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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 ± 1 mg/kg fresh weight) represented only 2% of the total FA concentration measured in WB (810 ± 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)

1. Introduction

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

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

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

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

2. Material and methods

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

Chemicals

Solvents, *i.e.* ethanol, methanol, diethyl ether, ethyl acetate and acetonitrile, as well as hydroxide chlorure and trifluoro acetic acid were purchased from Carlo Erba (Val de Reuil, France). Sodium hydroxide pellets (purity: 97%) were purchased from Prolabo (Paris, France). Phenolic acid standards, *i.e.* FA and CA (purity > 98.0%; HPLC grade) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Three salts used for the PBS solution; NaCl, KCl, and KH_2PO_4 were also from Sigma-Aldrich and $Na_2HPO_4.12H_20$ was ordered from Honeywell (Charlotte, North Carolina, USA).

Phenolic acid quantification in WB and BSFL fed with WB

BSFL rearing

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

Phenolic acid extraction

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

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

Quantification of the phenolic acids

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

Bioaccumulation factor

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

Estimation of the quantity of WB present in BSFL digestive tract

BSFL rearing

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

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

Calculation of the theoretical mass of FA present in the digestive tract of the larvae

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

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

(Eq 1)

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

(Eq 2)

Statistical analysis

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

3. Results

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

Phenolic acid content in WB and larvae

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

Table 1. Ferulic acid and caffeic acid content of wheat bran and BSFL reared on wheat bran.[1]

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

[1] BSFL: Black Soldier Fly Larvae; FW: Fresh weight; DW: Dry weight; ND: Not detected

Proportions of the different forms of FA in WB and in larvae

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

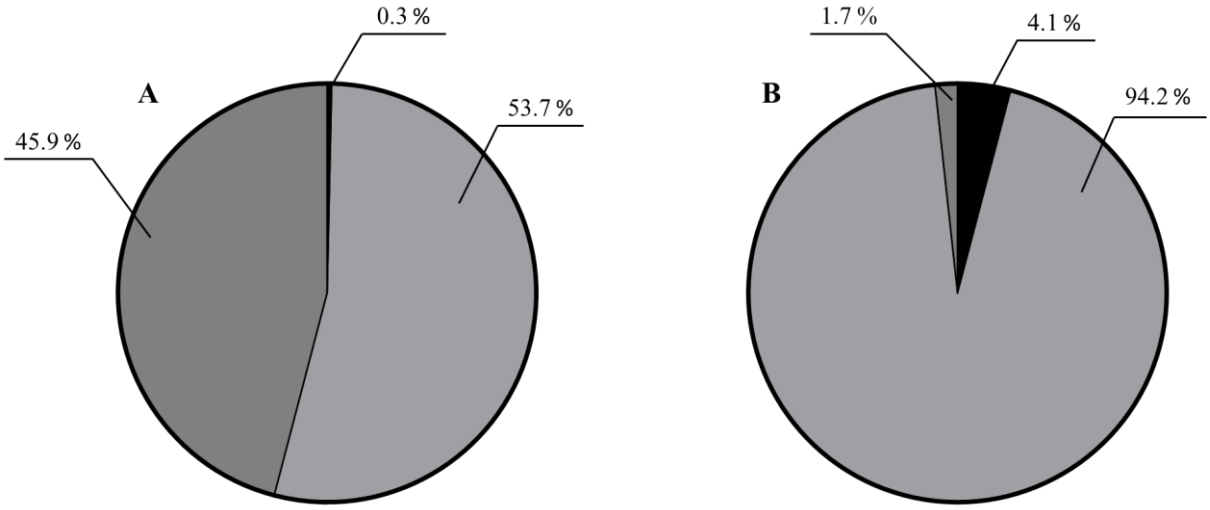

Figure 1. Distribution (% of mg/kg fresh weight) of the different ferulic acid forms in wheat bran (A) and wheat-bran fed BSFL (B).

: Free ferulic acid; : Soluble-bound ferulic acid; : Insoluble-bound ferulic acid

Free FA concentrations in WB and in larvae

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

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

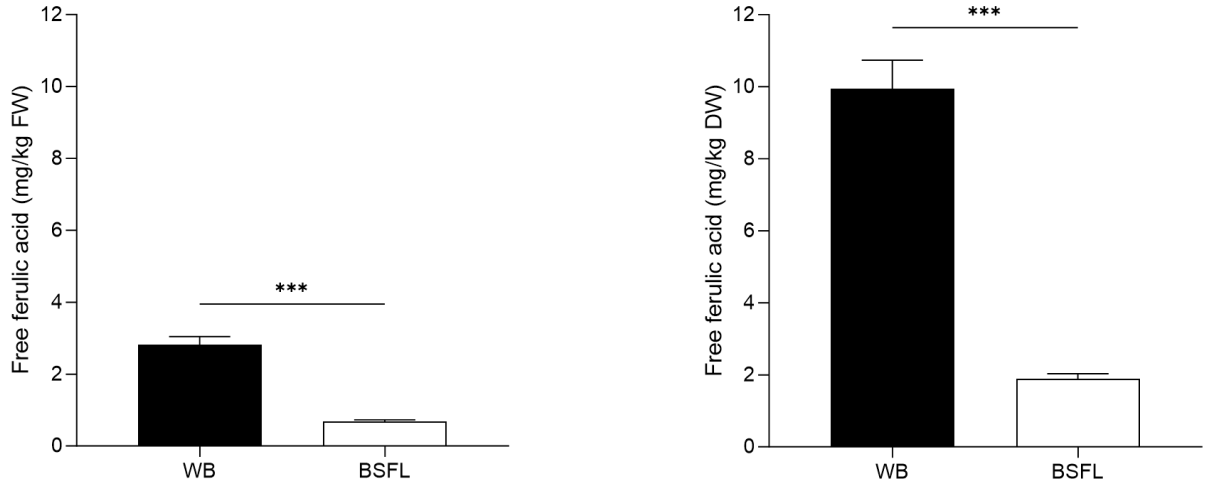

Figure 2. Free ferulic acid concentrations in wheat bran (black) and wheat-bran fed BSFL (white), expressed in fresh (A) and dry (B) weight. Bars represent means ± SEM (n=4). Asterisks indicate that the means are significantly different (*: p < 0.0005; unpaired t-test with Welch's correction).**

Bound FA concentrations in WB and larvae

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

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

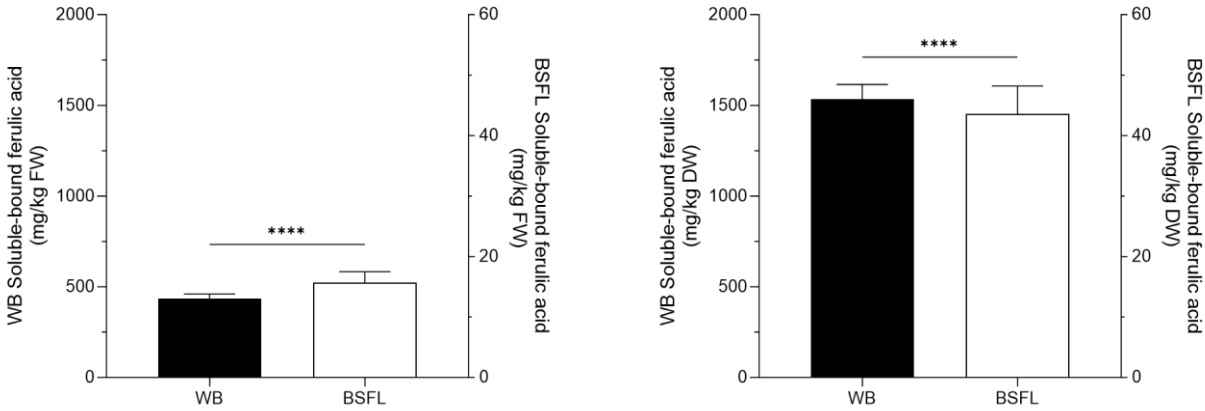

Figure 3. Soluble-bound ferulic acid concentrations in wheat bran (black) and wheat-bran fed BSFL (white) in fresh (A) and dry weight (B). Bars represent means ± SEM (n=4). The left scale represents the wheat bran concentration while the right scale is for

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

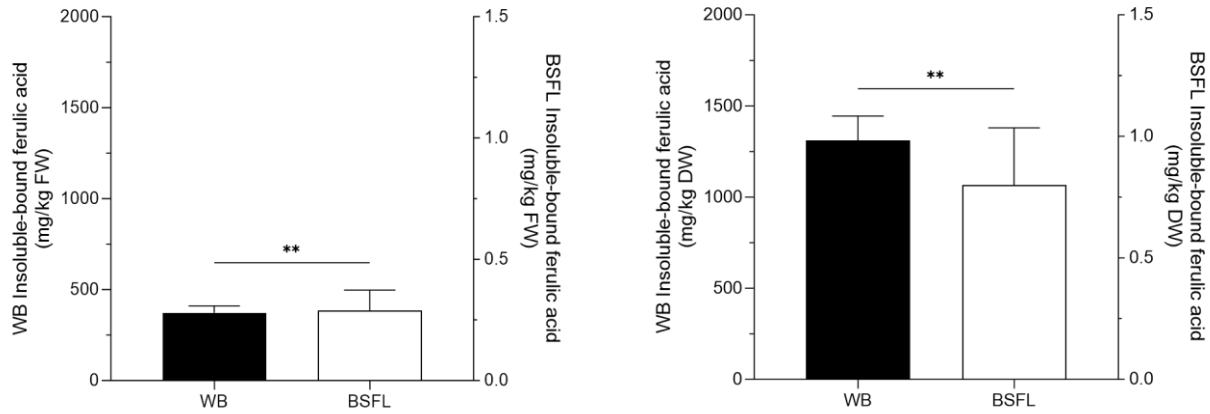

Figure 4. Insoluble-bound ferulic acid concentration in wheat bran (black) and wheat-bran fed BSFL (white) in fresh (A) and dry weight (B). Bars represent means ± SEM (n=3). The left scale represents the wheat bran concentration while the right scale is for BSFL. Asterisks indicate that the means are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

Bioaccumulation factors

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

CA concentrations in WB and in larvae

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

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

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

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

Theoretical mass of FA in non-fasted BSFL digestive tract

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

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

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

	BSFL mass (mg)	BSFL gut mass (mg)	BSFL gut proportion (%)
Fasted BSFL	111 ± 12	16 ± 2	14.4 ± 1.2
Non-fasted BSFL	144 ± 8*	26 ± 3*	17.8 ± 2.3

[1]BSFL: Black Soldier Fly Larvae

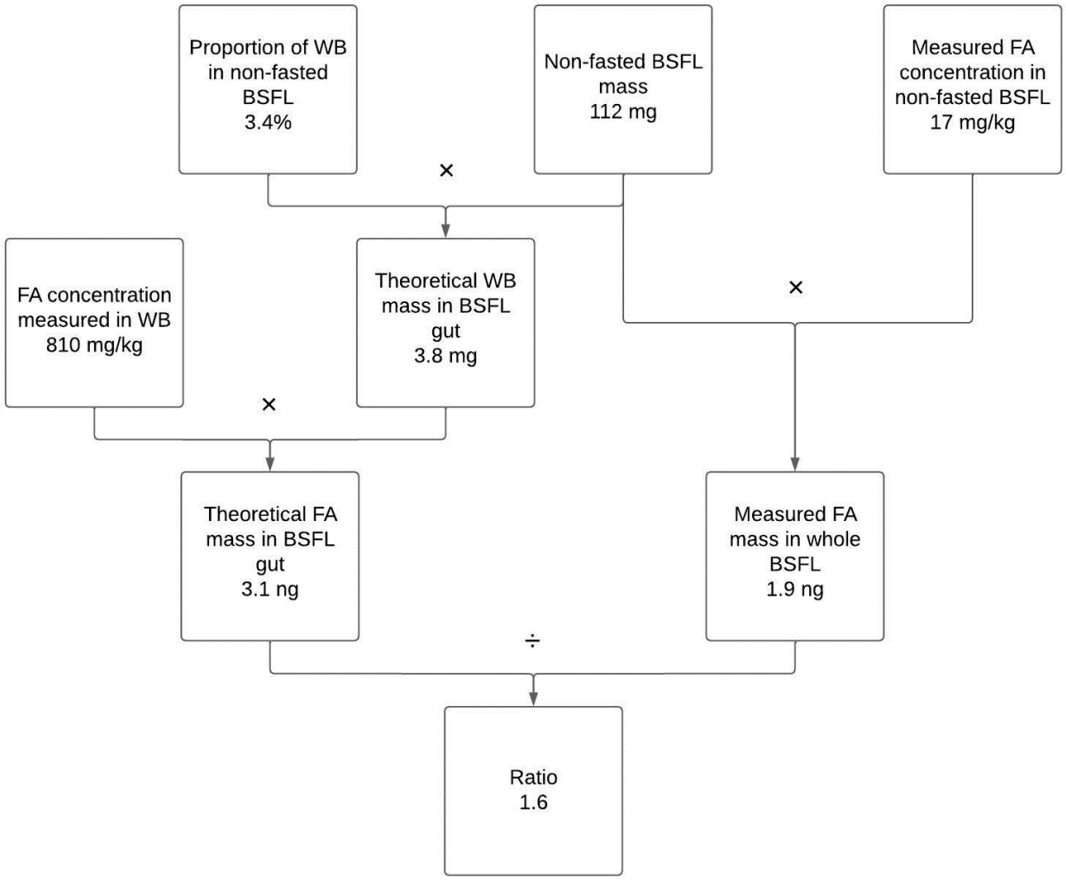

Figure 5. Comparison of the theoretical and measured quantity of ferulic acid in BSFL.

4. Discussion

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

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

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

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

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

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

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

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

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

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

5. Conclusion

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

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

Supporting information:

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