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## Ability of black soldier fly larvae to bioaccumulate tocopherols from different substrates and measurement of larval tocopherol bioavailability in vitro

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1 Research article

2

3 **Ability of black soldier fly larvae to bioaccumulate**  
4 **tocopherols from different substrates and measurement of**  
5 **larval tocopherol bioavailability *in vitro***

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21

22 **Abstract**

23

24 Edible insects are an emerging approach to provide sustainable proteins in feed. Black  
25 soldier fly larvae (BSFL) can also bioaccumulate micronutrients from various substrates. The  
26 purpose of this study was to assess whether BSFL can bioaccumulate significant  
27 concentrations of bioavailable  $\alpha$  and  $\gamma$ -tocopherol (TOC) from vitamin E (VE) rich substrates.  
28 BSFL were reared on VE rich substrates, e.g. wheat germ oil, bran, etc.  $\alpha$  and  $\gamma$ -tocopherol  
29 were quantified in larvae and substrates by HPLC. VE bioaccessibility was estimated using an  
30 *in vitro* model of digestion. Uptake efficiency of micellarized VE by intestinal cell was  
31 estimated using Caco-2 cells. BSFL were at least as rich in  $\alpha$ -TOC, but not  $\gamma$ -TOC, as the  
32 substrates they were reared on BSFL. VE bioaccessibility was almost always significantly  
33 lower in BSFL than in corresponding substrates. Conversely, VE uptake efficiency was either  
34 not significantly different or significantly higher in BSFLs than in substrates. Thus, VE  
35 enrichment of BSFL from VE rich substrates, in particular co-products such as brans and oil  
36 cakes, could be an innovative way to recycle VE and to provide significant amounts of  
37 sustainable VE in farm animal feed.

38

39 **Keywords:** tocopherol; insect; bioaccessibility; co-products.

40

41 **Conflicts of interest:** BC and DS work in the BioMiMetiC company. This company conducts  
42 research and development activities aimed at enhancing the value of entomo-conversion on a  
43 wide variety of organic materials generated in the area at all levels of the food value chain. All  
44 other authors have no conflict of interest to declare.

45

46 **Abbreviation:**

- 47 BSFL (black soldier fly larvae).
- 48 TOC (tocopherol)
- 49 VE (vitamin E)

## 50 1) Introduction

51 Vitamin E (VE) is a vital micronutrient that plays an important role in the health and  
52 well-being of both humans and animals. It is a powerful antioxidant that helps to protect cells  
53 from damages caused by free radicals and oxidative stress. In addition, it is important for  
54 immune function, vision, reproduction, and skin health. In human it is supposed to play a  
55 preventive role with respect to cardiovascular risk, immunomodulation, neuroprotection and  
56 hepatoprotection (Fata *et al.*, 2014; Galli *et al.*, 2017; Gupta and Suh, 2016; Sozen *et al.*,  
57 2019). When it comes to animal nutrition, it is particularly important for livestock, poultry,  
58 and aquaculture. In livestock, VE supplementation can improve fertility, reduce the incidence  
59 of mastitis in dairy cows (Politis, 2012), and enhance the immune response (Pinelli-Saavedra,  
60 2003). It can also improve meat quality by reducing lipid oxidation (Monahan *et al.*, 1992;  
61 Rey *et al.*, 2001) which can result in a longer shelf life and improved flavor. In poultry, it is  
62 important for immune function, reproductive performance (Surai, 1999), and egg quality  
63 (Cherian *et al.*, 1996). It is particularly important in aquaculture systems where fish and  
64 shrimp are fed diets high in polyunsaturated fatty acids, which are prone to oxidation. In  
65 addition to its benefits for animal health, VE is also important for food production. It is a key  
66 ingredient in many food products, including baked goods, cereals, and oils. VE is often added  
67 to these products as a natural preservative, as it helps prevent oxidation and spoilage. Overall,  
68 the interest of VE for food and feed is clear. It plays a vital role in both human and animals  
69 and it has additional interests in food and feed by promoting animal health and productivity  
70 and by improving food quality.

71 VE is a fat-soluble vitamin gathering two families which are the tocopherols (TOCs)  
72 ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) and the tocotrienols ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  as well). Nevertheless, the main forms of VE  
73 present in our diet are  $\alpha$ - and  $\gamma$ -TOCs. The first is the most consumed in Europe while the  
74 second is the most consumed in the US (Wagner *et al.*, 2004). One of the primary differences  
75 between these two TOCs is their ability to scavenge free radicals. While  $\alpha$ -TOC is considered  
76 to be the most potent lipid antioxidant in our diet and in our body (Niki, 2014),  $\gamma$ -TOC has  
77 been shown to have anti-inflammatory effects that are not observed with  $\alpha$ -TOC (Jiang *et al.*,  
78 2000; Jiang, 2014). Therefore, each TOC could be of interest depending on the biological  
79 effect targeted.

80  $\alpha$  and  $\gamma$ -TOCs are mainly found in plant foods containing high concentrations of  
81 unsaturated fatty acids, such as nuts, oilseeds and other common edible oils (de Camargo *et al.*  
82 *et al.*, 2019; Shahidi *et al.*, 2021). They are also abundant in the by-products of these plant  
83 foods, e.g. in brans and oil cakes. A completely innovative way of adding VE to farm animal  
84 feed could be to provide it via edible insects containing significant amounts of this vitamin.  
85 An advantage of this new way of providing this vitamin would be to provide a more  
86 sustainable form of this vitamin and to be able to recycle in the food chain VE that would be  
87 lost in waste or co-products rich in this vitamin and little or not used. Among the edible  
88 insects used in animal feed, the black soldier fly (BSF, *Hermetia Illucens*) is of particular  
89 interest. It has the ability to grow on a wide variety of organic substrates and it has been  
90 shown that it can bioaccumulate high concentrations of another fat-soluble vitamin, i.e.  
91 provitamin A (Borel *et al.*, 2021). It is therefore a perfect candidate for testing the possibility  
92 of having a larva enriched in this vitamin by cultivating it on substrates rich in this vitamin  
93 but which can be very different, e.g. vegetable oils, bran and oil cakes.

94 Although it has already been shown that BSF larvae (BSFL) contain  $\alpha$ -TOC (Finke,  
95 2013; Liu *et al.*, 2017; Shumo *et al.*, 2019), and can be enriched with  $\alpha$ -TOC if it is added to  
96 their diet (Liland *et al.*, 2017; Oonincx and Finke, 2021), it is not known whether there is an  
97 effect of the substrate matrix in which VE is provided to the larvae on the efficiency of the  
98 larvae to bioaccumulate this vitamin. It is also not known if the larvae have the same capacity

99 to bioaccumulate  $\alpha$  and  $\gamma$ -TOC and if these two forms of VE are bioavailable when they are  
100 incorporated into the insect matrix. To answer these many questions, we first raised different  
101 groups of BSFL on substrates rich in  $\alpha$  and/or  $\gamma$ -TOC. Then, the concentrations of these two  
102 forms of VE were measured and compared in the larvae and in substrates they were reared on.  
103 Finally, by using an *in-vitro* digestion model coupled with Caco-2 cell monolayers, we  
104 compared the bioaccessibility and intestinal cell uptake efficiency of these two forms of VE  
105 whether they came from the larval matrix or from the rearing substrate matrix.

## 106 **2) Material and methods**

### 107 *Chemicals*

108 Ethanol, *n*-hexane and methanol were purchased from Carlo Erba reagents (Peypin,  
109 France). The TOC standards used to identify and quantify TOCs by HPLC, i.e. DL-all-rac- $\alpha$ -  
110 TOC and (RRR)- $\gamma$ -TOC (HPLC purity  $\geq 96\%$ ), the internal standard used to calculate  
111 extraction yield, i.e. retinyl acetate, and the enzymes and the chemicals used for the *in vitro*  
112 digestions were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). For cell culture,  
113 Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, trypsin-  
114 ethylenediaminetetraacetic acid (EDTA), non-essential amino acids, penicillin/streptomycin,  
115 and phosphate-buffered saline (PBS) were purchased from Life Technologies (Villebon-sur-  
116 Yvette, France). Finally, fetal bovine serum (FBS) was from PAA (Vélizy-Villacoublay,  
117 France).

### 118 *Experimental food*

119 We first chose a series of substrates among the richest in  $\alpha$  and/or  $\gamma$ -TOC according to  
120 food tables and data from the literature (**Table 1**). Then we chose co-products rich in these  
121 two forms of VE because we had put forward the hypothesis that these co-products could be  
122 valorized via their VE supply for the larvae. This led us to work on a total of 10 vitamin E-  
123 rich substrates, and more specifically on two main types of different substrates, oils, e.g. corn  
124 oil, and solid substrates, e.g. rice bran. Some of these substrates, wheat germ oil (Emile Noel  
125 brand, France), corn oil (La Tourangelle brand, France), rice bran oil (Saldac brand, Italy),  
126 rice bran (Moulin Des Moines brand, France) and wheat bran (Priméal brand, France) were  
127 ordered via the internet. The brewer's spent grains were a kind gift from a local brewery  
128 (Brasserie de la Plaine, Marseille, France). The turnip rapeseed and corn oil cakes came from  
129 a local producer (Moulin Giraud, Ozan, France). Poultry feed (broiler grower, Evialis,  
130 Vedène, France) was provided by BioMiMetiC that used it to rear their BSFL and thus which  
131 was considered as a positive control. The other rearing substrates, i.e. rapeseed oil and white  
132 mushrooms (which were used as a negative control because they do not contain detectable  
133 amount of  $\alpha$  and  $\gamma$ -TOC), as well as foods used in the *in vitro* digestion experiments, i.e.  
134 potatoes, minced beef meat (with 5% of fat) and olive oil, were bought from local  
135 supermarkets.  
136

### 137 *BSFL farming*

138 As mentioned above, the BSFL were reared on either oils or solid substrates. As the  
139 larvae could not be reared in oil we previously mixed the studied vegetable oils with hydrated  
140 standard poultry feed to reach 10% of the total mass. For the by-products, only brewer's spent  
141 grains did not need to be hydrated with water. The other substrates were hydrated so that the  
142 relative humidity ranged from 60 to 70%, the ideal relative humidity for BSFL growth (Dzepe  
143 *et al.*, 2020). The rearing procedure was carried out at the BioMiMetiC company in Boulbon  
144 (France) as was described in detail in one of our previous studies (Borel *et al.*, 2021).  
145 Following a 5-day fasting period, the larvae were collected and stored at  $-80\text{ }^{\circ}\text{C}$ .  
146

147

148 *Assessment of the bioaccessibility of VE in rearing substrates and BSFL*

149 Before the experiment, 2 g of substrate or larva sample was ground with liquid  
150 nitrogen in a mortar grinder (Pulverisette 2, FRITSCH GmbH, Idar-Oberstein, Germany). The  
151 bioaccessibility of  $\alpha$  and  $\gamma$ -TOC was measured using an *in vitro* digestion model, as was  
152 described in detail in one of our previous studies (Borel et al., 2021). Briefly, samples were  
153 mixed with 6.7 g mashed potato, 1.2 g minced meat and 200 mg olive oil. The mixture was  
154 homogenized for 10 min at 37 °C in a rotary incubator (190 rpm) before adding 2.5 mL of  
155 artificial saliva and the mixture was once again incubated 10 min at 37 °C. After adjusting the  
156 pH to  $4 \pm 0.02$ , 2 mL pepsin were added to the mixture before incubation during 30 min.  
157 Then, pH was adjusted to  $6 \pm 0.02$  prior to the addition of 9 mL pancreatin-bile extract  
158 solution and 4 mL bile solution. The mixture was then incubated for 30 minutes and the  
159 digestate aliquots were collected. The remaining mixture was centrifuged at  $1,860 \times g$  for 1 h  
160 12 min at 10 °C. Aliquots of filtered supernatant, containing the mixed micelles were stored at  
161 80 °C until lipid extraction and quantification of vitamin E by HPLC.

162  
163 *Cell culture of Caco-2 and method for measuring the efficiency of uptake of VE by these cells*

164 The cell culture protocol, as well as the cytotoxicity assay were the same as previously  
165 described (Morand-Laffargue *et al.*, 2023). Regarding the measurement of the uptake  
166 efficiency of VE from the micellar fraction by Caco-2 clone TC7 cells, 1.5 mL of the micellar  
167 fractions obtained at the end of the *in vitro* digestion, previously diluted 5 times with DMEM  
168 to avoid the toxicity of bile salts towards the cells, were added on the cells. Solutions  
169 containing micellarized VE were incubated for 4 hours (Liu *et al.*, 2004) at 37 °C.

170  
171 *VE extraction*

172 For the rearing substrates and the BSFL, 250 mg of samples finely ground with liquid  
173 nitrogen in a mortar grinder (Pulverisette 2, FRITSCH GmbH, Idar-Oberstein, Germany)  
174 were first homogenized in 250  $\mu$ L of distilled water and 500  $\mu$ L of an ethanol solution  
175 containing retinyl acetate (as internal standard) at a concentration of 0.4 mg/L. In the case of  
176 *in vitro* digestion samples, i.e. digestates and mixed micelles, and for the recovered medium  
177 and scrapped cells, 500  $\mu$ L were added to 500  $\mu$ L of the same internal standard solution. Then  
178 a double extraction with hexane was performed as described previously (Borel *et al.*, 2021).  
179 Finally, the dry film was resolubilized in 200 to 2000  $\mu$ L of methanol, based on the theoretical  
180 VE concentration of the sample.

181  
182 *VE quantification*

183 The resolubilized extracts rich in VE were injected, the same day of their extraction,  
184 into the HPLC system. After an initial injection, injection volumes were adjusted to obtain  
185 signals within the calibration range. Injection volumes of 50 to 100  $\mu$ L for BSFL and rearing  
186 substrate samples, 25 to 50  $\mu$ L for digestate samples, 50 to 100  $\mu$ L for micellar fractions and  
187 180  $\mu$ L for recovered apical cell medium as well as scrapped cells were injected into the  
188 HPLC system. All the analyses were performed using a  $250 \times 4.6$  nm ZORBAX Eclipse  
189 XDB-C18 5- $\mu$ m column and the corresponding analytical guard column (Agilent  
190 Technologies, Santa Clara, CA, USA). The column was maintained at a constant temperature  
191 (40 °C). VE analysis was conducted with a 100% methanol mobile phase and flow rate was  
192 1.5 mL/min.

193 The HPLC system included an UltiMate 3000 system (Thermo Fisher Scientific, San  
194 Jose, CA, USA) and a fluorimetric detector. For fluorimetric analysis of TOCs, detection was  
195 set at 292 nm (excitation) and 325 nm (emission). Quantification was performed using  
196 Chromeleon software comparing peak area with the curves of the TOC standards.

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*Calculations*

VE bioaccessibility and uptake efficiency were calculated as:

$$\text{Bioaccessibility (\%)}: \frac{\text{amount of } \alpha \text{ or } \gamma \text{ TOC recovered in mixed micelles}}{\text{amount of } \alpha \text{ or } \gamma \text{ TOC recovered from the digesta}}$$

$$\text{Uptake efficiency (\%)}: \frac{\text{amount of } \alpha \text{ or } \gamma \text{ TOC recovered in cells}}{(\text{amount of } \alpha \text{ or } \gamma \text{ TOC recovered in cells}) + (\text{amount of } \alpha \text{ or } \gamma \text{ TOC in apical medium})}$$

*Statistics*

Results are expressed as means  $\pm$  SD or SEM. The homogeneity of variances ( $p > 0.05$ ) was assessed by the Levene’s test. In the case of variance heterogeneity, the data were log-transformed. Departures from normality were assessed using Q–Q plots of standardized residuals. Differences between groups were tested using several two-way ANOVA. When a significant effect of either the substrate matrix or the insect matrix was found by ANOVA, the comparison of means between the two matrices was carried out by pairwise comparisons using bilateral t-tests, with a Bonferroni adjustment. To compare the bioaccessibility of VE among the substrate groups or among the larval groups and to compare the VE amount in the micelles of larvae reared on the different substrates, one-way ANOVA were performed. Tukey-Kramer’s test was used as a post hoc test for pairwise comparisons. Pearson's correlation coefficients were calculated with 95% confidence intervals (CI) to assess possible correlations between certain variables. Values of  $p < 0.05$  were considered significant. All statistical analyses were performed using R version 4.1.1 for Windows (R Core Team, 2021). The different ANOVA were performed using the “anova\_test” function in the rstatix package v.0.7.2.

**Table 1:** Amount of  $\alpha$  and  $\gamma$ -TOC (mg/kg) in the rearing substrates selected in the study.

	$\alpha$ -TOC	$\gamma$ -TOC	Source
Rapeseed oil	181.0	383.5	(Wen <i>et al.</i> , 2020)
	189 – 240	370 – 510	(de Camargo <i>et al.</i> , 2019)
	202.9 $\pm$ 38.5	131.6 $\pm$ 24.2	Measured in this study
Corn oil	180 – 257	440 – 752	(de Camargo <i>et al.</i> , 2019)
	183.9	623.9	(Wen <i>et al.</i> , 2020)
	208.9 $\pm$ 27.0	339.9 $\pm$ 25.6	Measured in this study
Rice bran oil	151.8	64.4	(Wen <i>et al.</i> , 2020)
	7.3 – 159	2.6 – 80	(de Camargo <i>et al.</i> , 2019)
	91.2 $\pm$ 4.1	24.9 $\pm$ 2.7	Measured in this study
Wheat germ oil	1510 – 1920	traces – 523	(de Camargo <i>et al.</i> , 2019)
	1622.4	912.6	(Kumar and Krishna, 2015)
	1166.6 $\pm$ 343.0	152.9 $\pm$ 49.2	Measured in this study
Brewer’s spent grain (BSG)	8.8 – 11.0*	1.4 – 4.6*	(Badea <i>et al.</i> , 2018)
	1.7	3.6	(Bouillon, 2016)
	0.6 $\pm$ 0.05	0.2 $\pm$ 0.05	Measured in this study
Corn oil cake	12.9 $\pm$ 0.7	25.4 $\pm$ 1.5	Measured in this study
Poultry feed	7.0 $\pm$ 2.8	6.9 $\pm$ 1.3	Measured in this study
Rice bran	7 – 55*	4 – 23*	(Górnaś <i>et al.</i> , 2016)
	26.4 – 35.0*	20.9 – 41.0*	(Aguilar-Garcia <i>et al.</i> , 2007)
	27.4 $\pm$ 7.9	1.1 $\pm$ 0.2	Measured in this study
Turnip rapeseed cake	6.6 $\pm$ 0.3	12.6 $\pm$ 2.0	Measured in this study

	6 – 31*	0 – 1*	(Górnaś <i>et al.</i> , 2016)
Wheat bran	1.3 – 21.3*	0.9 – 6.9*	(Zhou <i>et al.</i> , 2004)
	n.d.	0.1 ± 0.02	Measured in this study
	0.75 ± 0.04	n.d.	(Barros <i>et al.</i> , 2008)
Mushrooms	n.d.*	n.d.*	(Glamočlija <i>et al.</i> , 2015)
	n.d.	n.d.	Measured in this study

222 Data are means ± SD. n.d.: not detected.

223 Data are expressed in fresh weight except when there is an asterisk which means that they are  
224 expressed in dry weight.

225

### 226 3) Results

227 *Concentrations of α- and γ-TOC in VE rich substrates and in larvae reared on these*

228 *substrates*

229 **Figure 1** shows the different concentrations of α- (**Figure 1.A**) and γ-TOC (**Figure**  
230 **1.B**), on a fresh weight basis, in substrates rich in these compounds and in BSFL reared on  
231 these substrates. Firstly, note that both the mushrooms and the larvae reared on this substrate  
232 did not contain detectable amounts of α- and γ-TOC. Looking at the graphs from left to right,  
233 we first see that for the corn oil, rapeseed oil and rice bran oil groups, α-TOC concentrations  
234 were not significantly different ( $p > 0.3$ ) between the substrates and the larvae ( $72.4 \pm 3.5$ ,  
235  $71.7 \pm 6.3$  and  $34.9 \pm 0.5$  mg/kg versus  $76.9 \pm 1.9$ ,  $82.1 \pm 9.2$  and  $38.7 \pm 1.6$  mg/kg,  
236 respectively). Conversely, for the same groups, γ-TOC concentrations were significantly  
237 ( $p < 0.001$ ) lower in the larvae than in the substrates ( $17.2 \pm 0.6$ ,  $11.1 \pm 1.0$  and  $4.9 \pm 0.2$   
238 mg/kg versus  $114.9 \pm 3.3$ ,  $48.1 \pm 3.4$  and  $13.0 \pm 0.3$  mg/kg, respectively). The α- and γ-TOC  
239 concentrations of the wheat germ oil substrate ( $719.1 \pm 86.1$  and  $101.8 \pm 12.0$  mg/kg,  
240 respectively) were significantly ( $p < 0.001$ ) higher than those of the larvae reared on this  
241 substrate ( $286.1 \pm 4.6$  and  $29.6 \pm 1.4$  mg/kg, respectively). Larvae reared on brewer's spent  
242 grains (BSG) contained significantly ( $p < 0.001$ ) more α- ( $23.2 \pm 0.3$  mg/kg) and γ-TOC ( $2.3 \pm$   
243  $0.2$  mg/kg) than the brewer's spent grains ( $0.6 \pm 0.02$  and  $0.2 \pm 0.02$  mg/kg, respectively). The  
244 α-TOC concentrations of corn oil cake and turnip rapeseed cake ( $12.9 \pm 0.4$  and  $6.6 \pm 0.1$   
245 mg/kg respectively) were significantly lower than those of larvae reared on these cakes ( $41.5$   
246  $\pm 1.7$  and  $13.4 \pm 1.7$  mg/kg, respectively). However, the opposite was observed for the γ-TOC  
247 concentration, since corn oil cake ( $25.4 \pm 1.7$  mg/kg) and turnip rapeseed cake ( $12.6 \pm 1.0$   
248 mg/kg) contained significantly ( $p < 0.001$ ) more γ-TOC than the larvae reared on these cakes  
249 ( $15.3 \pm 0.8$  and  $4.6 \pm 0.6$  mg/kg, respectively). Larvae reared on rice bran ( $59.6 \pm 1.7$  mg/kg)  
250 contained significantly ( $p < 0.001$ ) more α-TOC than rice bran ( $27.4 \pm 4.0$  mg/kg). The  
251 concentration of α-TOC in wheat bran was not detectable but the concentration in wheat bran  
252 fed larvae was  $4.4 \pm 0.7$  mg/kg. The γ-TOC concentrations of rice ( $1.1 \pm 0.1$  mg/kg) and  
253 wheat ( $0.1 \pm 0.01$  mg/kg) brans were significantly lower than those of larvae fed the same  
254 brans ( $3.4 \pm 0.4$  and  $4.4 \pm 0.7$  mg/kg respectively). The poultry feed contained significantly  
255 more γ-TOC but less α-TOC than the larvae that consumed it (for γ-TOC:  $6.9 \pm 0.6$  versus  $2.6$   
256  $\pm 0.7$  mg/kg, respectively, and for α-TOC:  $7.0 \pm 1.4$  versus  $16.7 \pm 4.8$  mg/kg, respectively).  
257 Finally, positive correlations have been highlighted between the α-TOC and γ-TOC  
258 concentrations of the substrates and those of the BSFL (Pearson's correlation coefficients = +  
259 0.98 (CIr (95% Confidence Interval of r): [0.91-0.99]) and + 0.85 (Cir: [0.47-0.96]),  
260 respectively,  $p < 0.01$ ). Supplementary **figures S1A** and **S1B** show α-TOC and γ-TOC  
261 concentrations on a dry weight basis.

262

263 **Figure 1. VE concentrations in rearing substrates and in larvae (mg/kg fresh weight).**

264



265 *Bioaccessibility of  $\alpha$ - and  $\gamma$ -TOC from VE rich substrates and larvae reared on these*  
 266 *substrates*

267 **Tables 2.A** and **2.B** respectively represent the bioaccessibility of  $\alpha$ - and  $\gamma$ -TOC from  
 268 the selected substrates and from the larvae fed on these substrates. Three types of comparisons  
 269 are available: comparison of the bioaccessibility of each form of VE in different substrates,  
 270 comparison of the bioaccessibility of each form of VE in larvae reared on different substrates,  
 271 and comparison of the bioaccessibility of each form of VE when present in larvae versus  
 272 when present in their corresponding substrate.

273 The first comparison shows that the bioaccessibility of these two forms of VE was  
 274 significantly different between certain substrates. The maximum amplitude being 7 times for  
 275  $\alpha$ -TOC (wheat bran vs corn oil), and 14 times for  $\gamma$ -TOC (rice bran vs corn oil). It is also  
 276 interesting to note that the bioaccessibility of these two forms of VE was the highest in rice  
 277 bran and wheat bran, and the lowest in oils, with the exception of wheat germ oil.

278 The comparison of the bioaccessibility of the two forms of VE in the larvae also shows  
 279 significant differences. The maximum amplitude being about 35 times for  $\alpha$ -TOC (larvae  
 280 reared on rice bran vs larvae reared on corn oil), and 36 times for  $\gamma$ -TOC (larvae reared on  
 281 rice bran vs larvae reared on corn oil as well).

282 The last comparison is that of the bioaccessibility of VE when it was present in the larvae and  
 283 in their corresponding substrates. A striking overall observation is that the bioaccessibility of  
 284  $\alpha$  and  $\gamma$ -TOC in the larvae was almost always significantly lower in the larvae than in their  
 285 corresponding substrates, with an amplitude of up to about 34 times for  $\alpha$ -TOC provided in  
 286 wheat germ, and up to about 11 times for  $\gamma$ -TOC provide in wheat germ as well. There are  
 287 however two exceptions: the turnip rapeseed cake for which the bioaccessibility of the two

Substrate	Bioaccess in substrates	Bioaccess in larvae	p-value**
Corn oil	$8.8 \pm 2.1^d$	$1.3 \pm 0.4^c$	0.002
Rapeseed oil	$16.6 \pm 5.3^{cd}$	$1.4 \pm 0.3^c$	<0.001
Rice bran oil	$9.2 \pm 2.3^d$	$1.7 \pm 0.4^c$	0.003

288 forms of VE was not significantly different between the larvae and the substrate, and the corn  
 289 oil cake for which the bioaccessibility of the two forms of VE was significantly higher in the  
 290 larvae than in the substrate.

291  
 292 **Table 2.** Bioaccessibility of  $\alpha$ - and  $\gamma$ -TOC<sup>1</sup> from larvae and their corresponding rearing  
 293 substrates.

Wheat germ oil	58.1 ± 6.9 <sup>a</sup>	1.7 ± 0.1 <sup>c</sup>	<0.001
Brewers' spent grain	21.2 ± 3.6 <sup>bc</sup>	2.2 ± 0.6 <sup>c</sup>	<0.001
Corn oil cake	28.9 ± 1.9 <sup>ac</sup>	41.0 ± 0.9 <sup>a</sup>	0.002
Rice bran	59.7 ± 0.8 <sup>a</sup>	45.1 ± 3.5 <sup>a</sup>	0.008
Turnip rapeseed cake	42.4 ± 4.1 <sup>ab</sup>	37.1 ± 5.1 <sup>a</sup>	0.4
Wheat bran	61.9 ± 1.0 <sup>a</sup>	7.6 ± 0.2 <sup>b</sup>	<0.001
Poultry feed	40.6 ± 8.5 <sup>ab</sup>	18.6 ± 2.0 <sup>ab</sup>	0.02

294 **A. α-TOC**

295 **B. γ-TOC**

Substrate	Bioaccess in substrates	Bioaccess in larvae	p-value**
Corn oil	3.6 ± 0.5 <sup>c</sup>	1.3 ± 0.4 <sup>f</sup>	0.009
Rapeseed oil	5.3 ± 1.4 <sup>c</sup>	1.8 ± 0.4 <sup>ef</sup>	0.01
Rice bran oil	5.3 ± 0.7 <sup>c</sup>	2.9 ± 0.4 <sup>de</sup>	0.02
Wheat germ oil	25.1 ± 4.2 <sup>b</sup>	2.2 ± 0.4 <sup>ef</sup>	<0.001
Brewers' spent grain	16.8 ± 2.6 <sup>b</sup>	2.6 ± 0.7 <sup>df</sup>	0.002
Corn oil cake	26.4 ± 2.1 <sup>ab</sup>	38.2 ± 1.9 <sup>ab</sup>	0.009
Rice bran	52.0 ± 1.3 <sup>a</sup>	46.9 ± 3.6 <sup>ab</sup>	0.2
Turnip rapeseed cake	36.0 ± 2.7 <sup>ab</sup>	37.0 ± 5.1 <sup>a</sup>	1.0
Wheat bran	49.1 ± 1.6 <sup>ab</sup>	8.2 ± 0.3 <sup>c</sup>	<0.001
Poultry feed	36.9 ± 7.3 <sup>ab</sup>	19.0 ± 1.8 <sup>bc</sup>	0.04

296 <sup>1</sup>Percent of α- or γ-TOC recovered in the micelle fraction relative to the quantity of these  
 297 compounds found in the digestate at the end of the *in vitro* digestion. Values are means ±  
 298 SEM (n=4). \*\*p-value of a two-way ANOVA for each TOC, followed by pairwise  
 299 comparisons using bilateral t-tests. In each column, means that bear different superscript  
 300 letters are significantly different (*p* < 0.05; ANOVA followed by Tukey's HSD test).  
 301

302 *Uptake efficiency of micellized VE by Caco-2 cells*

303 **Table 3** shows the absorption efficiency of α- and γ-TOC contained in the mixed  
 304 micelle fractions which were obtained after the *in vitro* digestions. The absorption efficiency  
 305 of α-TOC from micelles of larvae reared on wheat germ oil was about twice as high as that  
 306 from micelles from this substrate. In the case of rice bran, the absorption efficiency of α-TOC  
 307 from micelles of larvae was not significantly different from that of the substrate. The  
 308 absorption efficiencies of γ-TOC from *in vitro* digestions of larvae reared on wheat germ oil  
 309 or rice bran were not significantly different from their corresponding substrates, but note that  
 310 the means were higher in larvae than in the substrates (about +50%).

311 **Table 3.** VE uptake efficiency (%) by Caco-2 cells. <sup>1</sup>

	Micellar fraction from rearing substrate digestion	Micellar fraction from larva digestion	Substrate vs larvae comparison **p-value
<b>α-TOC</b>			
Wheat germ oil	16.2 ± 2.1	34.2 ± 3.6	0.005
Rice bran	24.1 ± 1.3	28.8 ± 3.8	0.29
<b>γ-TOC</b>			
Wheat germ oil	12.4 ± 2.3	19.7 ± 5.0	n.s****

Rice bran	20.3 ± 2.0	30.1 ± 5.6	n.s
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312 <sup>†</sup>Percentage of  $\alpha$ - or  $\gamma$ -TOC that was recovered in the Caco-2 cells relative to the quantity of  
313 these compounds in the mixed micelle-rich fractions deposited on the cells before incubation  
314 for 4 hours at 37°C. Values are means ± SEM (n=4). \*\* For  $\alpha$ -TOC,  $p$ -value of a two-way  
315 ANOVA followed by pairwise comparisons using bilateral  $t$ -tests. \*\*\*For  $\gamma$ -TOC, the result  
316 of the two-way ANOVA was non-significant ( $p = 0.7$ ) thus post-hoc tests were not carried  
317 out.

318 **Figure 2** shows the VE amount absorbed by the Caco-2 cells on a fresh weight basis. This  
319 value is of great interest to compare the theoretical amount of VE that each matrix can bring  
320 to the intestinal cell because, in addition to considering the cell uptake efficiency, it also  
321 considers the bioaccessibility of VE in the substrate or the larvae. The cellular amount of VE  
322 after digestion and cell uptake of micelles from wheat germ oil was significantly higher than  
323 that of larvae reared on wheat germ oil (for  $\alpha$ -TOC (**Figure 2A**),  $30.3 \pm 7.8 \mu\text{g}$  and  $3.3 \pm 0.5$   
324  $\mu\text{g}$ , respectively, and for  $\gamma$ -TOC (**Figure 2B**)  $3.7 \pm 1.2 \mu\text{g}$  and  $0.2 \pm 0.04 \mu\text{g}$ , respectively).  
325 Conversely to what was observed for wheat germ oil, cells incubated with micelles from  
326 digestion of larvae reared on rice bran had higher concentrations of  $\alpha$  and  $\gamma$ -TOC than cells  
327 incubated with micelles from digestion of rice bran, although this was not significantly  
328 different ( $p = 0.08$  for  $\alpha$ -TOC and  $p = 0.3$  for  $\gamma$ -TOC). Supplementary **figures S2A** and **S2B**  
329 show  $\alpha$ -TOC and  $\gamma$ -TOC cellular concentrations on a dry weight basis.

330  
331 **Figure 2. Amounts of VE taken up by Caco-2 cells incubated with micelle-rich fractions**  
332 **obtained after *in vitro* digestions of rearing substrates or larvae.**

#### 333 4) Discussion

334 The first objective of this study was to assess the ability of BSFL to bioaccumulate  $\alpha$ -  
335 and  $\gamma$ -TOC from substrates rich in these compounds but made up of very different matrices  
336 (de Camargo *et al.*, 2019; Franke *et al.*, 2010; Shahidi *et al.*, 2021). Indeed, these substrates  
337 were either solid, e.g. bran or cake, or liquid, i.e. vegetable oils. The first interesting result of  
338 this study is the fact that when BSFL were grown on white mushrooms, they did not contain  
339 VE. This result not only suggests that BSFL reared on a substrate without VE can apparently  
340 grow like larvae that have access to VE, but also shows that BSFL do not have the ability to  
341 synthesize VE. With regard to the substrates rich in VE, the first observation is that the  
342 contents of  $\alpha$  and  $\gamma$ -TOC that we measured agreed with the data in the literature, except in  
343 wheat bran where we found no  $\alpha$ -TOC and a very low concentration of  $\gamma$ -TOC compared to  
344 what has been reported in the literature (**Table 1**). This suggests that our extraction protocol  
345 was not adapted for this substrate. Regarding  $\alpha$ -TOC concentrations measured in the BSFL,  
346 they differed strongly depending on the rearing substrate, since they ranged from  $18.1 \pm 2.3$   
347  $\text{mg/kg}$  for BSFL reared on turnip rapeseed cake to  $286.1 \pm 4.6 \text{ mg/kg}$  for BSFL reared on  
348 wheat germ oil. This very high concentration, which is moreover largely higher than the  $\alpha$ -  
349 TOC concentration of all the VE-rich substrates, with the exception of wheat germ, has never  
350 been observed. Indeed, what has been reported in whole larvae is  $9.6 \text{ mg/kg}$  if reared on  
351 chicken manure and  $19.3 \text{ mg/kg}$  if reared on spent grain (Shumo *et al.*, 2019).

352 If we now compare the concentrations of  $\alpha$ -TOC in the larvae and in the substrates on which  
353 they were raised, we see that they are always higher in the larvae than in the substrates,  
354 sometimes significantly, with the exception of the larvae raised on the wheat germ oil. To  
355 explain this exception, we hypothesize that the very high amount of  $\alpha$ -TOC provided by  
356 wheat germ oil led the larvae to reach the maximum concentration of  $\alpha$ -TOC they can reach.  
357 Concerning  $\gamma$ -TOC, what was immediately striking was that the concentrations in the larvae  
358 are, 7 times out of 10, lower than in their corresponding substrate. In fact, it is likely that the

359 result should have been the same with the 3 other substrates, made of bran, because we  
360 deduced that our method of extracting the two VE species from these substrates was not very  
361 effective which must have led to underestimating their VE content. To support this  
362 hypothesis, we rely on the results of bioaccessibility which show that we can detect  $\alpha$  and  $\gamma$ -  
363 TOC in the micelles resulting from the digestion of wheat bran (**Table 2**) whereas we do not  
364 detect  $\alpha$ -TOC, and we detect very low concentration of  $\gamma$ -TOC, in wheat bran when we have  
365 extracted this substrate with organic solvents (**Figure 1**), even though wheat bran contains  
366 both forms of VE (**Table 1**). This difference in bioaccumulation of these two forms of VE in  
367 BSFL is quite remarkable. The most likely hypothesis is that, as has been described in  
368 *Drosophila*, which is a dipteran like BSF, this is due to a preferential degradation of  $\gamma$ -TOC,  
369 as compared to  $\alpha$ -TOC, by cytochrome P450 TOC- $\omega$ -hydroxylase (Parker and McCormick,  
370 2005). If this hypothesis is true, this suggests that  $\alpha$ -TOC has an essential function in the BSF  
371 and that it is important to provide it in its diet during its breeding, as is the case for other  
372 production animals.

373 After demonstrating that larvae can bioaccumulate very high concentrations of  $\alpha$ -TOC  
374 and that they can also be enriched in  $\gamma$ -TOC, the question arose of the bioavailability of these  
375 two forms of VE in the insect matrix. The first key step in the bioavailability of lipid  
376 molecules is their incorporation into the micelles during digestion. The rate of incorporation is  
377 called bioaccessibility. When we look at **Table 2** we can clearly see that, with one exception  
378 which is corn oil cake, the bioaccessibility of the two forms of VE was significantly lower  
379 when they were in the insect matrix than when they were in the plant matrix. We do not know  
380 the mechanism but we can propose two hypotheses. The first is that, as the insect's  
381 cuticle/exoskeleton contains a variety of compounds, e.g. chitin, proteins and lipids (Kramer  
382 *et al.*, 1995) , it is possible that a fraction of the VE was associated with one or several of  
383 these compounds and is therefore not bioaccessible. The second hypothesis, knowing that all  
384 these substrates contain not only VE but also phytosterols, which are well known to inhibit  
385 the micellization of cholesterol and fat-soluble vitamins (Fardet *et al.*, 2017), is that the  
386 phytosterols have strongly bioaccumulated in the larvae and/or are much more bioaccessible  
387 in the larvae than in their corresponding substrates. This effect of phytosterols could also  
388 explain why the bioaccessibility of VE in the oils was significantly lower than in the solid  
389 matrices. It can indeed be assumed that the bioaccessibility of phytosterols present in oils is  
390 greater than that of these compounds when they are present in plant matrices in which they  
391 can interact with dietary fibers. These hypotheses obviously need to be verified by dedicated  
392 studies.

393 Bioaccessibility gives an idea of the extractability of a bioactive from a matrix in  
394 which it is present. But, from a nutritional point of view, it is important to consider both this  
395 parameter and the content of bioactive of interest in the matrix. It is therefore relevant to  
396 compare the VE concentrations in the micelles which come from the digestions of the larvae  
397 having been reared on the different substrates (Supplemental **figures S3 and S4**). This  
398 comparison clearly shows that the larvae which give the highest amount of  $\alpha$  and  $\gamma$ -TOC in  
399 micelles are the two cakes, the rice bran and the chicken feed, substrates which were not the  
400 richest in VE.

401 The amount of VE in micelles is obviously a very important parameter of  
402 bioavailability, but we know that everything that is present in micelles is not necessarily  
403 absorbed by the intestinal cells, or it can be absorbed and then resecreted in the digestive  
404 lumen and is therefore not bioavailable to the body, as is the case for phytosterols (Ling and  
405 Jones, 1995). To get a better idea of what the in vivo bioavailability might be it is therefore  
406 essential to measure the absorption efficiency of VE present in micelles. And that's what was  
407 done with the Caco-2 model by choosing the oil and the solid substrate giving the greatest  
408 amount of  $\alpha$ -TOC in the micelles (**Table 3**). When we look at this table we see that the only

409 significant difference was that the VE uptake efficiency was twice as high from the micelles  
 410 resulting from the digestion of larvae reared on the substrate containing wheat germ oil than  
 411 from the micelles resulting from the digestion of this same substrate. Unfortunately, we do not  
 412 have a solid hypothesis to explain this difference.

413 But what is important from a nutritional point of view is the  
 414 quantity of VE which is found in the cells, i.e. which is actually bioavailable. This quantity  
 415 depends on the absorption efficiency by the cells but also on the amount of VE present in the  
 416 micelles. When we look at these data (**Figures 2A** and **2B**) we can clearly see that the  
 417 amounts of VE found in the cells which have been incubated with micelles from the digestion  
 418 of larvae which have been reared on the substrate containing wheat germ oil was 3 to 4 times  
 419 lower than the amount of VE found in cells that were incubated with micelles from larvae  
 420 reared on rice bran. This suggests that rice bran is a better source of bioavailable VE than  
 421 wheat germ oil, although the latter was the richest source of VE among all the studied  
 422 substrates.

423 Finally, in order to get an overview of the capacity of VE-rich BSFL to be used in the  
 424 animal feed industry as a significant source of VE, the amount of VE that could potentially be  
 425 provided by VE-rich BSFL to different livestock was calculated according to the VE needs of  
 426 the different animal species and to the recommended quantities of BSFL that are usually  
 427 incorporated in their diet (**Table 4**). This table suggests that these vitamin-rich larvae would  
 428 be of particular interest in poultry as they could cover all the vitamin requirements of laying  
 429 hens, broilers and turkeys.

430 In conclusion, this study first shows that BSFL can bioaccumulate both  $\alpha$ - and  $\gamma$ -TOC  
 431 from VE-rich substrates. Larvae  $\alpha$ -toc concentrations are mostly equal to or higher than those  
 432 of their rearing substrates. Conversely larvae  $\gamma$ -toc concentrations are generally lower than  
 433 those of their substrates suggesting a different metabolism of these two forms of VE in the  
 434 larvae. Regarding VE bioaccessibility it was generally lower in the insect matrix than in the  
 435 corresponding plant matrices. Nevertheless, most BSFL reared on by-products (brans and  
 436 press oil cakes) contained significant amounts of micellarized VE, which could lead to a  
 437 sustainable VE valorization of these by-products. Finally, adding VE rich BSFL in the diet of  
 438 some farm animals could provide a significant proportion of the needs of these animals in this  
 439 vitamin. The enrichment of larvae with VE could make it possible to recycle part of the VE  
 440 present in waste or co-products, it would make it possible to provide the natural forms of this  
 441 vitamin and would increase the economic value of these larvae.

442  
 443 **Table 4.** Proportion of the recommended VE dose that could come from the incorporation of  
 444 VE-rich larvae in animal feed.

Species	VE concentration that was added in the diet (mg/kg)	BSFL fraction provided in the diet	Recommended proportion of the BSFL fraction added in the diet (weight%)	mg of VE* provided by BSFL incorporated at the recommended proportion	VE % provided by BSFL
Broiler chicken ( <i>Gallus gallus domesticus</i> )	7 (National Research Council, 1994)	Prepupae	15% (Pieterse <i>et al.</i> , 2019)	9	128

Laying hens ( <i>Gallus gallus domesticus</i> )	3 (National Research Council, 1994)	Prepupae	20% (Tahamtani <i>et al.</i> , 2021)	12	400
Salmon ( <i>Salmo salar</i> )	40 (National Research Council, 1993)	Prepupae	50% (Sealey <i>et al.</i> , 2011)	30	75
Tilapia ( <i>Oreochromis niloticus</i> )	40 (National Research Council, 1993)	Prepupae	50% (Taufek <i>et al.</i> , 2021)	30	75
Turkey ( <i>Meleagris gallopavo</i> )	7 (National Research Council, 1994)	Prepupae	50% (Chia <i>et al.</i> , 2021)	30	428
Dog ( <i>Canis lupus familiaris</i> )	20 (National Research Council, 2006)	Partially defatted black soldier fly	20 % (Freel <i>et al.</i> , 2021)	12	60

445 \*Using the theoretical  $\alpha$ -TOC content of BSFL reared on rice bran, the BSFL group that  
446 provided the highest amount of  $\alpha$ -TOC in micelles.  
447

#### 448 **Supporting information:**

449 The costs of this project were covered equally by the own budget of P. Borel's research team,  
450 which came mainly from INRAE endowments, and by the BioMiMetiC company.

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456

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