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Research article

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

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Abstract

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

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

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

Abbreviation:

BSFL (black soldier fly larvae).
TOC (tocopherol)
VE (vitamin E)

1) Introduction

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

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

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

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

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

2) Material and methods

Chemicals

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

Experimental food

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

BSFL farming

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

Assessment of the bioaccessibility of VE in rearing substrates and BSFL

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

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

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

VE extraction

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

VE quantification

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

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

Calculations

VE bioaccessibility and uptake efficiency were calculated as:

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

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 ± 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 α and γ-TOC (mg/kg) in the rearing substrates selected in the study.

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

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

Data are means ± SD. n.d.: not detected.
Data are expressed in fresh weight except when there is an asterisk which means that they are expressed in dry weight.

3) Results

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

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

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

Bioaccessibility of α- and γ-TOC from VE rich substrates and larvae reared on these substrates

Tables 2.A and **2.B** respectively represent the bioaccessibility of α- and γ-TOC from the selected substrates and from the larvae fed on these substrates. Three types of comparisons are available: comparison of the bioaccessibility of each form of VE in different substrates, comparison of the bioaccessibility of each form of VE in larvae reared on different substrates, and comparison of the bioaccessibility of each form of VE when present in larvae versus when present in their corresponding substrate.

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

The comparison of the bioaccessibility of the two forms of VE in the larvae also shows significant differences. The maximum amplitude being about 35 times for α-TOC (larvae reared on rice bran vs larvae reared on corn oil), and 36 times for ɣ-TOC (larvae reared on rice bran vs larvae reared on corn oil as well).

The last comparison is that of the bioaccessibility of VE when it was present in the larvae and in their corresponding substrates. A striking overall observation is that the bioaccessibility of α and ɣ-TOC in the larvae was almost always significantly lower in the larvae than in their corresponding substrates, with an amplitude of up to about 34 times for α-TOC provided in wheat germ, and up to about 11 times for ɣ-TOC provide in wheat germ as well. There are however two exceptions: the turnip rapeseed cake for which the bioaccessibility of the two forms of VE was not significantly different between the larvae and the substrate, and the corn oil cake for which the bioaccessibility of the two forms of VE was significantly higher in the larvae than in the substrate.

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

Table 2. Bioaccessibility of α- and γ-TOC[1] from larvae and their corresponding rearing substrates.

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

A. α-TOC

B. ɣ-TOC

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

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

Uptake efficiency of micellarized VE by Caco-2 cells

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

Table 3. VE uptake efficiency (%) by Caco-2 cells. [1]

	Micellar fraction from rearing substrate digestion	Micellar fraction from larva digestion	Substrate vs larvae comparison **p-value
α-TOC			
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
γ-TOC			
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

[1]Percentage of α- or γ-TOC that was recovered in the Caco-2 cells relative to the quantity of these compounds in the mixed micelle-rich fractions deposited on the cells before incubation for 4 hours at 37°C. Values are means ± SEM (n=4). **For α-TOC, *p*-value of a two-way ANOVA followed by pairwise comparisons using bilateral t-tests. ***For γ-TOC, the result of the two-way ANOVA was non-significant (p = 0.7) thus post-hoc tests were not carried out.

Figure 2 shows the VE amount absorbed by the Caco-2 cells on a fresh weight basis. This value is of great interest to compare the theoretical amount of VE that each matrix can bring to the intestinal cell because, in addition to considering the cell uptake efficiency, it also considers the bioaccessibility of VE in the substrate or the larvae. The cellular amount of VE after digestion and cell uptake of micelles from wheat germ oil was significantly higher than that of larvae reared on wheat germ oil (for α-TOC (**Figure 2A**), 30.3 ± 7.8 µg and 3.3 ± 0.5 µg, respectively, and for γ-TOC (**Figure 2B**) 3.7 ± 1.2 µg and 0.2 ± 0.04 µg, respectively). Conversely to what was observed for wheat germ oil, cells incubated with micelles from digestion of larvae reared on rice bran had higher concentrations of α and ɣ-TOC than cells incubated with micelles from digestion of rice bran, although this was not significantly different (p = 0.08 for α-TOC and p = 0.3 for ɣ-TOC). Supplementary **figures S2A** and **S2B** show α-TOC and γ-TOC cellular concentrations on a dry weight basis.

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

4) Discussion

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

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

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

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

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

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

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

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

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

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

Table 4. Proportion of the recommended VE dose that could come from the incorporation of 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 *(Gallus gallus domesticus)*	7 (National Research Council, 1994)	Prepupae	15% (Pieterse *et al.*, 2019)	9	128

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

*Using the theoretical α-TOC content of BSFL reared on rice bran, the BSFL group that provided the highest amount of α-TOC in micelles.

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