



Fatty acid plasticity of black soldier fly (*Hermetia Illucens*) larvae reared on alternative feeding media: crude olive cake and processed animal protein

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ABSTRACT. The trial was performed to assess the effects of feeding media consisting of crude olive cake (COC) or processed animal protein (PAP) on the growth rate and the fatty acid composition of *Hermetia illucens* (black soldier fly) larvae (BSFL). The results showed a significant decrease in length and weight of larvae in the experimental groups reared on media containing 100% of PAP originating from poultry (PAP-P), mixed PAP (PAP-Mx) or COC compared to the control group fed on the basal diet (CON). The replacement of 50% of PAP with basal diet or wood shavings, significantly improved the length and weight of BSFL reared on both kinds of PAP; however not to the values obtained in the CON group. The addition of mineral and vitamin mixture into COC feeding significantly improved the growth of the BSFL, although it also did not attain the results of the basal diet. In comparison to the CON group, experimental groups containing 100% of PAP-P, PAP-Mx or COC were characterized by a decrease in the content of lauric acid and saturated fatty acids, and an increase in the content of oleic acid and monounsaturated fatty acids (MUFA). Especially high content of MUFA was observable in the COC group. The long-chain polyunsaturated fatty acid content was very low in all groups. In conclusion, with the usage of alternative feeding media, like PAP or COC, significant plasticity in the fatty acid profile of *Hermetia illucens* larvae could be achieved.

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Introduction

The European Parliament adopted a resolution in 2011 on the European Union (EU) protein deficit: what is the solution for a long-standing problem? (2010/2111(INI)) (European Parliament, 2012). The resolution was based on the fact that more than 80% of the protein requirements of livestock reared in the EU is imported from non-EU countries. Since then, the rearing of insects as an alternative source

of protein for feed has become an area of intensive scientific research.

The *Hermetia illucens* (L.) (black soldier fly, BSF) has emerged as one of the most interesting subjects of research, in the area of insects as alternative protein sources (Wang et al., 2017). As of 2018, insects, including BSF larvae (BSFL) are allowed as fish feed. Adult BSF are not considered pestiferous or invasive and they do not bite or sting (Sheppard et al., 2002; Čičková et al., 2015). Due to their

behaviour and antimicrobial properties, they are not considered as biological vectors of specific pathogens, unlike most fly species (Furman et al., 1959; Sheppard et al., 2002; Choi et al., 2012).

The BSFL could be easily reared in a short period of time (2 to 3 weeks) using a huge diversity of feeding substrates, ranging from chicken feed to human or animal faeces (Sheppard, 1983; Green and Popa, 2012; Zheng et al., 2012; Lalander et al., 2013; Banks et al., 2014; Nguyen et al., 2015; Webster et al., 2016). The BSFL feed conversion ratio is very low and could be as low as 1.4 when they are fed a high-protein, high-fat diet, which makes it superior to most other insects (Ooninx et al., 2015). The survival rate of the BSFL is very high and does not depend significantly on the diet (Ooninx et al., 2015), whereas the survival rate of other insects may be greatly decreased when using specific diets (Starčević et al., 2017).

EU legislation on animal proteins in feed drastically changed after the outbreak of transmissible spongiform encephalopathies in the 1980s and 1990s. Nowadays, it is hard to expect that insects reared in feeding media containing human or animal faeces, or similar waste products, will be permitted to be used as animal feed in the EU. Therefore, it is essential to investigate how to maintain cheap and efficient production of BSFL by using permitted and safe by-products.

Crude olive oil cake (COC) is a by-product of olive oil extraction. It is obtained by mechanical extraction and contains residual oil and stones. The chemical composition of COC is characterized by a high amount of crude fibre, which makes it adequate as feed only for animals that can digest large amounts of fibre (e.g., ruminants, rabbits) (Tortuero et al., 1989). Although COC can be used as animal feed, fertilizer or as an energy source (Molina-Alcaide and Yáñez-Ruiz, 2008; Rodríguez et al., 2008), large quantities still remain accessible for use as insect feed in countries with high production of olive oil (Mediterranean basin). Processed animal protein (PAP) are animal proteins processed from any category 3 animal by-products as defined by EU legislation. Since 2001 there has been a ban on the use of PAP in animal feed, but the possibility of using PAP has been constantly discussed at the EU level (EFSA, 2007; EFSA Panel on Biological Hazards, 2011; ESFA Panel on Biological Hazards et al., 2018).

Our trial was performed to assess the effects of feeding media consisting of COC or PAP on the growth rate and the fatty acid composition of BSFL, as well as the effects of the protein content in feeding media on the BSFL growth rate. Additionally, the

possibilities of improving the growth of BSFL in experimental media containing PAP or COC by the addition of basal diet, wood shaving or mineral-vitamin mixture was investigated.

Material and methods

Rearing of insects and experimental design

Hermetia illucens eggs and adults were obtained from the certified commercial breeder 'Insektarij' in Zagreb (Croatia). Black soldier fly eggs were incubated inside a closed container (47 cm length × 32 cm width × 23 cm height) at 37.5 ± 2 °C and ~90% relative humidity. Strips of corrugated cardboard filled with the eggs were placed over 2 kg of basal diet (Table 1) mixed with water (80%). The strips were not in contact with the substrate. After the eggs had hatched, the larvae were weighed (1.0 g), counted and transferred into non-transparent plastic boxes (38 cm length × 28 cm width × 20 cm

Table 1. The composition¹ of the basal diet and by-products

Indices	Basal diet	PAP-P	PAP-Mx	COC
Ingredients, g/kg				
crude protein	222	680	531	78.2
crude fat	30.4	140	130	124
crude fibre	46.5	–	–	381
calcium	10.4	52.4	116	73.2
phosphorus	5.0	29.7	58.9	8.7
lysine	12.2	32.1	30.3	0.3
methionine	6.5	9.3	85.7	0.4
crude ash	54.4	130	260	82.6
Fatty acid composition, % of total fatty acids				
C10:0 (capric)	0.02	0.03	0.32	0.00
C12:0 (lauric)	0.91	0.16	0.49	0.00
C14:0 (myristic)	0.54	0.98	3.30	0.00
C15:0 (pentadecanoic)	0.02	0.15	0.40	0.00
C16:0 (palmitic)	16.97	30.85	29.18	13.64
C16:1n7 (palmitoleic)	0.69	5.32	2.38	0.92
C17:0 (heptadecanoic)	0.23	0.28	0.78	0.1
C18:0 (stearic)	4.39	10.29	18.72	1.84
C18:1n9 (oleic)	29.10	44.77	39.59	73.31
C18:1n7 (<i>trans</i> -vaccenic)	1.31	2.02	2.32	0.00
C18:2n6 (linoleic)	43.63	5.15	2.03	9.06
C18:3n3 (linolenic)	1.82	0.00	0.02	0.83
C20:1n9 (<i>cis</i> -11-eicosenoic)	0.37	0.00	0.43	0.00
C20:5n3 (eicosapentaenoic, EPA)	0.00	0.00	0.02	0.00
C22:6n3 (docosahexaenoic, DHA)	0.00	0.00	0.02	0.00

¹ the samples were analysed according to the AOAC International (1995); PAP-P – processed animal protein originating from poultry; PAP-Mx – mixed processed animal protein; COC – crude olive cake

height) with transparent lids, to prevent the larvae from escaping. The boxes contained basal diet or experimental feeding media. Groups were formed using four different feeding media: 1. basal diet (chicken feed; CON group); 2. processed animal protein of poultry origin (PAP-P); 3. processed animal protein of mixed origin (PAP-Mx); and 4. crude olive cake (COC). Additional groups were formed by mixing PAP feeding media with the basal diet (BD) or wood shavings (WS), and COC with mineral-vitamin mix (MV) (Table 2). Different protein levels (22, 25, 30, 40 and 50%) were achieved by mixing the BD and egg white powder protein (Myprotein, The Hut Group, Cheshire, UK). Every group had 3 replicates. All feeding media were thoroughly mixed with water (60% moisture). The thickness of feeding media was 5 cm in all groups and replicates. The experiments were conducted in temperature- and moisture-controlled environment (temperature 26 ± 2 °C; relative humidity ~80%).

Table 2. Experimental groups based on the used percentage of feeding media

Experimental group	Basal diet	PAP-P	PAP-Mx	COC	MV	WS
CON	100	0	0	0	0	0
COC	0	0	0	100	0	0
COC+MV	0	0	0	95	5	0
PAP-P	0	100	0	0	0	0
PAP-P+BD	50	50	0	0	0	0
PAP-P+WS	0	50	0	0	0	50
PAP-Mx	0	0	100	0	0	0
PAP-Mx+BD	50	0	50	0	0	0
PAP-Mx+WS	0	0	50	0	0	50

PAP-P – processed animal protein originating from poultry; PAP-Mx – mixed processed animal protein; COC – crude olive cake; MV – mineral-vitamin mixture; WS – wood shavings

Measurements

After 14 days of rearing, the larvae were washed in water and individually measured. They were weighed using an electronic balance and their length was determined on photographs by measuring software (IC Measure, The Imaging Source Europe GmbH, Bremen, Germany).

Fatty acid analyses

All chemicals were HPLC grade and were obtained from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany). The lipids were extracted according to Folch et al. (1957) using a chloroform/methanol mixture (2:1, v/v). Total lipids were extracted from homogenized whole larvae with the addition of butylated hydroxytoluene as an

antioxidant (30 mg per 100 ml) and nonadecanoic acid (C19:0) as an internal standard.

Transmethylation was performed using 2M KOH in methanol at room temperature. The analysis of fatty acid methyl esters was performed using a Shimadzu GC-MS QP2010 Ultra Gas Chromatograph Mass Spectrometer (Shimadzu, Kyoto, Japan) with a BPX70 capillary column (0.25 mm internal diameter, 0.25 µm film thickness, 30 m long; SGE, Austin, TX, USA) and helium as the carrier gas, as previously described (Starčević et al., 2017). The injector temperature was 250 °C, the injection volume was 1 µl, the split ratio was 1:80 and linear velocity was 35 cm/s. The oven program was set as: initial temperature 40 °C, held for 3 min, then increased at the rate of 20 °C/min up to 130 °C, then increased at 1.5 °C/min up to 200 °C, and then increased at 45 °C/min up to 250 °C and held for 10 °C. The results of fatty acid profile were expressed as the percentage of total fatty acids.

Statistical analyses

The data were analysed using one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test in order to determine statistical differences between group means. Data were analysed using Statistica software ver. 12 (StatSoft, Tulsa, OK, USA). The statistical significance was set at $P < 0.05$.

Results

The first part of the trial examined the influence of four different feeding media (basal diet (CON), PAP-P, PAP-Mx and COC) in a 100% concentration on the BSFL length and weight. The results showed a significant decrease in length and weight (Figure 1a and 1b, respectively) in all experimental groups compared to the CON group. The complete absence of the early prepupae stage (reddish to brownish colour) in the PAP fed groups was observed (Figure 2).

Additional groups were introduced in the second part of the experiment by mixing the PAP feeding media with 50% of the BD or WS, while the COC feeding media was enriched with MV. The 50% of BD, as well as WS, significantly improved the length and weight of BSFL reared in PAP-P (Figure 3) and PAP-Mx (Figure 4). The improvement of the BSFL weight was better in the PAP feeding media mixed with the BD compared to the WS.

To elucidate further the role of protein content in feeding media, the influence of protein contents, ranging from 22 to 50%, on the BSFL length and weight was examined. The results showed

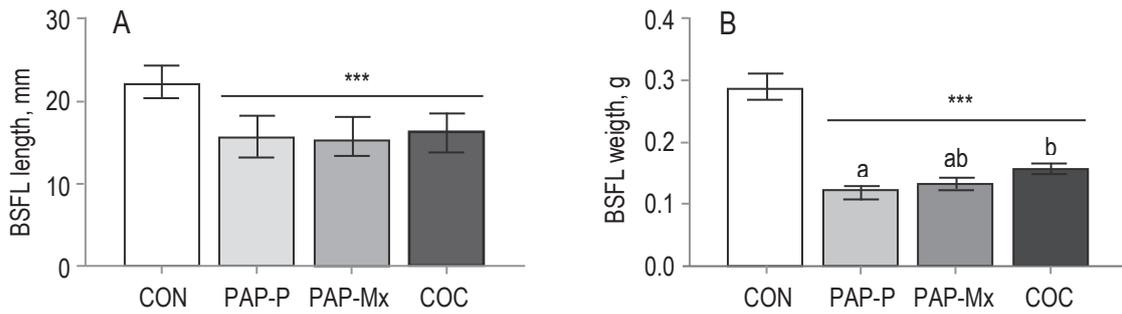


Figure 1. The influence of dietary treatment with processed animal protein (PAP) or crude olive cake (COC) on *Hermetia illucens* (black soldier fly) larvae (BSFL) length (A) and weight (B)

Groups: CON – control group fed basal diet (100%); PAP-P – group fed on processed animal protein originating from poultry (100%); PAP-Mx – group fed on mixed processed animal protein (100%); COC – group fed on crude olive cake (100%); *** – $P < 0.001$ for each experimental group vs CON one; a–b – bars with different superscripts differ significantly

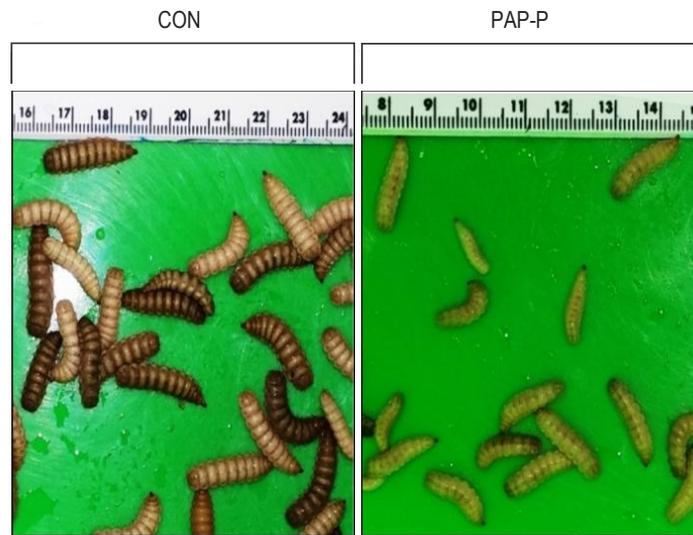


Figure 2. *Hermetia illucens* (black soldier fly) larvae (BSFL) development: BSFL fed basal diet (CON, 100% basal diet) developed normally (creamy white younger larvae and reddish to brownish mature larvae), while there were no mature larvae when processed animal protein originating from poultry (PAP-P, 100%) was used as feeding media

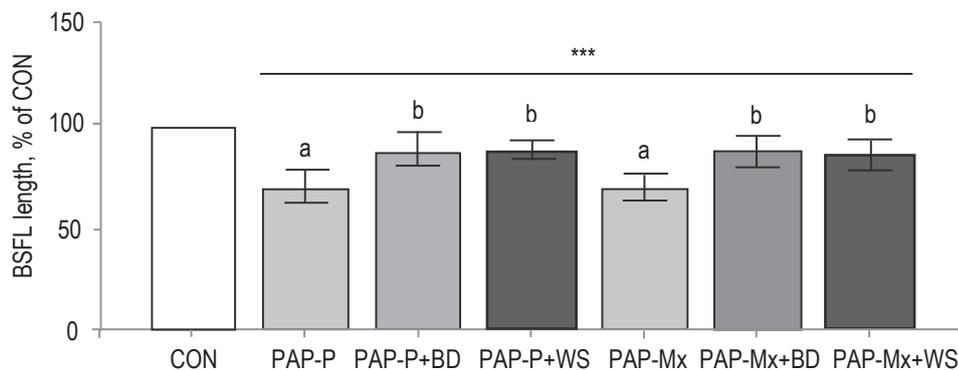


Figure 3. The influence of mixing the processed animal protein (PAP) with 50% of basal diet (BD) or wood shavings (WS) on the length of *Hermetia illucens* (black soldier fly) larvae (BSFL)

Groups: CON – control group fed on BD (100%); PAP-P – group fed on processed animal protein originating from poultry (100%); PAP-P+BD – group fed on PAP-P (50%) mixed with BD (50%); PAP-P+WS – group fed on PAP-P (50%) mixed with WS (50%); PAP-Mx – group fed on mixed processed animal protein (100%); PAP-Mx+BD – group fed on PAP-Mx (50%) mixed with BD (50%); PAP-Mx+WS – group fed on PAP-Mx (50%) mixed with WS (50%); *** – $P < 0.001$ for each experimental group vs CON one; a–b – bars with different superscripts differ significantly within the same dietary treatment (PAP-P and PAP-Mx, respectively)

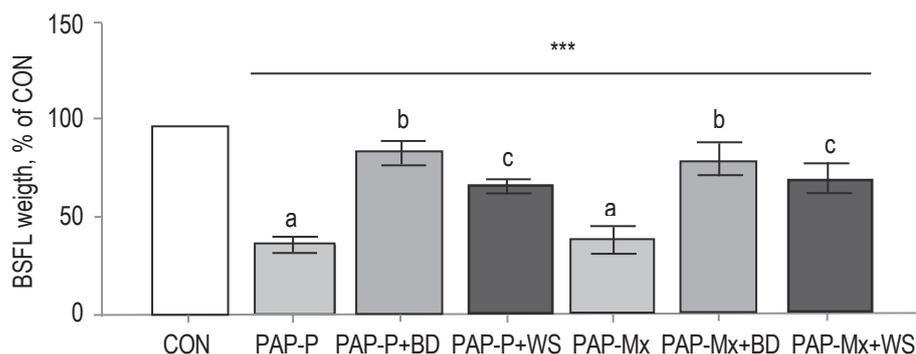


Figure 4. The influence of mixing the processed animal protein (PAP) with 50% of basal diet (BD) or wood shavings (WS) on the weight of *Hermetia illucens* (black soldier fly) larvae (BSFL)

Groups: CON – control group fed on BD (100%); PAP-P – group fed on processed animal protein originating from poultry (100%); PAP-P+BD – group fed on PAP-P (50%) mixed with BD (50%); PAP-P+WS – group fed on PAP-P (50%) mixed with WS (50%); PAP-Mx – group fed on mixed processed animal protein (100%); PAP-Mx+BD – group fed on PAP-Mx (50%) mixed with BD (50%); PAP-Mx+WS – group fed on PAP-Mx (50%) mixed with WS (50%); *** – $P < 0.001$ for each experimental group vs CON one; a–c – bars with different superscripts differ significantly within the same dietary treatment (PAP-P and PAP-Mx, respectively)

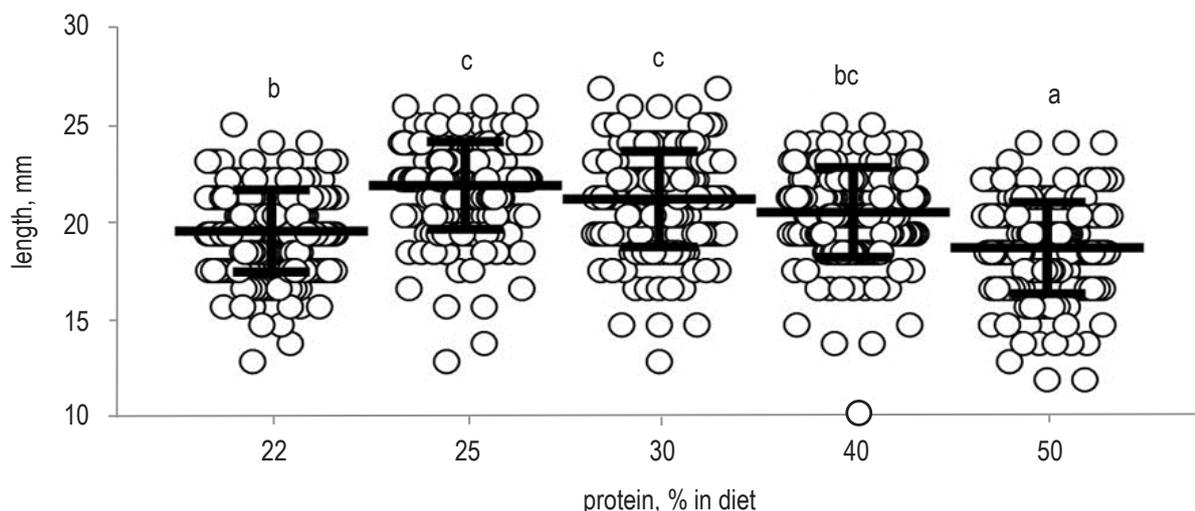


Figure 5. The influence of different levels of crude protein obtained by mixing basal diet with egg white powder protein on the length of *Hermetia illucens* (black soldier fly) larvae (BSFL); a–c – protein levels with different superscripts differ significantly at $P < 0.05$

significant improvement in the growth of BSFL with 25 and 30% of protein, then no change with 40% of protein and then a significant decrease with 50% of protein in the feeding media in comparison to 22% protein level in feeding media (Figure 5).

With the COC feeding media supplemented with the mineral and vitamin mixture (MV), the growth of the BSFL significantly improved in comparison with COC feeding media, although it did not attain the results of the CON group (Figure 6).

The fatty acid profile of the experimental BSFL is presented in Table 3. In comparison to the CON group, experimental groups were characterized by a decrease in the content of lauric acid and saturated fatty acids (SFA), and an increase in the content of oleic acid and monounsaturated fatty acids (MUFA).

Especially high content of MUFA was observable in the COC group. An additional characteristic of the PAP groups was an increase in the palmitoleic acid and small amounts of EPA, which was not detectable in other groups. The polyunsaturated fatty acid (PUFA) content was very low in all groups, with the lowest value in the PAP-P group. Between the PAP groups, there was a significant difference in linoleic acid content, with almost 4-fold lower values in the PAP-Mx compared to the PAP-P group.

The plasticity of the BSFL fatty acid profile under the influence of different feeding media is assessed in Figure 7. It is observable that the highest plasticity could be seen for lauric acid (11–43%) and oleic acid (11–54%), followed by linoleic and linolenic acids.

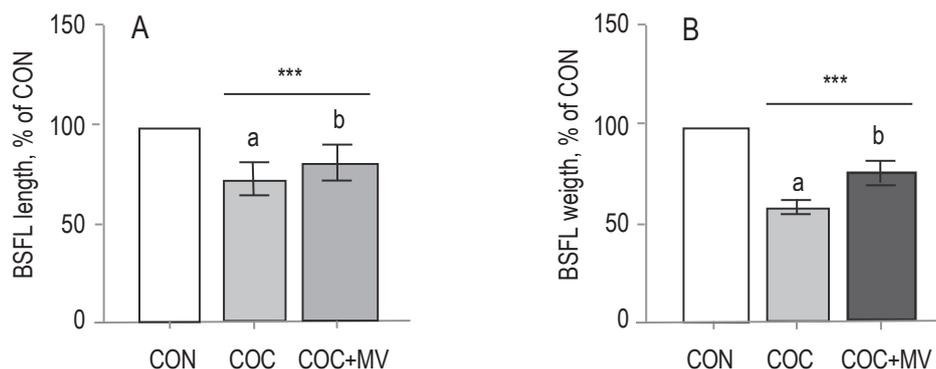


Figure 6. The effect of the crude olive cake (COC) alone or supplemented with a mineral and vitamin mixture (MV) on *Hermetia illucens* (black soldier fly) larvae (BSFL) length (A) and weight (B)

Groups: CON – control group fed on BD (100%); COC – group fed on crude olive cake (100%); COC+MV – group fed on COC (95%) mixed with MV (5%); *** – $P < 0.001$ for each experimental group vs CON one; a–b – bars with different superscripts differ significantly within the same dietary treatment (COC)

Table 3. The influence of oil addition on the fatty acid profile of *Hermetia illucens* larvae

Fatty acids	CON	PAP-P	PAP-Mx	COC
C10:0 (capric)	0.59 ± 0.05	0.24 ± 0.03***	0.29 ± 0.04***	nd
C12:0 (lauric)	36.5 ± 1.75	14.17 ± 1.31***	17.4 ± 0.65***	11.85 ± 1.56***
C14:0 (myristic)	8.08 ± 0.26	3.66 ± 0.23***	5.16 ± 0.11***	2.09 ± 0.08***
C16:0 (palmitic)	18.0 ± 0.79	19.0 ± 0.49	18.0 ± 0.41	12.69 ± 0.34***
C16:1n7 (palmitoleic)	1.44 ± 0.42	6.77 ± 0.22***	8.38 ± 0.37***	1.26 ± 0.05
C18:0 (stearic)	2.90 ± 0.34	4.35 ± 0.02**	4.48 ± 0.29**	1.40 ± 0.01
C18:1n9 (oleic)	14.7 ± 0.86	31.5 ± 0.34***	40.1 ± 0.71***	54.12 ± 1.05***
C18:1n7 (trans-vaccenic)	0.52 ± 0.17	0.88 ± 0.03*	0.93 ± 0.06**	0.82 ± 0.10*
C18:2n6 (linoleic)	16.3 ± 1.74	18.6 ± 0.68**	4.80 ± 0.61***	12.29 ± 0.05*
C18:3n3 (linolenic)	0.84 ± 0.08	0.60 ± 0.01**	0.23 ± 0.06***	0.44 ± 0.03**
C20:1n9 (cis-11-eicosenoic)	nd	nd	nd	nd
C20:5n3 (eicosapentaenoic, EPA)	nd	0.06 ± 0.00***	0.07 ± 0.02***	nd
C22:6n3 (docosahexaenoic, DHA)	nd	nd	nd	nd
n6	16.3 ± 1.74	18.6 ± 0.68**	4.80 ± 0.61***	12.2 ± 0.05*
n3	0.84 ± 0.08	0.66 ± 0.02*	0.33 ± 0.09***	0.44 ± 0.03**
n6/n3	19.4 ± 0.95	28.2 ± 1.47**	15.12 ± 3.04	28.58 ± 1.75**
MUFA	16.7 ± 0.68	39.2 ± 0.55***	49.4 ± 1.07***	59.14 ± 1.19***
PUFA	17.1 ± 1.81	19.2 ± 0.67**	5.13 ± 0.66***	12.73 ± 0.08**
SFA	66.1 ± 2.04	41.5 ± 1.06***	45.4 ± 0.91***	28.11 ± 1.26***

CON – control group fed basal diet (100%); PAP-P – group fed on processed animal protein originating from poultry (100%); PAP-Mx – group fed on mixed processed animal protein (100%); COC – group fed on crude olive cake (100%); nd – not determined (below quantification level); MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; SFA – saturated fatty acids; *** – $P < 0.001$, ** – $P < 0.01$ and * – $P < 0.05$ for each experimental group vs CON

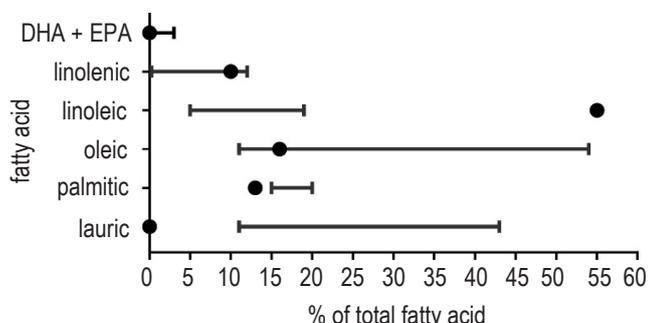


Figure 7. The plasticity of the fatty acid profile of *Hermetia illucens* (black soldier fly) larvae (BSFL) reared on different feeding media. The minimum and maximum range presented in the graph is composed from data including feeding media with processed animal protein originating from poultry (PAP-P), mixed processed animal protein (PAP-Mx), crude olive cake (COC) as well as 5% linseed oil, fish oil and coconut oil from our unpublished data. The average fatty acid profile of soybean meal (●) is included in the graph

Discussion

Different by-products are especially interesting as feed for production animals due to their much higher viability in comparison to the traditional animal feed (e.g., grains). One-third of the world's cereal production is used as livestock feed each year (Foley et al., 2011). With the increasing demand for meat, demand for animal feed is also growing. Additionally, livestock farming has a great impact on the environment (e.g., deforestation, soil erosion, water pollution, greenhouse gas emission) (van Huis, 2013). The acceptance of by-products as feed depends on the efficacy of conversion into body mass by the production animal (Oonincx et al., 2015), as well as on legal issues that may emerge in relation to some by-products (e.g., PAP). Over the last two decades, a substantial amount of scientific data has been obtained on rearing BSFL. Nevertheless, a large amount of data, especially on the influence of feeding media on the nutritional composition of BSFL, is still missing. That gap in knowledge is the result of the poorly understood metabolism of BSFL, as well as the lack of nutritional trials on the plasticity of BSFL chemical composition (Oonincx et al., 2015).

The first part of the present trial compared the growth rates of BSFL fed on chicken feed, which is usually used on commercial farms, with feeding media consisting completely of processed animal protein from poultry or of mixed origin or crude olive cake. The data showed a reduction in BSFL growth in all groups based on examined by-products compared to the control diet during the 14-day trial.

In the second part of the trial, we addressed the main problems visible in the previous trial. The probable cause of the decreased growth in the PAP media was the muddy texture of the PAP mixed with water. The texture prevented movement, which led to the escape of BSFL from the media. Additionally, the unfavourable composition of media (excess protein, low content of fermentable components) contributed to the growth decrease. After mixing the PAP feeding media with 50% of the basal diet, a significant improvement was observed, indicating that an imbalance in the composition of the feeding media caused a decrease in the BSFL growth. After mixing the PAP feeding media with wood shavings, a similar significant increase in growth was visible, indicating that improvement in the feeding media texture may influence BSFL growth. Nevertheless, the improvement in the growth was much higher after the addition of basal diet than after the addition

of wood shavings, indicating that improvement in the texture is not an adequate strategy, without balancing the composition of the feeding media. To assess the possible negative influence of protein excess further, we tested the influence of different protein contents on the BSFL growth. The results showed that 50% of protein in the feeding media leads to decreased BSFL growth compared to 22% present in chicken feed, which shows that high-protein by-products could affect BSFL growth.

The feeding media consisting of COC had an appropriate texture for BSFL, therefore, the more important problem could be dietary imbalance, mainly high-fibre content and low content of some minerals. The high-fibre content in the BSFL feeding media lowered BSFL growth, survival and development time, although BSFL can degrade cellulose, hemicellulose and lignin (Rehman et al., 2017). After the addition of the mineral and vitamin mixture to the COC feeding media, we observed a significant improvement in BSFL growth. These data show that COC could be an interesting and viable feeding media for BSFL, and that its performance may be improved even more after balancing the mineral content.

After the initial production trial, we tested the fatty acid profile of BSFL reared in different media to address the plasticity of the fatty acid profile. The most evident change in all experimental groups was the substitution of lauric acid with oleic acid. The high increase in oleic acid content was the consequence of its high content in the experimental feeding media. There is scientific evidence showing that the substitution of SFA with oleic acid has beneficial health effects (Lopez-Huertas, 2010; Sales-Campos et al., 2013; Palomer et al., 2018). In contrast, the content of beneficial n3 and n6 polyunsaturated fatty acids (PUFA) was not improved in our trial by using the experimental feeding media. As a consequence, the n6/n3 ratio was very high in all experimental groups, indicating that only the addition of oils containing a high amount of PUFA can significantly decrease the n6/n3 ratio to desirable levels (unpublished data).

Conclusions

In conclusion, our results showed that crude olive cake (COC) and processed animal protein (PAP) after modifications could be interesting feeding substrates for the rearing of *Hermetia illucens* (black soldier fly) larvae (BSFL). By using these feeding media, high plasticity in the lipid profile of

BSFL could be achieved with increased quantities of oleic and lauric acid. The fat composition of BSFL could add significant value to the BSFL utilization as the source of oils for different industries.

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