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### ► To cite this version:

Marie Papin, L. Morand-Laffargue, C. Sabran, D. Sabatier, A. Sefah, et al.. Black soldier fly larvae (*Hermetia illucens*) do not bioaccumulate ferulic and caffeic acids from wheat bran. *Journal of Insects as Food and Feed*, 2024, pp.1-12. 10.1163/23524588-00001338 . hal-04780999

**HAL Id: hal-04780999**

<https://hal.inrae.fr/hal-04780999v1>

Submitted on 13 Nov 2024

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# Black Soldier Fly larvae (*Hermetia illucens*) do not bioaccumulate ferulic and caffeic acids from wheat bran

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## Abstract

As recently shown, black soldier fly larvae (BSFL) are capable of bioaccumulating high concentrations of vitamin E and carotenoids, but the potential bioaccumulation of polyphenols remains unknown. Wheat bran (WB), a common breeding substrate for BSFL, is particularly rich in ferulic acid (FA) and also contains caffeic acid (CA). Numerous studies suggest that these polyphenols have beneficial effects on human and animal health. BSFL ability to bioaccumulate these bioactive compounds was assessed by comparing their concentration in WB and BSFL raised on WB. The three forms of FA and CA, *i.e.* free, soluble-bound and insoluble-bound, were extracted from WB and BSFL and quantified by HPLC-UV. No form of CA was detected in BSFL. The three forms of FA were detected but the total FA concentration in BSFL ( $17 \pm 1$  mg/kg fresh weight) represented only 2% of the total FA concentration measured in WB ( $810 \pm 38$  mg/kg fresh weight). Since our larvae were not fasted, another experiment was carried out to find out if the small amount of FA found in the larvae was the FA contained in WB present in their digestive tract. The digestive tracts of fasted and non-fasted larvae at the end of the rearing period were weighted. And it was calculated that all the FA measured in the larvae could be the FA present in their digestive tract. Moreover, the distribution of the three different forms of FA differed between WB and BSFL. Therefore, we have demonstrated that not only BSFL do not significantly bioaccumulate FA from WB but that they apparently metabolize it. Regarding CA as a precursor of FA, the same phenomenon might have happened. However, assuming an equivalent bioaccumulation factor as FA, the amounts of CA theoretically transferred in larvae were too low to be detected.

**Keywords:** polyphenols, edible insects, diptera, larval digestive tract mass

**Conflict of interest:** AS and DS work in the BioMiMetiC company. This company conducts research and development activities aimed at valorizing insect-based bioconversion of a wide variety of organic materials generated at all levels of the food value chain. All other authors have no conflict of interest to declare.

**Abbreviations:** BSFL (black soldier fly larvae); CA (caffeic acid); FA (ferulic acid); WB (wheat bran)

49  
50

## 1. Introduction

51 Black Soldier Fly larvae (BSFL, *Hermetia illucens*) (Diptera : Stratiomyidae) can be reared  
52 on almost all kinds of organic waste (e.g. biowaste and agricultural by-products) with a high  
53 conversion rate to transform organic waste into BSFL biomass (Scieuzo *et al.*, 2022; Surendra  
54 *et al.*, 2020). They are recognised to be rich in macronutrients, especially in proteins and  
55 lipids (37-50% and 7-39% in dry weight, Dörper *et al.*, 2021). Hence, BSFL have been more  
56 and more used for biowaste recycling to create value-added products. They especially make a  
57 suitable alternative source of protein for animal feed (Dörper *et al.*, 2021; Lu *et al.*, 2022;  
58 Wang and Shelomi, 2017). BSFL also contains micronutrients such as minerals, e.g. calcium,  
59 copper, iron, manganese, depending on the substrate they are reared on (Lu *et al.*, 2022).  
60 Recently, it was demonstrated that BSFL can bioaccumulate significant concentrations of fat-  
61 soluble vitamins and carotenoids from plant substrates they are reared on (Borel *et al.*, 2021;  
62 Morand-Laffargue *et al.*, 2023a, 2023b, 2023c). This suggests that BSFL could bioaccumulate  
63 valuable concentrations of other phytochemicals. Among all the phytochemicals contained in  
64 plants, polyphenols hold a special place because numerous studies have suggested that they  
65 can have beneficial effects on health, whether in humans (Mithul Aravind *et al.*, 2021;  
66 Ruskovska *et al.*, 2020) or in livestock (Wang *et al.*, 2020). Yet, the presence of polyphenols  
67 in different insect species, including BSF, has been reported (Baigts-Allende *et al.*, 2021;  
68 Nino *et al.*, 2021a). But to the best of our knowledge, the scientific literature about phenolic  
69 compounds in insects, and more particularly edible insects, is still really scarce and no study  
70 has been dedicated to assess whether some polyphenols can significantly bioaccumulate in  
71 insects reared on polyphenol rich substrates. One of the most used substrate for raising BSFL  
72 is wheat bran (WB) (Mannaa *et al.*, 2024). It contains most of the micronutrients and  
73 bioactive compounds of the grain (Hemery *et al.*, 2007). Among these bioactive, polyphenols,  
74 and more particularly phenolic acids, occupy a special place (Boudaoud *et al.*, 2021), and  
75 ferulic acid, FA (4-hydroxy-3-methoxycinnamic acid), makes up 70% of phenolic acids  
76 (Boudaoud *et al.*, 2021; Zhou *et al.*, 2004a). WB also contains caffeic acid, CA (3-(3,4-  
77 Dihydroxyphenyl)-2-propenoic acid), which is a precursor of FA. The fact that WB is  
78 extremely rich in these two phenolic acids makes it very interesting from a health point of  
79 view. Indeed, FA is said to have many beneficial effects for health, including neuroprotective,  
80 anti-inflammatory and antitumoral ones (Neto-Neves *et al.*, 2021; Palani Swamy and  
81 Govindaswamy, 2015; Yeh *et al.*, 2020). In addition, it seems that FA esters improve  
82 memory, not only in flies, but also in mice, suggesting the possibility that they also improve  
83 this cognitive function in humans, although clinical studies are needed to demonstrate this  
84 (Michels *et al.*, 2018). This allows for better understanding of its health effects in farmed  
85 animals. Indeed, it has been shown to increase antioxidant capacity in weaned piglets (Wang  
86 *et al.*, 2020) and maintain intestinal integrity in broilers (Tang *et al.*, 2023). All the  
87 mechanisms of action of FA are not known but it is accepted that its antioxidant activity is  
88 predominant. It was shown to have the highest antioxidant activity of all phenolic acids tested  
89 in a previous study (Laddomada *et al.*, 2015). Concerning CA and its derivatives, antioxidant,  
90 anti-inflammatory, anticancer and vasorelaxant properties were put forward (Khan *et al.*,  
91 2016; Silva and Lopes, 2020).

92 FA and CA exist under three forms in WB: free, conjugated soluble-bound and insoluble-  
93 bound phenolic acids. Conjugated soluble-bound phenolic acids are covalently bound through  
94 ester or ether bonds to low-molecular mass compounds, such as sugars or fatty acids, and are  
95 soluble in aqueous/organic solvents. The insoluble-bound phenolic acids are covalently bound  
96 to structural components of the cell wall (Rocchetti *et al.*, 2022), through ester or ether bonds,  
97 and are not soluble due to interactions with macromolecules such as cellulose, proteins and

98 lignin. In WB, phenolic acids are mostly found in the bound form (Wang *et al.*, 2013) which  
99 would explain their very low bioavailability in this cereal matrix, as shown in rats and *in vitro*  
100 (Adam *et al.*, 2002; Mateo Anson *et al.*, 2009). It has in fact been shown that the  
101 bioavailability of FA is 10 to 20 times lower when it is associated with a cereal matrix than  
102 when it is ingested in free form.

103 BSFL is commonly raised on WB, rich in FA and CA, compounds of nutritional interest due  
104 to their potential health effects both on livestock and humans. Knowing that BSFL is capable  
105 of bioaccumulating other phytochemicals with health effects, the aim of this study is to  
106 determine if it can bioaccumulate significant quantities of these phenolic acids. BSFL were  
107 therefore raised on this substrate for 7 days and the concentration of the different forms of FA  
108 and CA in the larvae were measured. The bioaccumulation factors of the different forms of  
109 FA and CA at the end of the rearing period were also calculated.

## 110 **2. Material and methods**

111 Two experiments were conducted in this study: one dedicated to the measurement of the  
112 concentrations in two phenolic acids (FA and CA) in WB and WB fed BSFL, and the other  
113 one dedicated to the estimation of the quantity of WB present in BSFL digestive tract, when  
114 non-fasted.

### 115 **Chemicals**

116 Solvents, *i.e.* ethanol, methanol, diethyl ether, ethyl acetate and acetonitrile, as well as  
117 hydroxide chloride and trifluoro acetic acid were purchased from Carlo Erba (Val de Reuil,  
118 France). Sodium hydroxide pellets (purity: 97%) were purchased from Prolabo (Paris,  
119 France). Phenolic acid standards, *i.e.* FA and CA (purity > 98.0%; HPLC grade) were  
120 purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Three salts used for the  
121 PBS solution; NaCl, KCl, and KH<sub>2</sub>PO<sub>4</sub> were also from Sigma-Aldrich and Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O  
122 was ordered from Honeywell (Charlotte, North Carolina, USA).

### 123 **Phenolic acid quantification in WB and BSFL fed with WB**

#### 124 *BSFL rearing*

125 The rearing procedure was conducted as previously described (Borel *et al.*, 2021). Briefly,  
126 eggs were put in a hatchery in a dark incubation room at 28 ± 1°C, humidity ratio: 65-70%.  
127 After hatching, larvae were fed with the standard juvenile BioMiMetiC food, *i.e.* organic-  
128 poultry feed. After 8.5 d of growth, larvae were separated from the frass and aliquots of about  
129 7,000 larvae (evaluated by weighting and knowing the average weight of a larva) were  
130 prepared. WB (Moulins Soufflet, Corbeil-Essonnes, France) was then used as the rearing  
131 substrate. It was mixed with tap water to obtain a 70% humidity ratio substrate. Seven  
132 kilograms of substrate were put in the growing tray with the aliquot aforementioned. The tray  
133 was placed in the incubation room at 28 ± 1°C, 70% humidity ratio for 7 d in darkness. Larvae  
134 were collected at the end of the rearing period and frozen to death. They were stored at -20°C  
135 until analysis.

136

#### 137 *Phenolic acid extraction*

138 The three forms of phenolic acids present in WB, *i.e.* free, soluble-bound and insoluble-  
139 bound, were extracted following the method described by Zhang *et al.* (2012) with the minor  
140 modifications detailed below. Samples were extracted in quadruplicates and stored at -20°C  
141 until analysis. Phenolic acid extraction began with that of the free phenolic acids. WB and  
142 larvae were first crushed using a mortar (Pulverisette 2, FRITSCH GmbH, Idar-Obstein,  
143 Germany) with liquid nitrogen and stored at -20°C before extraction. Free phenolic acids were

144 extracted from 250 mg of WB and 100 mg of larvae using 5 mL of an 80% chilled ethanol  
145 solution. Tubes were agitated using a tube agitator (Vibrax, IKA; Staufen im Breisgau,  
146 Germany) at 1500 rpm during 10 min at ambient temperature and centrifuged at 1,257 g  
147 during 10 min at 4°C. Supernatants were transferred into another glass tube and the extraction  
148 was repeated once more on the residue. Supernatants were then pooled, evaporated to dryness  
149 at 45°C under nitrogen and dissolved in 2 mL of methanol so as to be injected into HPLC.  
150 After extracting the free forms, the extraction procedure was continued to extract the bound  
151 forms. In the tubes containing the residues of the previous extraction, 3.75 mL of ultrapure  
152 water and 1.25 mL of 6 M NaOH were added. Tubes were agitated overnight with the tube  
153 agitator at low speed and ambient temperature with two plastic beads each (2 mm diameter).  
154 The pH was adjusted to 2 +/- 1. The extraction of the soluble-bound phenolic acids was  
155 carried out twice using 3.75 mL of a solution of diethyl ether/ethyl acetate (50:50, V/V).  
156 Tubes were centrifuged at 1,257 g during 10 min at 4°C. Supernatants were collected and  
157 pooled before evaporation under gentle nitrogen flow. The extraction of the insoluble-bound  
158 phenolic acids was carried out as described by Zhang et al. (2012). Briefly, the tubes with the  
159 residues were heated at 85°C and 100 rpm in a water bath with 1.25mL of 6M HCl. After  
160 cooling at ambient temperature, pH was adjusted to 2 and the insoluble-bound phenolic acids  
161 were extracted twice with a solution of diethyl ether/ethyl acetate (50:50, V/V). All samples  
162 were dissolved in 2 mL methanol so as to be injected into HPLC.

163

#### 164 *Quantification of the phenolic acids*

165 The high-performance liquid chromatography system comprised a Dionex separation module  
166 (P680 HPLC Pump and ASI-100 Automated Sample Injector) and a Dionex UVD340U  
167 photodiode array detector (Thermo Scientific Dionex, Sunnyvale USA). Analysis were  
168 performed on an Agilent Zorbax Eclipse XDB-C18 (250 x 4.6 mm, 5 µm) column coupled  
169 with C18 5-µm Zorbax guard column. The solvent A of the mobile phase consisted in water  
170 with 0.05% trifluoroacetic acid. The solvent B consisted in 30% acetonitrile, 10% methanol,  
171 59.95% water and 0.05% trifluoroacetic acid. The flow rate was 1.0 mL/min, the oven was set  
172 at 25°C and the run time was 39 min. The gradient program used was: 12-38% B for 9 min,  
173 38-70% for 7 min, 70-85% for 8 min, 85-12% for 10 min and 12% for 5 min. Between 2 to 10  
174 µL for WB extracts and 20 to 180 µL for larvae extracts were injected in the HPLC apparatus.  
175 Detection was done at 325 nm. The identification and quantification of phenolic acids were  
176 carried out by comparing the retention times and areas with the authentic standards described  
177 in the chemicals paragraph. Standards were used to create calibration curves. Standard FA  
178 solution was prepared at 10 mg/L in ethanol and CA solution at 1 mg/L in ethanol and  
179 injected in different volumes the same day.

180

#### 181 *Bioaccumulation factor*

182 The bioaccumulation factor was calculated as the percentage ratio of the concentration in  
183 fresh weight of each form of FA measured in larvae divided by the concentration of the same  
184 form measured in the fresh weight substrate (WB).

185

### 186 **Estimation of the quantity of WB present in BSFL digestive tract**

#### 187 *BSFL rearing*

188 For the experiment designed to assess the quantity of substrate remaining in the digestive tract  
189 of larvae at the time of slaughter, BSFL were reared as mentioned above. After 8.5 d of  
190 growth, an aliquot of larvae was placed in a growing tray and larvae were reared under  
191 standard BioMiMetiC conditions with a mix of apple (Mesfruits, Cavaillon, France) and WB

192 (30-70% respectively, humidity ratio: 70%) for 4.5 days. After reception, ten larvae were  
193 given WB (humidity ratio: 70%) and ten others were fasted for 1.5 d. They were reared at  
194 26°C, humidity ratio: 70%. After 1.5 days, all larvae were rinsed with tap water to remove  
195 any rest of substrate and weighted. BSFL were then dissected following Bonelli *et al.* (2020)  
196 with slight modifications. Briefly, BSFL were anaesthetized on ice during at least 10 min and  
197 dissected to recover their digestive tract. A 10% PBS solution was prepared to clear the  
198 digestive tract from lipids surrounding it. After wiping dry the digestive tracts, their masses  
199 were determined.

200

#### 201 *Calculation of the theoretical mass of FA present in the digestive tract of the larvae*

202 An estimation of the theoretical quantity of feed (WB) present in BSFL digestive tract when  
203 slaughtered was necessary because the phenolic acid assays were carried out on larvae which  
204 were not fasted. With this number, the theoretical quantity of FA present in BSFL digestive  
205 tract can be estimated. Firstly, the proportion of the digestive tract in fasted and non-fasted  
206 BSFL was calculated by dividing the digestive tract mass by the BSFL mass. Secondly, using  
207 Equation 1, it was possible to calculate the proportion of the mass of the larvae which was due  
208 to WB present in the digestive tract. This percentage was used to calculate the theoretical  
209 mass of FA in the larval digestive tract (Equation 2). Finally, this mass was compared to the  
210 total mass of FA measured in the larvae by calculating the ratio between the theoretical mass  
211 of FA in the digestive tract and the mass of FA measured in the whole larvae. If this ratio was  
212 approximately equal to 1, it meant that the FA measured in the whole larvae corresponded to  
213 that present in the WB from their digestive tract. If the ratio was less than 1, it meant that  
214 there was more FA in the whole larvae than in their digestive tract. However, if the ratio was  
215 greater than 1, it meant that there was less FA in the whole larvae than it should be, based on  
216 the amount of WB present in their digestive tract.

217 Proportion of WB in BSFL = Proportion of gut in non-fasted BSFL – Proportion of gut in  
218 fasted BSFL

219 (Eq 1)

220 Theoretical mass of FA in BSFL gut = Proportion of WB in BSFL × BSFL mass × FA  
221 concentration measured in WB

222 (Eq 2)

### 223 **Statistical analysis**

224 Data are expressed as means ± SEM. Unpaired t-test were carried out to assess the differences  
225 between the means. Previously to those tests, normality of residues was tested using Shapiro-  
226 Wilk tests and homogeneity of variances using Brown-Forsythe tests. In case of  
227 inhomogeneity of variances, the Welch's correction was applied. P-values below 0.05 were  
228 considered significant. All statistical analysis were performed using GraphPad Prism version  
229 10.0.3 for Windows (GraphPad Software, Boston, Massachusetts USA).

## 230 **3. Results**

231

232 The aim of this work was to study the capacity of BSFL to bioaccumulate two phenolic acids  
233 present in WB. Thus, the concentrations of the different forms of two phenolic (FA and CA)  
234 acids in WB and in larvae that were fed WB during 7 days were compared. Furthermore, to  
235 overcome differences in water content of the substrate and larvae, the results are presented  
236 both in fresh and dry matter.

237

238 **Phenolic acid content in WB and larvae**

239 The total FA and CA content in WB and larvae that were fed with WB are summarised in  
 240 **Table 1**. Three main observations can be made. The first is that, in WB, the FA concentration  
 241 is much higher than the one of CA; around 157 times higher in FW. The second is that the  
 242 concentration of FA in larvae is much lower than the one observed in WB (around 50 times  
 243 lower). The third is that CA was not detected in the larvae.

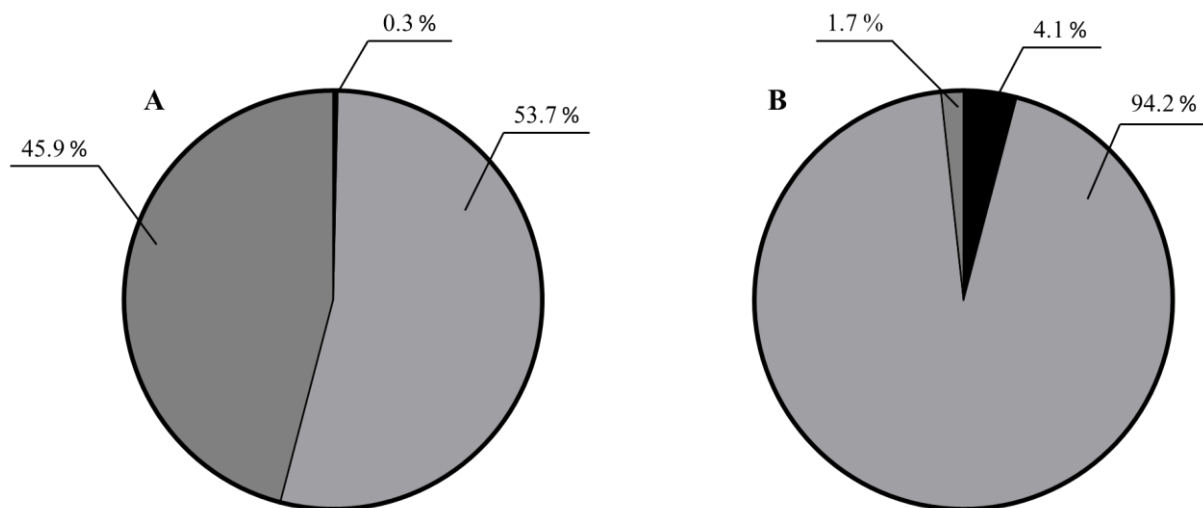
244  
 245 **Table 1. Ferulic acid and caffeic acid content of wheat bran and BSFL reared on wheat**  
 246 **bran.**<sup>1</sup>

	Ferulic acid (mg/kg)		Caffeic acid (mg/kg)	
	FW	DW	FW	DW
<b>Wheat bran</b>	810 ± 38	2 853 ± 135	5 ± 1	18 ± 3
<b>BSFL</b>	17 ± 1	46 ± 2	ND	ND

<sup>1</sup> BSFL: Black Soldier Fly Larvae; FW: Fresh weight; DW: Dry weight; ND: Not detected

248  
 249 **Proportions of the different forms of FA in WB and in larvae**

250 The pie charts in **Figure 1** express the proportions of the different forms of FA in WB and  
 251 larvae. In WB (**1A**), 0.3% FA is present as its free form, 53.7% as its soluble bound form, and  
 252 45.9% as its insoluble-bound form. In larvae (**1B**) the distribution is different with 4.1% FA  
 253 as its free form, 94.2% as its soluble-bound form, and 1.7% as its insoluble-bound form.  
 254 Therefore, the proportion of insoluble-bound form was considerably lower in larvae than in  
 255 WB and conversely the proportions of the soluble-bound and free forms were higher in larvae  
 256 than in WB.



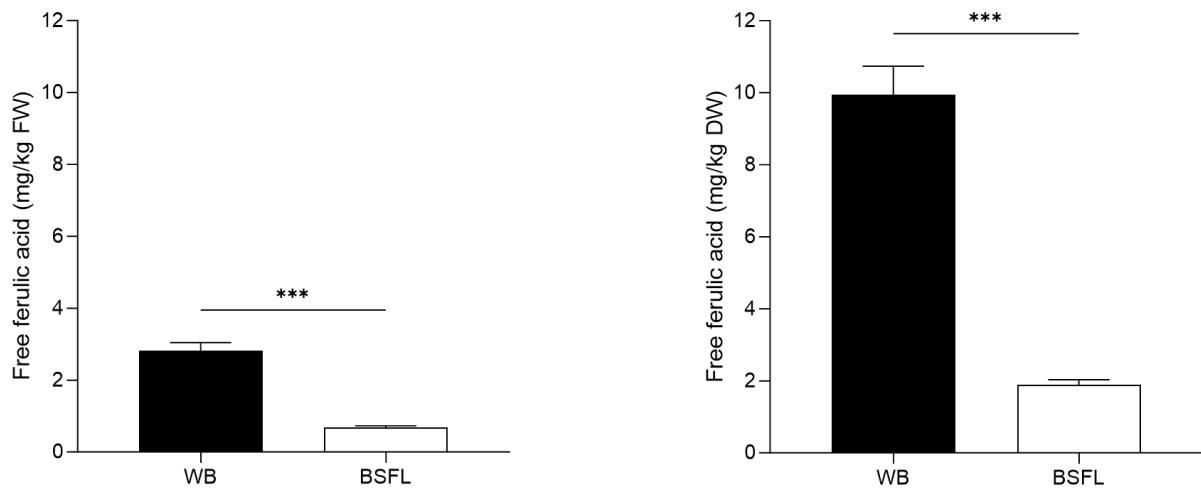
258  
 259 **Figure 1. Distribution (% of mg/kg fresh weight) of the different ferulic acid forms in**  
 260 **wheat bran (A) and wheat-bran fed BSFL (B).**

261 **■ : Free ferulic acid; ■ : Soluble-bound ferulic acid; ■ : Insoluble-bound ferulic acid**

262  
 263 *Free FA concentrations in WB and in larvae*

264 As can be seen in **Figure 2A** the concentrations of free FA measured in the larvae are  
 265 significantly lower than those measured in WB ( $0.68 \pm 0.02$  vs  $2.82 \pm 0.11$  mg/kg fresh

266 weight). In other words, BSFL are about 4 times less rich in free FA than the substrate they  
 267 were reared on. Expressing the results in dry matter does not fundamentally change the results  
 268 (Figure 2B).  
 269

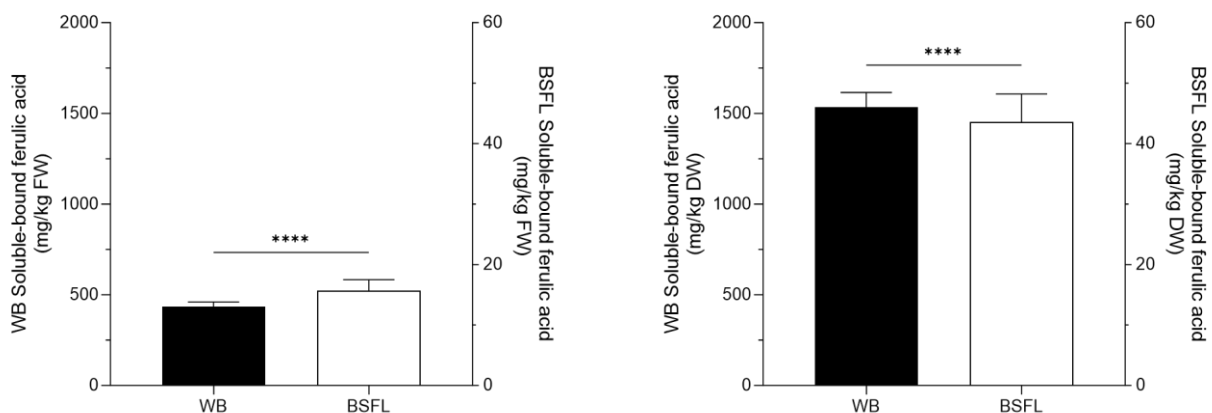


270  
 271 **Figure 2. Free ferulic acid concentrations in wheat bran (black) and wheat-bran fed**  
 272 **BSFL (white), expressed in fresh (A) and dry (B) weight. Bars represent means  $\pm$  SEM**  
 273 **(n=4). Asterisks indicate that the means are significantly different (\*\*\*:  $p < 0.0005$ ;**  
 274 **unpaired t-test with Welch’s correction).**

275  
 276 *Bound FA concentrations in WB and larvae*

277 **Figure 3A** shows that the soluble bound form was found in the larvae, but the difference in  
 278 concentration between the larvae and the substrate is even greater than for the free form.  
 279 Indeed, there was about 28 times less soluble-bound FA in the larvae than in WB ( $15.7 \pm 0.9$   
 280 and  $435 \pm 13$  mg/kg, respectively). The expression of concentrations relative to dry weight  
 281 (**Figure 3B**) increases the difference since there is approximately 35 times less of this form in  
 282 the larvae than in the substrate.

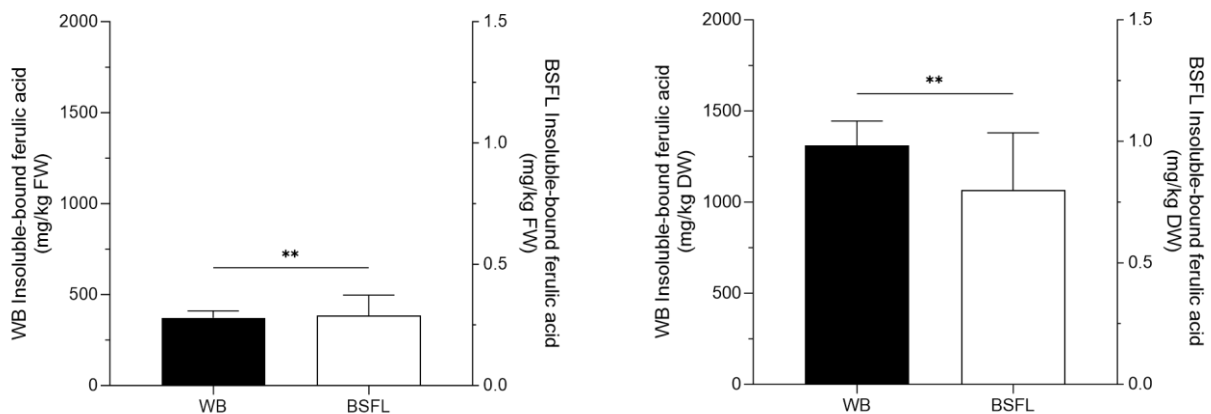
283 Concerning the insoluble bound form, **Figure 4A** shows that the concentration in WB ( $372 \pm$   
 284  $22$  mg/kg) is about 1290 times higher than the concentration in larvae reared on WB ( $0.29 \pm$   
 285  $0.04$  mg/kg). **Figure 4B** shows an even higher difference between the two concentrations,  
 286 indeed, when expressed in dry weight, WB is about 1600 times richer in insoluble bound form  
 287 than the larvae.



288  
 289 **Figure 3. Soluble-bound ferulic acid concentrations in wheat bran (black) and wheat-**  
 290 **bran fed BSFL (white) in fresh (A) and dry weight (B). Bars represent means  $\pm$  SEM**  
 291 **(n=4). The left scale represents the wheat bran concentration while the right scale is for**



292 **BSFL. Asterisks indicate that the means are significantly different (\*\*\*\*: p < 0.0001;**  
 293 **unpaired t-test with Welch's correction).**



294 **Figure 4. Insoluble-bound ferulic acid concentration in wheat bran (black) and wheat-**  
 295 **bran fed BSFL (white) in fresh (A) and dry weight (B). Bars represent means ± SEM**  
 296 **(n=3). The left scale represents the wheat bran concentration while the right scale is for**  
 297 **BSFL. Asterisks indicate that the means are significantly different (\*\*: p < 0.005;**  
 298 **unpaired t-test with Welch's correction).**

300 *Bioaccumulation factors*

302 Overall, the bioaccumulation factor of total FA was 0.0206 (0.0169-0.0252). This factor was  
 303 higher for the free form: 0.2413 (0.1953-0.2755). Concerning the soluble bound form, the  
 304 factor was 0.0360 (0.0294-0.0435) and it was 0.00077 (0.00042-0.00106) for the insoluble  
 305 bound form.

306 *CA concentrations in WB and in larvae*

307 As indicated in the paragraph on the phenolic acid content of WB and larvae, CA was  
 308 detected in WB but not in larvae. Furthermore, no free CA was detected in WB and the  
 309 soluble bound and insoluble bound forms were present in similar concentrations, whether  
 310 expressed in fresh or dry weight (**Table 2**).

311 **Table 2. Concentrations of the different caffeic acid forms measured in wheat bran in**  
 312 **mg/kg.**

	FW	DW
<b>Free caffeic acid (mg/kg)</b>	ND	ND
<b>Soluble-bound caffeic acid (mg/kg)</b>	2.4 ± 0.5	7.9 ± 2.4
<b>Insoluble-bound caffeic acid (mg/kg)</b>	2.8 ± 0.3	9.7 ± 0.9

FW: Fresh weight; DW: Dry weight; ND: Not detected

316 *Theoretical mass of FA in non-fasted BSFL digestive tract*

317 **Table 3** gives the mean BSFL masses, the mean BSFL digestive tract masses, and the  
 318 proportions of digestive tract in fasted and non-fasted BSFL. Fasted larvae were significantly  
 319 lighter than their non-fasted counterparts (111 ± 12 vs 144 ± 8 mg, respectively). The mean  
 320 larval digestive tract mass for fasted larvae was 16 ± 2 mg, while for non-fasted larvae it was  
 321 26 ± 3 mg. A significant difference between the digestive tract masses was also observed; as  
 322

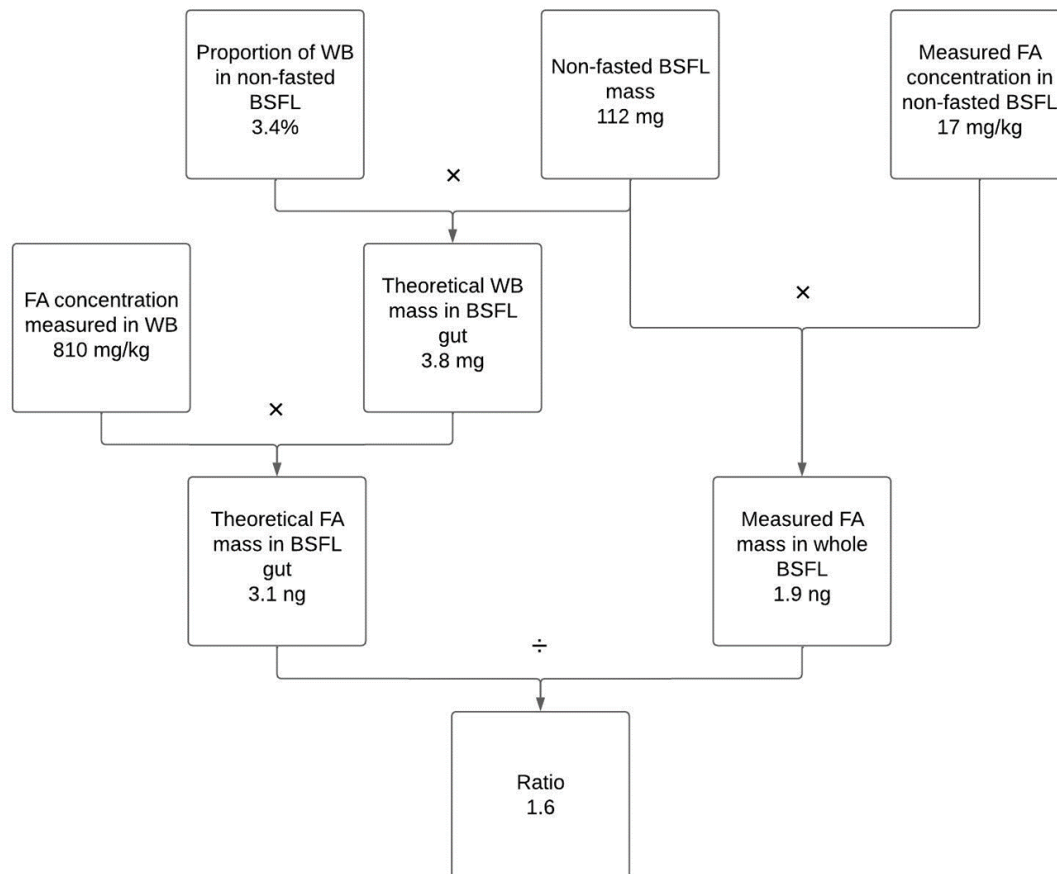
323 expected non-fasted larvae were heavier than fasted one by a factor of 1.7. The digestive tract  
 324 represented  $14.4 \pm 1.2\%$  of the total mass of fasted larvae, whereas it represented  $17.8 \pm 2.3\%$   
 325 of the total mass of non-fasted larvae. Therefore, the proportion of WB present in the  
 326 digestive tract of larvae represented approximately 3.4% of the larval mass. With this  
 327 proportion, it was possible to estimate the mass of WB present in the digestive tract of larvae  
 328 reared on WB, approximately 4 mg per larva (**Figure 5**). The digestive tract of a larva  
 329 contains, in theory, approximately 3 ng of FA, as calculated knowing the concentration of FA  
 330 in the WB. As explained in the materials and methods, the ratio between the FA mass  
 331 theoretically present in the digestive tract of a larva and the FA mass contained in the entire  
 332 larva was made. This ratio was equal to 1.6, meaning that the quantity of FA found in a larva  
 333 was approximately 1.6 times lower than the minimum quantity of FA which should have been  
 334 found in the larva, considering only what was present in its digestive tract.

335 **Table 3. Summary of the results of the dissection experiment: masses (mg) of fasted and**  
 336 **non-fasted BSFL<sup>1</sup> and of their digestive tracts, and proportion of digestive tract in BSFL**  
 337 **(%). Data are expressed as means  $\pm$  SEM (n=10). Means with \* in the same column are**  
 338 **significantly different ( $p < 0.05$ ; unpaired t-test).**

	BSFL mass (mg)	BSFL gut mass (mg)	BSFL gut proportion (%)
<b>Fasted BSFL</b>	$111 \pm 12$	$16 \pm 2$	$14.4 \pm 1.2$
<b>Non-fasted BSFL</b>	$144 \pm 8^*$	$26 \pm 3^*$	$17.8 \pm 2.3$

<sup>1</sup>BSFL: Black Soldier Fly Larvae

340



341  
342 **Figure 5. Comparison of the theoretical and measured quantity of ferulic acid in BSFL.**  
343  
344

#### 345 **4. Discussion**

347 **The WB used in this study contained concentrations and forms of FA and CA**  
348 **consistent with the literature.**

349 The main objective of this study was to determine whether BSFL can bioaccumulate  
350 significant quantities of FA and/or CA. So, the first thing checked was whether the  
351 concentrations and different forms of these molecules in the WB used in our study were  
352 consistent with what had been described in the literature.

353 Firstly FA, is the main phenolic acid in WB. Its concentration in WB,  $0.81 \pm 0.38$  mg/g FW  
354 ( $2.85$  mg/g  $\pm 0.13$  DW), was slightly higher than those given by Boudaoud *et al.* (2021) who  
355 reported concentrations ranging between  $1.36 \pm 0.08$  and  $2.51 \pm 0.21$  mg/g DW. It was also  
356 higher than those reported in Trego WB and in WB from different countries (Zhou and Yu,  
357 2004; Zhou *et al.*, 2004b). Nevertheless, other studies have reported even higher  
358 concentrations than the one found here, ranging between 13.5 and 32.2 mg/g DW (Liyana-  
359 Pathirana and Shahidi, 2006; Verma *et al.*, 2009). Therefore, the FA concentration in our WB  
360 source was consistent with mean concentrations observed in other WB sources.

361 Regarding the distribution of the different forms of FA, it was predominantly found in bound  
362 forms, *i.e.* 99.65%. This agrees with the few articles which have quantified the different forms  
363 of FA and which have observed that the vast majority (>99% ) of FA in wheat was in the  
364 bound forms (Boz, 2015; Li *et al.*, 2008) and confirmed in WB by Boudaoud *et al.* (2021) and  
365 Verma *et al.* (2009). Furthermore, these bound forms were equally distributed between the

366 soluble and insoluble form (53.7% soluble-bound and 45.9% insoluble-bound), which is also  
367 close to what has been described (Verma *et al.*, 2009). Finally, concerning the content of free  
368 FA, our value of  $9.95 \pm 0.4$  mg/kg DW was in agreement with Kim *et al.* (2006) and Verma *et*  
369 *al.* (2009). The proportions of the different forms of FA in WB samples from this study are  
370 also in agreement with the literature.

371 Secondly, CA is also present in WB, although in much smaller quantities than FA. The  
372 concentration found (17.6 mg/kg DW) was again in agreement with previous observations.  
373 Indeed, Verma *et al.* (2009) found concentrations ranging between 6.8 and 29.0 mg/kg DW.  
374 Concerning the bound forms of CA, similar proportions of soluble-bound and insoluble-  
375 bound forms were measured, in opposition with the Verma team who did not detect any CA  
376 from the alkaline hydrolysis fraction, *i.e.* insoluble-bound forms. Finally, the free CA was not  
377 detected. It was also the case for Kim *et al.* (2006) for three out of the four WB tested. On the  
378 contrary, Verma *et al.* (2009) found concentrations ranging between 0.4 and 1.8 mg/kg. It is  
379 likely that these very small concentrations were below the detection limits of our analytical  
380 method and our equipment. In summary, given the extremely low concentrations of CA in  
381 WB, the values given by different laboratories are subject to very strong variations.

382 In brief, the WB used in this study had concentrations of these two phenolic acids  
383 representative of those expected in this substrate. In addition, the proportions of the different  
384 forms of the main compound, *i.e.* FA, were also generally very close to those which have  
385 been described in the literature. It is therefore reasonable to say that our results on the  
386 potential bioaccumulation of these phenolic acids by BSFL would be similar with other WB  
387 samples.

388

### 389 **Not only BSFL do not bioaccumulate FA but they metabolize it.**

390 Regarding the main objective of the study, the concentrations of FA and CA in the larvae *vs*  
391 in the WB were investigated. While it was expected to have approximately equal or higher  
392 concentrations of these phenolic acids in the larvae *vs* the substrate, as had been observed for  
393 other antioxidant phytochemicals, *i.e.* carotenoids and vitamin E (Borel *et al.*, 2021; Morand-  
394 Laffargue *et al.*, 2023a, 2023b, 2023c), very low concentrations of FA in the larvae were  
395 observed and CA was not detected. Indeed, the FA concentration in BSFL represents only 2%  
396 of the FA concentration observed in WB. This proportion was surprisingly small, even though  
397 the bioavailability of FA is extremely low when it is ingested associated with a cereal matrix  
398 (Adam *et al.*, 2002; Mateo Anson *et al.*, 2009). Then, since the larvae were not fasted, the  
399 hypothesis was that the measured FA concentration in BSFL corresponds to the FA present in  
400 the WB remaining in the digestive tract of BSFL. Therefore, another experiment was set up to  
401 assess the quantity of WB remaining in BSFL digestive tract. It was not possible to recover  
402 sufficient quantities of the digestive contents of the larvae to allow adequate quantification of  
403 FA, without being sure that it was not contaminated by intestinal mucosa which could contain  
404 absorbed FA. Then the FA content present in the lumen of the digestive tract, and not  
405 absorbed by the larvae, was estimated by a theoretical calculation, as explained in the material  
406 and method. The digestive tracts of 15.5-days old fasted and non-fasted larvae were  
407 weighted. To the best of our knowledge, this study is the first to provide the average mass of  
408 the digestive tract of BSFL, and to indicate the proportion that it represents in fasted and non-  
409 fasted larvae (**Table 3**), despite the publication of several articles in which the digestive tracts  
410 of BSFL were extracted (Bonelli *et al.*, 2020; Genta *et al.*, 2006). The distribution of BSFL  
411 gut masses in **Table 3** was large and the normalisation by the BSFL mass only reduced a little  
412 bit the variability of the data. It seems then that the gut mass is not entirely mass-dependant  
413 for BSFL. Different levels of WB ingestion between larvae might explain this result. A full  
414 digestive tract represented 18% of BSFL mass. This experimental value is very close to the

415 theoretical value calculated by Mark Finke (in acknowledgments) using the vitamin A gut-  
416 loading method published by Boykin and Mitchell, (2021) of 20.2%.  
417 Our theoretical calculation showed that the digestive tract of non-fasted larvae could contain a  
418 sufficient quantity of WB to provide the quantity of FA which was found in the whole non-  
419 fasted larvae. This supports our hypothesis that FA is not absorbed by the BSFL.  
420 The metabolism of FA, and likely CA, in the larvae is supported by the modification of the  
421 proportions of the different forms of this phenolic acid in the larvae in comparison with the  
422 substrate. More precisely, the proportion of free FA increases from 0.35% in WB to 4.1% in  
423 the larvae, the proportion of soluble-bound FA increases from 53.7% in WB to 94% in the  
424 larvae and the proportion of insoluble-bound FA decreases from 45.9% in WB to 1.7% in the  
425 larvae. It therefore appears that, in the larvae, the insoluble-bound forms were more  
426 metabolized than the soluble-bound and free forms. There are obviously degradation  
427 metabolites which must have been produced from these different forms because the sum of  
428 these different forms represents only 2% of the quantity of FA present in WB and this  
429 hypothesis is supported by the observation that some phenolics are oxidised in insects (Appel,  
430 1993). Further studies investigating the degradation products of FA by BSFL are required.  
431 Obviously, this conclusion is valid for the parent molecule, but the hypothesis that FA  
432 degradation metabolites have bioaccumulated in the larvae cannot be ruled out. Only a study  
433 with FA labelled with an isotope could allow us to reject this second hypothesis.  
434 Concerning CA, there are two possible explanations for its absence of detection in larvae. The  
435 first is that its concentration in the larvae was below the detection limit of our device. Indeed,  
436 its concentration in WB was approximately 160 times lower than that of FA. And, if the CA  
437 bioaccumulation factor is close to that of FA, which is plausible given that their chemical  
438 formula differs only from one methyl group, 160 times less CA than FA should be present in  
439 the larvae, *i.e.* around 0.03 mg/kg FW. The second hypothesis is that, like FA, it was  
440 metabolized by larvae, since these molecules are chemically close. These results open the  
441 question of the potential degradation of other polyphenols by BSFL, further work is thus  
442 required to investigate this.  
443 At this point of the discussion, the question that comes into mind is ‘why BSFL metabolize  
444 FA?’. Hydroxycinnamic acids, such as FA and CA, are molecules generally generated by  
445 plants to protect them from insects (Niveyro *et al.*, 2023; Rani and Devanand, 2013). This  
446 induces that those molecules are potentially toxic for BSFL as for other insects. In particular,  
447 FA in wheat was shown to contribute to plant defence against *Sitobion avenae* (Feng *et al.*,  
448 2021). Concerning other edible insect than BSFL, only *Acheta Domesticus* phenolic  
449 compounds were mapped (Nino *et al.*, 2021b) and suggest similar metabolization. Indeed,  
450 both CA and FA were present but in lower concentrations than in the diet. Additional research  
451 is then required on other edible insects such as *Tenebrio Molitor*, to extrapolate those results.  
452 However, contrary to other studies (Felton *et al.*, 1989; Yang *et al.*, 2017), no larval growth  
453 issues were observed in this study. This enhances the hypothesis that natural selection of  
454 BSFL has established a mechanism for eliminating these molecules in order to overcome their  
455 toxicity. The hypothesis is that the metabolism is carried out either by the microbiota present  
456 in the digestive tract of the larva (Cheng *et al.*, 2018; Genta *et al.*, 2006; Jiang *et al.*, 2021) or  
457 by digestive enzymes secreted by the larva in its digestive tract, or located on the apical  
458 membrane of the intestinal cells of the larva. Detoxification enzymes, such as glutathione S-  
459 transferases, carboxylesterases, cytochrome P450s (Yang *et al.*, 2017), and the insect  
460 prophenoloxidase (Jiang *et al.*, 2021; Wu *et al.*, 2015), were described to metabolize phenolic  
461 compounds into less toxic metabolites. This hypothesis is the more likely one, considering the  
462 literature on FA. However, only dedicated studies to BSFL will be able to decide between  
463 these different hypotheses.

## 465 5. Conclusion

466 This study shows that BSFL do not bioaccumulate significant concentrations of FA and CA  
 467 from WB. On the contrary, our results suggest that FA, and likely CA, are metabolized in the  
 468 digestive tract of the larvae. Our data do not allow us to investigate if the larva carries out this  
 469 metabolism *via* its microbiota, its digestive enzymes or a combination of them. Nevertheless,  
 470 the fact that this metabolism is so significant suggests that BSFL would benefit from it. FA,  
 471 among other phenolic acids is recognized to contribute to the defence mechanisms of plants  
 472 against herbivores. It is thus very likely that FA is toxic for BSFL and that they have  
 473 developed a way to degrade it.

474 The consequences of these results are twofold. First of all, concerning the breeding of BSFL,  
 475 if the hypothesis that FA is toxic for the larvae is true, substrates very rich in FA could exceed  
 476 the elimination capacities of this phenolic acid by the larvae and therefore affect their growth.  
 477 This nevertheless remains to be verified with a dedicated study linking the growth rate of the  
 478 larvae to the concentration of FA in the substrate. The second consequence of our  
 479 observations is that, contrary to what has been shown for other phytochemicals such as  
 480 carotenoids and vitamin E, BSFL cannot be enriched in these phenolic acids and we cannot  
 481 hope to use them as a significant source of these bioactives for farm animals and, indirectly,  
 482 for humans.

483

### 484 Supporting information:

485 The costs of this project were covered by INRAE Metaprogramme Bioeconomy for urban  
 486 areas (BETTER) and by the BioMiMetiC company.

487

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 489 has been applied by the authors to the present document and will be applied to all subsequent versions up to the Author Accepted  
 490 Manuscript arising from this submission.

491



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493

## 494 Acknowledgements

495 The authors thank Dr Mark D. Finke (Domestic LLC, Arizona, US) for his help with  
 496 theoretical calculations on the proportion of digestive tract in BSFL.

497

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