental, economic and human impacts. Because of the rapid growth in the human population, which will affect the rising cost of feed, it is predicted that by 2050 the demand for animal protein will rise by 25–70 percent (Fitriana, Laconi, Astuti & Jayanegara, 2022). In return, this will accelerate the issues of food waste management. Therefore, the agri-food industry needs to develop novel circular economy systems to solve or minimize both issues: higher demand of food and larger generation of by-products.

Among the different agro-industrial by-products, spent coffee grounds (SCG) and blood meal were chosen due to their relevance and complimentary nutritional composition. First, coffee is the second most traded product in the world and, consequently, the coffee industry produces large amounts of waste, SCG being one of the most relevant by-products. It is a fine-particulate solid material, with high moisture content, high organic load and acidity (Mussatto, Machado, Martins & Teixeira, 2011). SCG are a source of phenolic compounds with antioxidant and anti-inflammatory properties that can be used as food supplements in the food industry (Low, Rahman & Jamaluddin, 2015). Second, meat processing facilities are responsible for blood meal generation which is a by-product of the meat industry obtained by dehydrating blood (Miller, 1992). Blood meals are good sources of high biological value proteins, minerals and vitamins. In addition, they could be used as total protein improvers in staple foods and provide some amino acids that are markedly deficient in vegetable proteins. It is an alternative source of protein and provides a good energy input in the formulated diet (Jedrejek, Levic, Wallace & Oleszek, 2016; Takakuwa et al., 2022). However, blood meal use as animal feed in the EU is very

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https://doi.org/10.1016/j.foodres.2024.115151
Received 10 April 2024; Received in revised form 20 September 2024; Accepted 25 September 2024
Available online 27 September 2024

limited due to risk associated with certain transmissible spongiform encephalopathies (Commission Regulation (EU) 2021/1372).

The term "co-digestion" in black soldier fly larvae refers to the ingestion and digestion of two or more substrates simultaneously with complementary characteristics, which improves the yield and efficiency of the larvae growing process (Zhang et al., 2023). One of the main benefits of co-digestion is the diversification of substrates, allowing the use of a wide range of organic resources and avoiding dependence on a single feed source (Gómez, Cuetos, Cara, Morán & García, 2006; Lin et al., 2011). Another advantage is that through co-digestion the substrate can adapt the nutritional to be more suitable for a given biological such as BSFL (Fernandez-Bayo, Yazdani, Simmons & VanderGheynst, 2018; Palma, Fernández-Bayo, Putri & VanderGheynst, 2020). Fischer et al. (2021) showed that co-digestion of a SCG and dough (1:1) blend produced significantly higher gross and net production of BSFL compared to both substrates tested individually. In this sense, the co-digestion of SCG and/or blood meal can help to take advantage of their nutritional content and contribute to the sustainable production of protein and other valuable compounds.

Most of the proteins consumed from animal sources is causing severe environmental damage and water scarcity due to the high impact of livestock, pig and poultry production (Dopelt, Radon & Davidovitch, 2019). Sustainable protein production could be achieved by using insects as the main source, thus minimizing natural resources use, reducing pressure on ecosystems and environmental impact. Conventional foods tend to have lower protein content than insects, with the exception of beef and some fish (Hawkey, Lopez-Viso, Brameld, Parr & Salter, 2021). Due to their high levels of protein and well-balanced amino acids, insects have attracted the attention of many researchers as food ingredients for supplements for people, mainly athletes and children (Melgar-Lalanne, Hernández-Álvarez & Salinas-Castro, 2019). Among the most studied insect meal used as protein substitutes black soldier fly (*Hermetia illucens*) larvae (BSFL) are one of the most promising alternatives for human consumption (Bessa, Pieterse, Marais & Hoffman, 2020). The black soldier fly is a detritivorous insect native of tropical, subtropical and warm areas of the Americas. However, it can now be found in different parts of the world due to human interest (Tomberlin, Sheppard & Joyce, 2002). It contributes exponentially to the circularity and sustainability of agricultural and food systems because it is efficient in reducing the volume of organic by-products while converting them into protein-rich biomass and soil fertilizer (Kim et al., 2021). Authors claim that BSFL should be considered as animal feed, stating that regardless of their low environmental impact, they have a good nutritional composition since the last larval stage of the black soldier fly contains 42 % protein content and 35 % fat in dry matter (Lu et al., 2022). Furthermore, fatted and defatted BSFL have a rich essential amino acid profile. Particularly, it has been shown that BSFL are richer in valine, histidine and isoleucine compared with fish meal and soybean meal (Lu et al., 2022). They have also shown higher levels of leucine, lysine levels than soybean and comparable levels of methionine and tryptophan than soybean (Lu et al., 2022). Digestibility of larvae protein has already been studied in mammals (Kim et al., 2023) and fish (Weththasinghe et al., 2021). Studies show that BSFL can be used as highly bio-available protein source for human nutrition (Traksele et al., 2021). However, there is a need to confirm these results, particularly because BSFL composition is strongly influenced by the feed used to rear them (Bonelli et al., 2020).

Replacing common animal feeds such as soybean with BSLF has shown some benefits. For example, it prevents the soybean meal-induced intestinal enteritis in rainbow trout and has some immunological benefits (Kumar et al., 2021). Kawasaki et al. (2019) argued that BSFL meal was a potential ingredient for poultry feed. Furthermore, increasing BSFL meal in the diet of broilers did not compromise growth and meat quality of the animal, and resulted in a 23 % decrease in feed cost per bird and higher net marginal benefits (Nampijja et al, 2023).

Taking all these information into account, the aim of this work is to evaluate the potential of two different food by-products individually or co-digested as a feedstock for BSFL to obtain a meal with a high protein content. SCG was characterized by having high C content whereas blood meal was characterized by having high N content. Furthermore, this paper also aims to unravel the potential benefits of BSFL meal for human nutrition. To test this, simulated *in vitro* digestion and fermentation were carried out to measure the release of antioxidant capacity and production of short chain fatty acids through fermentation by the gut microbiota.

2. Materials and methods

2.1. Chemicals

$CaCl_2(H_2O)_2$, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, NaOH, HCl, NaH_2PO_4, $K_2S_2O_8$, Na_2S, $FeCl_3{\cdot}6H_2O$, AcONa, Na_2CO_3, α-amylase, porcine pepsin, pancreatin, bile acids, tryptone, cysteine, resazurin, DPPH, methanol, ABTS, TPTZ, Folin-Ciocalteu reagent, formic acid and acetonitrile were of analytical grade and purchased from Sigma Aldrich (Darmstadt, Germany), except for porcine pancreatin, which was sourced from Alpha Aesar (UK).

2.2. Production of larvae meal

SCG were collected from the cafeteria of the Faculty of Pharmacy (Granada, Spain) and air-dried before storage, the blood meal was original from Far Pro Modena S.p.A (Spilamberto, Italy), and the chicken feed was bought at a local store (Cereales y Piensos Herben, Santa Fe, Spain). As described in the label, the chicken feed was a mix of corn, soy, wheat and minerals with levels of protein, fat, fiber and ashes of 16 %, 3.75 %, 3 % and 14 %, respectively. The BM used (PROTESAN®, Deham, Iowa, United States) was from non-ruminant animals in order to avoid risk of prions transmission. The total C and N were measured in CN analyzer LECO TruSpec (LECO Instrumentos S.L., Madrid, Spain).

Four treatments were used as feedstock: 100 % spent coffee grounds (SCG), 100 % blood meal, a blend of 24 % blood meal and 76 % SCG (dry weight equivalent), and 100 % of chicken feed as a control, as the larvae feed and develop well on this substrate (Palma et al., 2019). Their total C, N and the C/N ratios are described in Table 1.

A total of 12 containers were prepared (3 replicates per substrate), all with 135 g of substrate (dry weight) to have feeding rate of 0.125 g/day/larvae (Gold, Tomberlin, Diener, Zurbrügg & Mathys, 2018) at a moisture content between 50 and 68 % (adapted to reach 100 % of the fiber saturation point of each substrate). The larvae were then selected (a total of 1200 larvae) to introduce 100 larvae per container. The larvae were selected one-by-one, were about one week-old and measured between 5/6 mm. Larvae were weighed and measured before feeding and finally introduced into the different container.

The containers with larvae and substrates were incubated at 30 °C for 10 days in a climatic chamber maintaining in the dark. The larval feeding period ended after 9 days, when control larvae were close to pupation. At that time, larvae were removed from their containers and fasted for 24 h. The, larvae were sized, weighed and placed in a freezer at −15 °C causing an unaggressive death. After 48 h in the freezer, the jars were baked at 60 °C for another 48 h. After this period, larvae were put into a desiccator and crushed. The process of obtaining larvae meal

Table 1
Total C (%) and N (%) concentration in a dry weight basis, and C/N ratio of the substrates used to feed the larvae.

Substrates	%N	%C	C/N
SCG	2.2	47.4	21.5
Blood meal	14.6	49.7	3.4
Blood meal + SCG	5.3	48.0	9.0
Control	2.3	38.2	17.0

was carried out using a porcelain mortar.

2.3. Protein content

Total carbon, hydrogen, nitrogen and traces of sulphur were determined with a Thermo Scientific Flash 2000 model elemental analyser. The protein content was calculated from the nitrogen content (Protein content (%) = nitrogen content (%) × 4.67). This protein conversion factor (Kp) was specifically estimated for insects including BSFL (Janssen et al., 2017).

2.4. In vitro digestion and fermentation

According to the previously outlined protocols (Pérez-Burillo, Rufián-Henares & Pastoriza, 2018; Pérez-Burillo et al., 2021), samples were submitted to *in vitro* gastrointestinal and *in vitro* fermentation in triplicate. Larvae meals were added to falcon tubes together with simulated salivary fluid (1:1, w/v) composed of salts and α-amylase (75 U/mL). The mix was kept at 37 °C for 2 min in oscillation. Right after, 5 mL of simulated gastric fluid was added, simulating the gastric juices content in salts and pepsin (2000 U/mL). The mix was kept at 37 °C for 2 h, at pH 3 in oscillation. Finally, 10 mL of simulated intestinal fluid was added, simulating the intestinal juices content in salts, bile salts and enzymes (here we used 67.2 mg/mL pancreatine). The mix was kept at 37 °C for 2 h, at pH 7, in oscillation. Once the intestinal phase was finished, tubes were kept in ice to stop enzymatic reactions and thereafter centrifuged at 3500 rpm for 10 min. The supernatant, which represents the fraction available for absorption in the small intestine, was stored in 1 mL tubes at −80 °C until analysis. The solid pellet served as the *in vitro* fermentation substrate and represents the undigested portion that enters the large intestine.

The *in vitro* fermentation was carried out using fecal samples from five healthy donors (who had not taken antibiotics for three months prior to the assay, with a mean (Body Mass Index = 21.3); the fecal samples were pooled together to reduce inter-individual variability. The fermentation was carried out at 37 °C for 20 h. Once the *in vitro* fermentation was finished, tubes were kept in ice to stop microbial reactions and thereafter centrifuged at 3500 rpm for 10 min. The supernatant, which represents the fraction available for absorption in the large intestine, was stored in 1 mL tubes at −80 °C until analysis. The solid pellet, which stands for the portion that wasn't fermented and expelled with feces, was duly disposed of.

In vitro gastrointestinal digestion and fermentation resulted in two fractions: fermentation supernatant, which is for large intestine absorption, and digestion supernatant, which is for small intestine absorption. Antioxidant capacity was assessed in both fractions, with the sum of them taken into consideration as total antioxidant capacity.

2.5. Antioxidant tests

Antioxidant capacity of those two fractions was studied. The sum of the antioxidant capacity of the two fractions was considered the total antioxidant capacity that a given larvae meal could exert (Pastoriza, Delgado-Andrade, Haro & Rufián-Henares, 2011).

$TEAC_{DPPH}$ assay (Trolox equivalent antioxidant capacity against DPPH radicals). The method followed the protocol of Yen and Chen (Yen & Chen, 1995) and it was adapted to a microplate reader (Cytation 5, Agilent Technologies). In brief, 20 μL of either digestion or fermentation supernatant were added to a 96 well plate and mixed with 280 μL DPPH reagent, all in duplicate. This reagent was prepared at a concentration of 74 mg DPPH/L methanol. The antioxidant reaction was monitored for 1 h. Calibration curve was prepared with Trolox at a concentration ranging from 0.01 to 0.4 mg/mL. Results were expressed as mmol Trolox Equivalent/Kg larvae meal.

$TEAC_{FRAP}$ assay (Trolox equivalent antioxidant capacity referred to reducing capacity). The method to measured ferric reduction capacity of samples followed the protocol of by Benzie and Strain (Benzie & Strain, 1996) and it was adapted to a microplate reader (Cytation 5, Agilent Technologies). In brief, 20 μL of either digestion or fermentation supernatant were added to a 96 well plate and mixed with 280 μL FRAP reagent, which was prepared the day of the experiment. The antioxidant reaction was monitored for 30 min. Calibration curve was prepared with Trolox at a concentration ranging from 0.01 to 0.4 mg/mL. Results were expressed as mmol Trolox Equivalent/Kg larvae meal.

$TEAC_{ABTS}$ assay (Trolox equivalent antioxidant capacity against ABTS·+ radicals). It measures scavenging capacity of samples against the artificial radical ABTS. The antioxidant capacity was estimated in terms of radical scavenging activity following a procedure previously described (Re et al., 1999) and adapted to a microplate reader (Cytation 5, Agilent Technologies). Briefly, 20 μL of either digestion or fermentation supernatant were added to a 96 well plate and mixed with 280 μL ABTS reagent, which was prepare the day before according to Re et al. (1999). The antioxidant reaction was monitored for 30 min. Results were expressed as mmol Trolox Equivalent/kg larvae meal.

Total phenolic content was also analyzed in the supernatant obtained after *in vitro* gastrointestinal digestion representing the fraction available for absorption in the small intestine and in the soluble phase (supernatant) obtained after *in vitro* microbial fermentation representing the fraction available for absorption in the large intestine. The sum of both fractions would be the total amount of phenolics released. Total phenolic content was estimated following the procedure described by Singleton and Rossi (Singleton & Rossi, 1965) with few modifications and adapted to a microplate reader (Cytation 5, Agilent Technologies). Briefly, 30 μL of either digestion or fermentation supernatant were added, in triplicate, to a 96 well plate and mixed with 15 μL of Folin-Ciocalteu reagent, 60 μL of sodium carbonate 10 % (w/v), and 195 μL of milli-Q water. The antioxidant reaction was monitored for 60 min. The results obtained are expressed as mg Gallic acid equivalents (GAE) per Kg of larvae meal.

2.6. Short chain fatty acids determination

The determination of short chain fatty acids (SCFAs) atty acids was carried out by high performance liquid chromatography (HPLC) as described in a previous work (Panzella et al., 2017). The sample did not require any pre-treatment prior to injection. Briefly, fatty acid standards were prepared in the mobile phase at concentrations ranging from 5 to 10 000 ppm. The mobile phase, consisting of a mixture of two solutions and delivered at a flow rate of 0.250 ml/min. Specifically, the mobile phase consisted of 99 % of a first solution (being ultrapure water acidified with 1 % formic acid) and 1 % of a second solution (being acetonitrile acidified with 1 % formic acid). After the fermentation process, 1 mL of supernatant was centrifuged to remove solid particles, filtered through a 0.22 μm nylon filter and finally transferred to a vial for HPLC analysis. The HPLC was an Accela 600 (Thermo Scientific) equipped with a quaternary pump, an autosampler and a UV–Vis photodiode array detector (PDA) set at 210 nm. The column was a reversed-phase Accucore™ C18 (Thermofisher) with a particle size of 2.6 μm and 150 mm long. The analysis was performed in duplicate and the data presented are the mean values expressed as millimolar (mM) concentration of each fatty acid.

2.7. Statistical analysis

First, the Shapiro-Wilk normality test was carried out, obtaining a non-normal distribution of the samples. Statistical differences were calculated using the unpaired Kruskal Wallis test with 95 % confidence. Then, to make comparisons between the different groups, the Conover-Iman test was carried out. Multivariate principal component analysis (PCA) was performed to explore the differences between the groups. Statgraphics Plus, version 5.1, was used to perform all statistical analyses.

3. Results

3.1. Larvae weight and length

The average survival rate of the larva of the experiment was not significantly different in all treatments (97.67 ± 0.58, 101.00 ± 4.36, 97.33 ± 1.53, and 98.67 ± 8.14, for the control, blood meal, SCG with blood meal, and SCG, respectively, P > 0.05). Larvae fed with regular chicken feed (control) had the highest larval biomass (Fig. 1). Statistical analysis showed that larvae fed with blood meal, SCG and the mixture of both substrates had a significantly lower weight ($p < 0.05$) than larvae fed with regular feed (Fig. 1).

3.2. Protein content

In terms of protein content, statistically significant differences ($p < 0.05$) were found between the group of larvae fed with blood meal and those fed with regular feed, with the former having higher protein content. Statistically significant differences were also found between the meal of the group of larvae fed with the substrate mixture and that of the larvae fed with regular feed, with a higher protein content in the meal of larvae fed with the substrate mixture (Fig. 2).

3.3. Antioxidant capacity

3.3.1. DPPH assay

After *in vitro* digestion and fermentation, as well as after the sum of both (total antioxidant capacity), the same significant differences were found ($p < 0.05$). Fig. 3**A** shows that the digestion, fermentation, and total antioxidant capacity of the larval meal fed with chicken feed was significantly lower than that of the other groups. When comparisons were made between the different groups, taking into account the total antioxidant capacity, statistically significant differences were found between all groups of meals, except between larval meals fed with blood meal and those fed with the mixture of substrates. The larval meal with the highest reported antioxidant capacity was that of larvae fed with SCG, being significantly higher than that of the other groups.

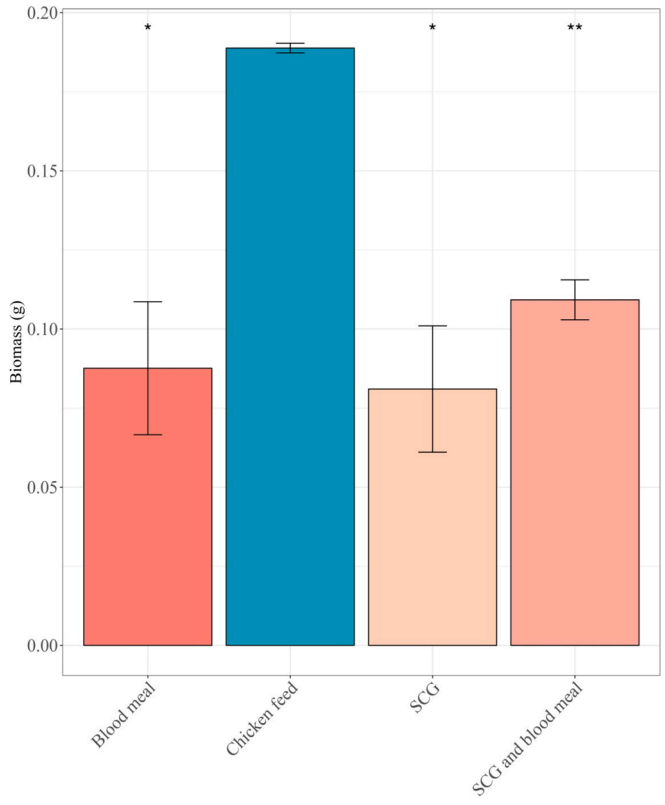

Fig. 1. Increment of biomass per larvae in grams of larvae after being fed with blood meal, spent coffee grounds (SCG) and a mixture of both (Blood meal + SCG) and chicken feed (Control). Statistical analysis was performed via Kruskal-Wallis test using larvae fed with chicken feed as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

3.3.2. FRAP assay

For the FRAP assay, the same significant differences obtained with the DPPH assay were found after digestion and in total antioxidant capacity ($p < 0.05$, Fig. 3B). Larval meals fed with blood meal, SCG and the mixture of both reported significantly higher antioxidant capacity than larval meals fed with feed ($p < 0.05$). Notably, the larval meal fed with SCG reported the highest antioxidant capacity. As for the measurements after fermentation, the only statistically significant difference ($p < 0.05$) found was between the meal from larvae fed with SCG and that from larvae fed with chicken feed, the former being higher.

When comparisons were made between the different groups, taking into account the total antioxidant capacity, statistically significant differences were found between all groups of meals, except between larval meals fed with blood meal and those fed with the mixture of substrates. The larval meal that reported the highest antioxidant capacity was that of larvae fed with SCG, being significantly higher than the rest of the groups, as in the DPPH test (Fig. 3**B**).

3.3.3. ABTS assay

After *in vitro* digestion, statistically significant differences were found ($p < 0.05$, Fig. 3**C**), with the antioxidant capacity of the meal from larvae fed with chicken feed showing the lowest antioxidant capacity. After *in vitro* fermentation, no statistically significant differences were found. In the results of the total antioxidant capacity, statistically significant differences were found, with the antioxidant capacity of the meal from larvae fed with blood meal and the meal from larvae fed with SCG, being higher than that of the meal from larvae fed with chicken feed. When comparisons were made between the different groups, taking into account the total antioxidant capacity, statistically significant differences were found between all meal groups, except between the meal from larvae fed with SCG and the meal from larvae fed with blood meal. Also, no statistically significant differences were found between larval meal fed with chicken feed and larval meal fed with a mixture of both substrates. The larval meal with the highest reported antioxidant capacity was that of larvae fed with SCG, being significantly higher than the other groups, as in the DPPH and FRAP assay (Fig. 3**C**).

3.3.4. Total phenolic content (Folin-Ciocalteu assay)

Statistically significant differences ($p < 0.05$, Fig. 3**D**) were found after *in vitro* digestion and fermentation and in total antioxidant capacity. The phenolic content in the meal from larvae fed with chicken feed was significantly lower than in the other meal groups. When comparisons were made between the different groups, taking into account the total phenolic content after the sum of digestion and fermentation, the same statistically significant differences were found as described above. Between groups of larval meals fed with substrates there were no statistically significant differences. The larval meal with the highest total phenolic content was reported from larvae fed with blood meal, being significantly higher than the rest of the groups (Fig. 3**D**).

3.3.5. Differences in antioxidant capacity in the in vitro digestion and fermentation phases

Fig. 4 depicts the contribution (%) to total antioxidant capacity of the phases obtained after *in vitro* digestion and fermentation. As can be observed, the antioxidant capacity after *in vitro* fermentation was substantially lower compared to that reported after *in vitro* digestion. While the fraction belonging to *in vitro* fermentation does not reach 25 % in any

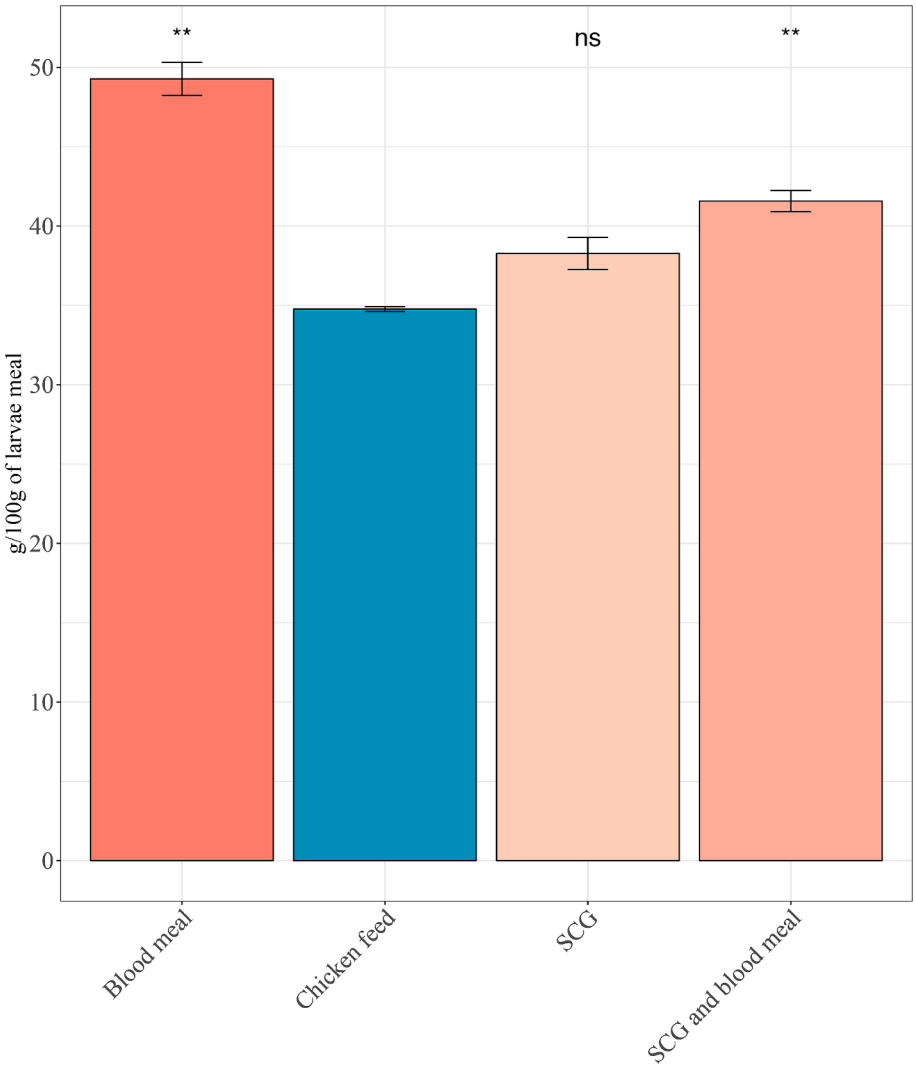

Fig. 2. Protein content in g/100 g of larval meal depending on the substrate fed to the larvae. Statistical analysis was performed via Kruskal-Wallis test using larvae fed with chicken feed as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

of the cases, *in vitro* digestion contributes more than 75 % to the total antioxidant capacity. It should be noted that in all four methods of antioxidant capacity determination, including the determination of total polyphenols, the meal from larvae fed with chicken feed showed a higher contribution to the total antioxidant capacity in the *in vitro* fermentation fraction compared to the other meals. Even so, this was not even 25 %.

3.4. Short chain fatty acids production

Meal from larvae fed with chicken feed was kept as the reference group and the statistically significant differences ($p < 0.05$) found were as follows (Fig. 5). The larval meal fermentation samples fed with blood meal reported significantly higher contents of acetic, lactic, propionic, butyric, succinic and total fatty acids than the larval meal fermentation samples fed with chicken feed. On the contrary, fermentation samples of larval meal fed with the substrate mixture (blood meal + SCG) reported lower contents of acetic, butyric, lactic, succinic and total fatty acids than fermentation samples of larval meal fed with chicken feed, significantly. Other significant differences ($p < 0.05$) were found for the meal from larvae fed with SCG. These samples, after fermentation, reported a higher content of butyric and succinic acid than the fermentation samples of meal from larvae fed with chicken feed.

When comparisons between groups were carried out, more significant differences were found ($p < 0.05$). For acetic acid as well as for the sum of all fatty acids (total fatty acids) differences were found between all larval meal groups except between larval meal fed with chicken feed and larval meal fed with SCG.

For butyric and lactic acids, statistically significant differences ($p < 0.05$) were found between larval meals fed with blood meal and larval meal fed with SCG, separately. Also, between larval meal fed with the mixture of both substrates and only with blood meal, and finally, as shown in Fig. 5, between larval meal fed with feed and larval meal fed with the mixture of substrates. For propionic and succinic acids, significant differences ($p < 0.05$) were also found between almost all meal groups. For succinic acid, differences were found between all groups except between the meal from larvae fed with chicken feed and the meal from larvae fed with the substrate mixture. For propionic acid, significant differences were also found between all groups of meals except between the meal from larvae fed with chicken feed and the meal from larvae fed with SCG and with the mixture of both substrates, as can be seen in Fig. 5.

Fig. 6 is a Principal Component Analysis (PCA) that depicts the four groups of larval meals, clearly differentiated. Weight, length, protein content, total antioxidant capacity (with the DPPH, FRAP and ABTS methods), total phenolic content and quantity of all SCFAs were taken into account. This figure shows graphically that the characteristics of the different larval meals depend on the previous feeding of the larvae. In

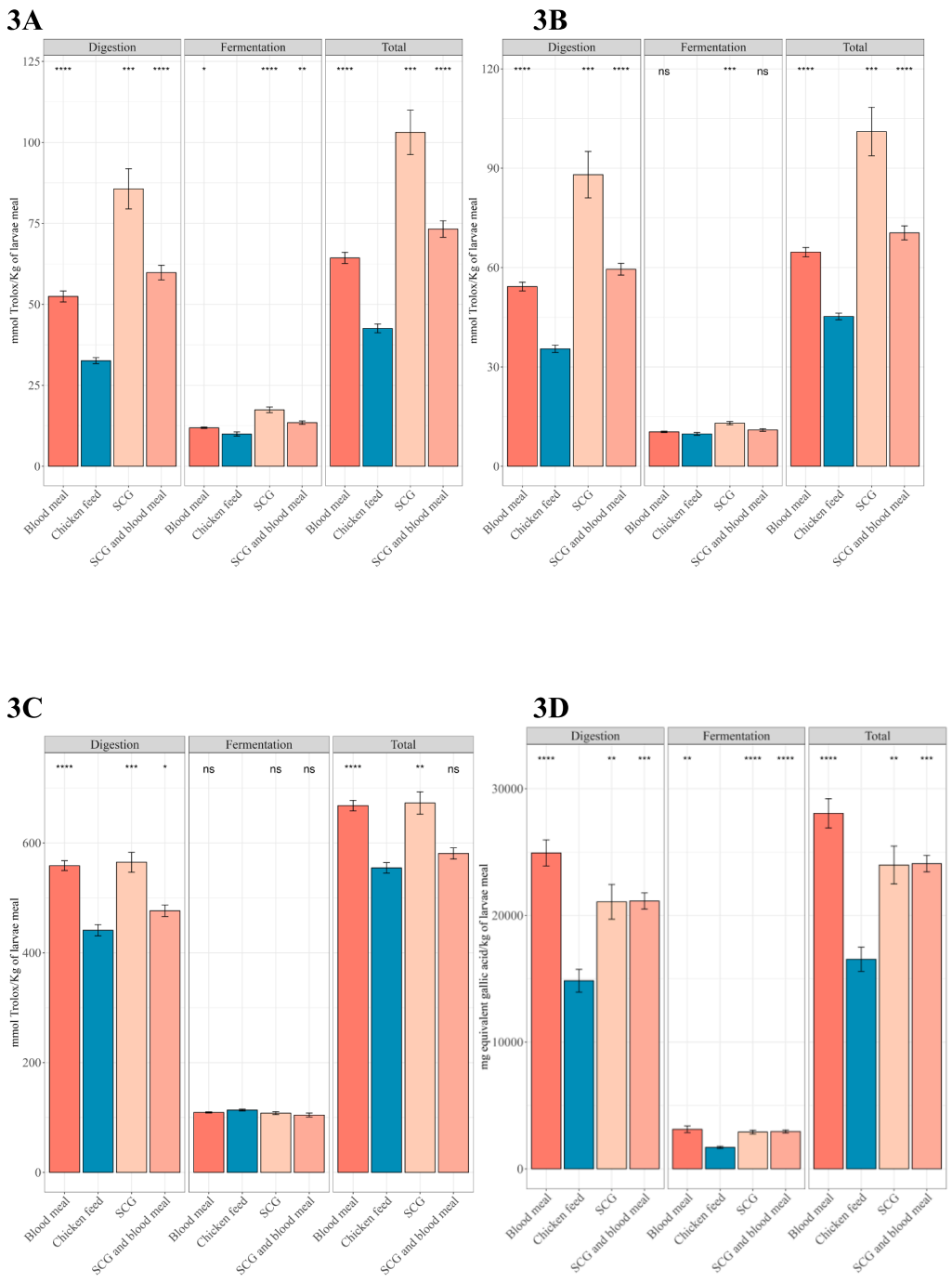

Fig. 3. Antioxidant capacity of digested-fermented larval meals depending on the substrate provided to feed them for **(A) $TEAC_{DPPH}$, (B) $TEAC_{FRAP}$, (C) $TEAC_{ABTS}$ and (D) Folin-Ciocalteu**. Statistical analysis was performed by Kruskal-Wallis test using larval meal fed with chicken feed as reference group. Statistical labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

general, the PCA explained a 76.3 of variability, being 41.2 % for the first component and a 35.1 % for the second one. In the group of larvae fed with blood meal, SCFAs were the more influential factor. The variables FC and FRAP were more influential in the SCG-fed larval meal group. Finally, weight was the variable that most influenced the results of the larval meal fed with chicken feed.

4. Discussion

The largest increment of the biomass was observed in the control. However, the survival rate and the positive increment of larvae biomass observed in the isolated feeds, and particularly in the mixture, confirms their potential application as feeding substrate for black soldier fly larvae. In a previous study larvae were fed with SCG, donut dough and a mix of both; these authors found that larvae fed with SCG only were lighter and shorter than those fed with the donut dough and with the mix (Fischer, Romano & Sinha, 2021). In addition, it has been found that SCG do not support optimal larval growth due to the high amount of non-digestible fiber, as well as the presence of caffeine, tannins and the deficiency of nutrients such as some amino acids and fatty acids during the roasting and preparation process using high temperatures (Fischer et al., 2021; Permana & Putra, 2018). Similarly, in this study, low larvae

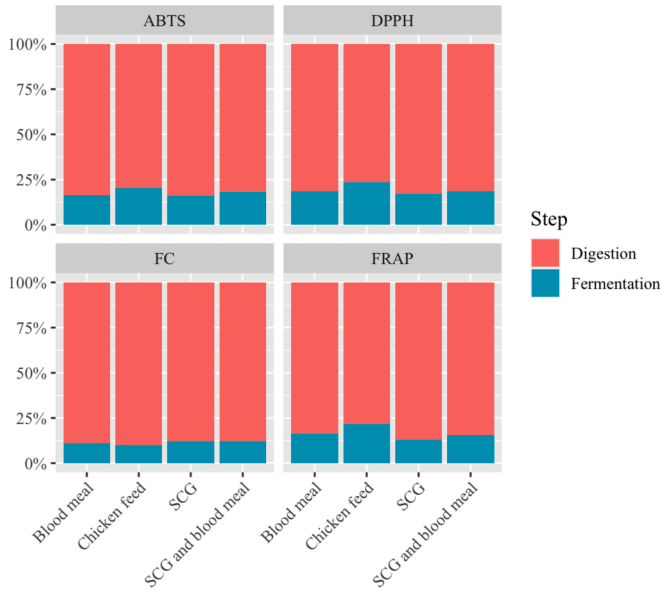

Fig. 4. Contribution (%) to the total antioxidant capacity of each fraction depending on the type of larvae meal.

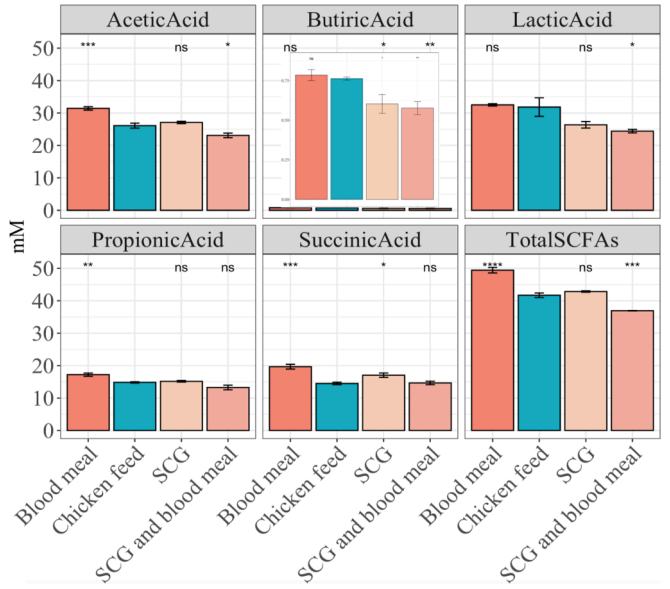

Fig. 5. Short chain fatty acids of digested-fermented larval meals depending on the substrate provided to feed them. Statistical analysis was performed via Kruskal-Wallis test using meal from larvae fed with chicken feed as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

growth was observed when blood meal was used as the sole feeding substrate. To our knowledge, no other studies have used this substrate before. One of the attributes responsible for the low larvae growth could have been the unbalance C/N ratio as observed in previous studies (Beesigamukama et al., 2021). The mix of blood meal and SCG slightly increased the carbon to nitrogen ratio, which may have contributed to increasing the larvae growth. However, this increase was not enough to reach the same level of biomass as the control substrate, meaning that other nutrients in the feedstock may have been involved. For instance, it has been observed that young larvae grown on chicken feed-based diets containing various carbohydrate additives (D glucose, sucrose, D (−) fructose, corn and wheat starch, D (+) galactose, D (+) mannose, D (+) xylose, D (−) arabinose and xylan from beechwood) showed different effects (Cohn, Latty & Abbas, 2022). Diets with galactose, arabinose, and xylose produced adverse effects on process performance compared with the control (chicken feed). On the other hand, BSFL fat content was increased by wheat starch and decreased by galactose and xylan compared to the control. This highlights the need for further characterization of the initial feedstock to better understand the impact on BSFL performance.

The heaviest larvae were those fed with chicken feed, yet they had the lowest protein content. In a study by Permana & Putra (2018), fly larvae were fed with different substrates, and as in the present study, the heaviest larvae had the least amount of protein. Therefore, although the chicken feed makes them larger, the high N content of the blood meal (Table 1) provides them with other nutrients that make them higher in protein. Protein values of blood meal-feed larvae were higher than those found in the literature (Lu et al., 2022). Fischer et al. (2021) also found that SCG led to significantly lower crude protein in BSFL prepupae as well as lower growth compared to BSFL fed dough that had higher crude protein and faster growth. Finally, other studies comparing prepupae reared in a wide range of feedstocks did not observe strong changes in crude protein content (ranging between 39 and 44 %). Lalander et al. (2019) did not observe any significant factor significantly correlated with the protein content of the larvae despite the size of the larvae/prepupae varied considerably (70–250 mg/larvae).

In terms of antioxidant capacity, the group of larvae fed with SCG stood out. For the latter application, the antioxidant capacity of SCG can be attributed to polyphenols, particularly chlorogenic acids such as caffeic acid, nicotinic acid and trigonelline, which is converted to nicotinic acid under heat treatment (Campos-Vega, Loarca-Piña, Vergara-Castañeda & Oomah, 2015; Gigliobianco et al., 2020; Jeszka-Skowron, Stanisz & De Peña, 2016; Panusa et al., 2013; Taguchi, Sakaguchi & Shimabayashi, 1985). However, for the Folin-Ciocalteu method, the group of larvae fed with blood meal stood out, showing also the highest protein content. In the DPPH, FRAP and ABTS methods, the results were similar, which leads us to hypothesize that the total phenolic content may be overestimated in the Folin-Ciocalteu method due to the reaction of those proteins present in blood meal with the Folin-Ciocalteu reagent. In a previous study (Navajas-Porras et al., 2022), the antioxidant capacity of foods of animal origin was studied after digestion and fermentation *in vitro*. In that paper it was found that there was a significant correlation between the protein content of the food and the values obtained from the Folin-Ciocalteu method (and that these were overestimated), which could explain our current results (Navajas-Porras et al., 2022).

Regarding SCFAs production, larvae fed with blood meal produced the highest amount of these acids in humans after *in vitro* digestion and fermentation. These SCFAs are produced by intestinal bacteria, and are well known to provide a broad range of health benefits to both terrestrial and aquatic animals that may include enhancing and improving gut health (Egnew et al., 2021). Muturi, Dunlap, Ramirez, Rooney & Kim (2019) carried out a study with a species of mosquito, finding that the composition of the microbiota of the mosquitoes changed as they fed on human blood (Muturi et al., 2019). This leads us to hypothesize that the high protein content of blood meal produces a higher metabolism at the level of the gut microbiota and therefore, the production of SCFAs is higher. It should be noted that the use of blood meal in the food chain is limited due to the possible presence of prions and disease transmission in case they are from ruminant animals (and this is why we selected a blood meal source from non-ruminant animals). Ofori & Hsieh (2014) argued that the use of whole blood and/or blood-derived proteins as ingredients in animal feed and in the human food chain poses only a minimal threat to food safety in terms of disease transmission, exposure to blood allergens and blood-borne pathogens. Any threat posed by their use would be no different from that posed by other foods of animal origin (Ofori & Hsieh, 2014).

In the last years, there has been a growing interest in antioxidant

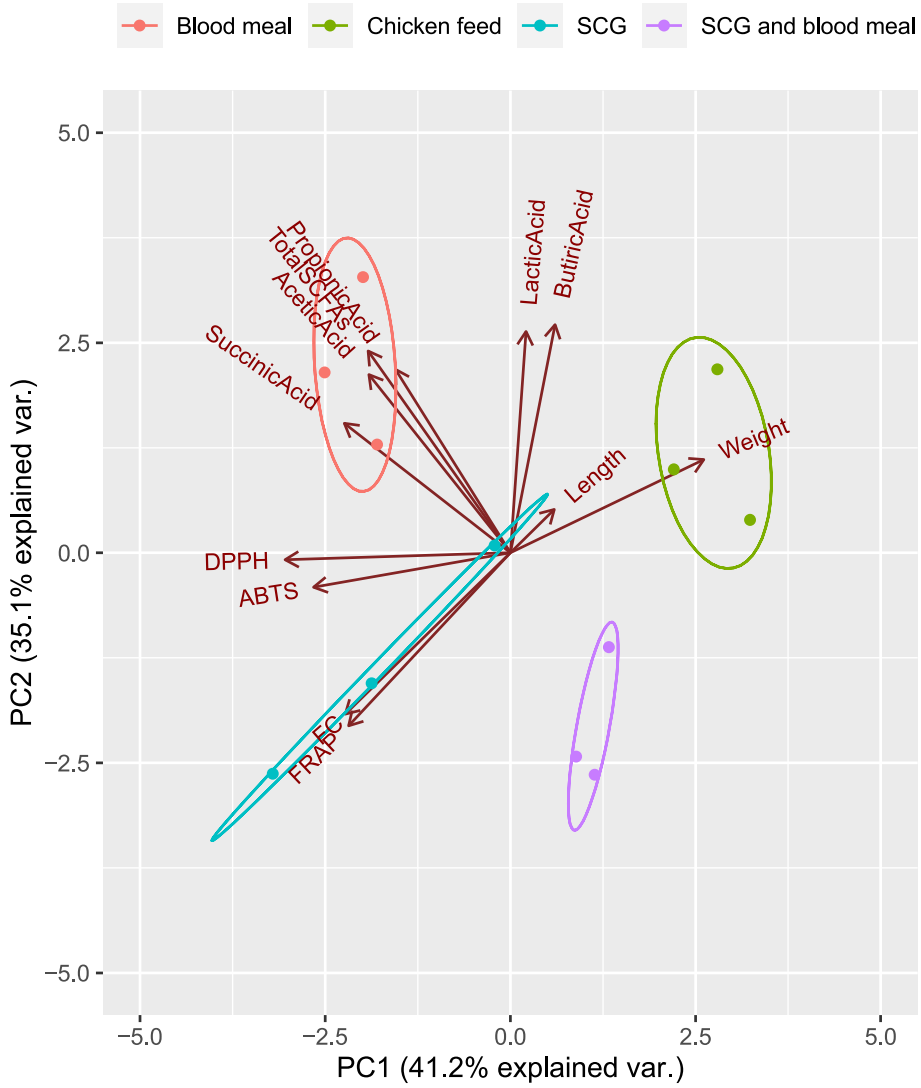

Fig. 6. Principal Component Analysis (PCA) taking into account weight, length, protein content, total antioxidant capacity and fatty acid content.

compounds present in the diet due to their benefits against chronic and degenerative diseases and the ageing process (Franco, Interdonato, Cordaro, Cuzzocrea & Di Paola, 2023). In the world population there is also a widespread preference for consuming natural antioxidants over synthetic ones. The consumption of natural antioxidants from the diet is a considerable boost to endogenous antioxidant defenses (Lourenço, Moldão-Martins & Alves, 2019). In this sense, it is important to highlight the effect of *in vitro* digestion and fermentation over the release of antioxidant capacity, being higher after *in vitro* digestion than after fermentation (Fig. 4). In another study carried out with foods of animal origin (Navajas-Porras et al., 2021) the opposite behavior was observed, being released more antioxidant capacity after *in vitro* fermentation; this may be due to the digestibility of the macronutrients found in feed and larval meals. From the findings of this work, larval meals are highly digestible, so all bioactive compounds with antioxidant capacity were released after *in vitro* fermentation, leaving the microbiota with hardly any substrates. However, in foods such as meat or fish, due to their complex matrix, they are not as highly digestible and many substrates reach the large intestine, where the resident microbiota metabolize these nutrients and release bioactive compounds such as carnitine and carnosine (Navajas-Porras et al., 2021).

5. Conclusions

The survival and increment of larvae biomass in the substrates confirm that blood meal and/or spent coffee grounds can be used to feed black soldier fly larvae. However, the lower biomass observed in the treatments compares to the chicken feed control highlights a need to improve the efficacy of the process. In this study, a step further was taken targeting the digestibility of the BSFL macronutrients. It was found that their weight was higher when fed with chicken feed (our control feed), but the protein content, antioxidant capacity and production of fatty acids after *in vitro* fermentation were higher in larvae fed with blood meal and/or SCG. These, in turn, are food by-products that could be used for the purpose of feeding larvae to improve their nutritional quality. Although larval meals fed with SCG reported a higher antioxidant capacity (related with the phenolic compounds of coffee), that from larvae fed with blood meal-fed larvae achieved the best results. Even so, the use of blood meal in the food chain is limited by the potential for disease transmission, which is currently being increasingly studied as it is a by-product that could provide high protein content in the diet.

Funding

This work was supported by the Plan propio de Investigación y Transferencia of the University of Granada under the program

"Intensificación de la Investigación, modalidad B".

CRediT authorship contribution statement

Beatriz Navajas-Porras: Writing – original draft, Formal analysis. **Adriana Delgado-Osorio:** Investigation, Formal analysis. **Daniel Hinojosa-Nogueira:** Formal analysis, Data curation. **Silvia Pastoriza:** Writing – review & editing, Methodology, Investigation. **María del Carmen Almécija-Rodríguez:** Writing – review & editing, Formal analysis. **José Ángel Rufián-Henares:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization. **Jesús D. Fernandez-Bayo:** Writing – review & editing, Supervision, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank the support of the Unit of Excellence 'UNETE' from the University of Granada (reference UCE-PP2017-05). The authors also thank the cafeteria of the Faculty of Farmacy of the University of Granada for providing the spent coffee grounds. Funding for open aceess charge: Universidad de Granada/CBUA.

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