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Research article

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Abstract

Black soldier fly larvae (BSFL) are increasingly used to recycle and convert food waste into feed. We attempted to assess whether they can bioaccumulate lutein, a xanthophyll used as a food coloring, and whether it is then sufficiently bioavailable for an economically relevant incorporation of BSFL into feed. Vegetables and larvae lutein concentrations were measured by HPLC. Lutein bioaccessibility was estimated by *in vitro* digestion and lutein absorption efficiency by Caco-2 cells. BSFL were at least as rich, and sometimes richer ($p < 0.05$), in lutein than the vegetables they were reared on. For example, the larvae reared on kale contained 160.2 ± 3.4 mg/kg vs 23.0 ± 3.5 mg/kg of lutein, on a fresh weight basis, for the kale substrate. For the same substrate, lutein bioaccessibility was not statistically different between BSFL and the substrate (respectively 14.8 ± 1.2 % and 16.2 ± 2.8 %; $p = 0.7$). Finally, by considering the lutein concentration in BSFL enriched in lutein and in lutein-rich substrates, as well as the bioaccessibility and intestinal absorption efficiency of lutein contained in these matrices, it was estimated that consumption of lutein-enriched larvae would lead to a theoretical amount of absorbed lutein about 2 to 13 times higher compared to that following the consumption of an equal quantity of lutein-rich vegetables. Thus, BSFL can be used as a sustainable and concentrated source of bioavailable lutein for feed and, indirectly, for food.

Keywords: edible insects, circular economy, nutritional quality, xanthophyll, bioaccessibility.

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.

Abbreviation:
BSFL (black soldier fly larvae).

1) Introduction

Lutein is a xanthophyll, i.e. an oxygenated carotenoid, which is found in the human diet mainly from green leafy vegetables such as parsley, kale or spinach (Perry *et al.*, 2009) but this phytochemical can also be found in eggs, algae or marigold extracts. It exerts very varied biological effects, whether in photosynthetic plants, where it is involved in photosynthesis (Dall'Osto *et al.*, 2006; Demmig-Adams *et al.*, 2022), in the animal kingdom, where it can participate in the coloring of certain animals (Langi *et al.*, 2018; Nabi *et al.*, 2020), or even in humans, where it seems to be involved in visual function (Feng *et al.*, 2019; Johnson, 2014). Lutein-rich feed ingredients are used to improve egg yolk and broiler meat color (Langi *et al.*, 2018). Lutein is therefore a molecule of interest for the feed and food industry and as a consequence, strategies to obtain it at lower costs are sought after.

Nowadays, products used to feed farm animals, such as soy, whose cultivation is one of the main causes of deforestation in the world, or fishmeal produced from wild fish, which further exacerbate overfishing, are contributing to an unsustainable food system. In the context of climate change and the need to find more sustainable nutrient sources, the incorporation of insects into livestock and human diets is recommended by FAO and EFSA (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) *et al.*, 2021; FAO, 2021). Indeed, insect farming has many advantages over traditional animal husbandry, e.g. lower greenhouse gas production and water consumption per kg protein produced (Allegretti *et al.*, 2018; FAO, 2021). Moreover, some insects, such as black soldier fly larvae (BSFL), i.e. *Hermetia Illucens*, can also be fed a wide variety of organic wastes (Amrul *et al.*, 2022; Nguyen *et al.*, 2015). Consequently, black soldier flies (BSF) are increasingly used to recycle and valorize farm waste and/or co-products from feed and food industries (Ites *et al.*, 2020; Siddiqui *et al.*, 2022). The larvae obtained are either used as is or transformed into a defatted meal and oil before being incorporated into the feed of various production animals (Sánchez-Muros *et al.*, 2014; Secci *et al.*, 2018). They are very good sources of protein and fat, but they also contain many micronutrients and phytochemicals, including carotenoids (Borel *et al.*, 2021; Finke, 2013; Van Huis *et al.*, 2013).

Although insects, like other animals, generally cannot synthesize carotenoids, they can concentrate selected carotenoids from their diet into specific tissues with the help of carotenoid binding proteins and active transport mechanisms (Heath *et al.*, 2013). Consequently, several studies, e.g. hemipteran insects, dragonflies or silk moth, have reported accumulation of xanthophylls, such as lutein, astaxanthin or zeaxanthin (Maoka *et al.*, 2020, 2021; Yuasa *et al.*, 2014). Concerning the BSFL, they do not contain provitamin A carotenoids if they are raised on substrates that do not contain any (Borel *et al.*, 2021), and therefore they are not a priori capable of synthesizing them. However, they can bioaccumulate carotenoids when they are raised on substrates that contain them (Borel *et al.*, 2021; Leni *et al.*, 2022).

The aim of this study was first to investigate whether BSFL can be enriched with significant amounts of lutein and potentially later with lutein from plant co-products of the food industry, promoting the circular economy. The second objective of this study was to assess whether this sustainable source of lutein was bioavailable enough in order to provide animals, when incorporated into their feed, with a quantity of lutein adapted to their production needs, e.g. allowing satisfactory coloring of the egg yolk of laying hens. To answer this question, we first measured the bioaccumulation of this pigment in larvae reared on vegetables among the richest in lutein. Secondly, we compared the bioaccessibility of lutein in these larvae with that of lutein in the rearing vegetables with an *in vitro* digestion model. Finally, we compared the intestinal uptake efficiency of lutein in the micelles from the digestion of either the larvae or the rearing substrates using Caco-2 cells. Indeed, the

combination of the measurement of bioaccessibility and intestinal uptake efficiency constitute a good estimate of the relative bioavailability of carotenoids in different food matrices.

2) Material and Methods

Chemicals

Solvents used for HPLC (ethanol, *n*-hexane, methanol, dichloromethane, water and methyl tert-butyl ether) were purchased from Carlo Erba reagents (Peypin, France). Lutein and echinenone (HPLC purity > 95%) were from CaroteNature GmbH (Münsingen, Switzerland). Other chemicals and enzymes were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, trypsin-ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin, non-essential amino acids and phosphate-buffered saline (PBS) were purchased from Life Technologies (Villebon-sur-Yvette, France). Fetal bovine serum (FBS) was from PAA (Vélizy-Villacoublay, France).

Experimental foods

Lutein-rich vegetables (de Azevedo-Meleiro and Rodriguez-Amaya, 2005; Humphries and Khachik, 2003) were chosen as substrates for BSFL rearing : parsley (*Petroselinum sativum*), kale (*Brassica oleracea var. acephala*), endive (*Cichorium intybus var. foliosum*) and broccoli (*Brassica oleracea var. asparagoides*). White mushrooms (*Agaricus bisporus*), which do not contain detectable amounts of lutein (based on literature and verified by HPLC), were used as control substrate. These vegetables and the foods used for the *in vitro* digestion experiments, i.e. potatoes, minced beef meat with 5% fat and olive oil, were bought from a local supermarket.

BSFL farming

BSFL were reared on the above-mentioned substrates, which were ground using a kitchen chopper. The rearing protocol was performed by BioMiMetiC in Avignon, France. The rearing procedure was conducted as previously described (Borel *et al.*, 2021).

Measurement of dry matter

Larva dry matter was determined by measuring water loss after 72 h of freeze-drying. For the substrates, dry matter values were taken from the Ciqual database (https://ciqual.anses.fr/ Accessed 20.06.22).

In vitro digestions to assess lutein bioaccessibility

The in vitro digestion experiments used to measure lutein bioaccessibility were carried out in quadruplicate as previously described (Borel *et al.*, 2021). Before the experiment, 1 g of substrate or larva sample was ground with liquid nitrogen in a mortar grinder (Pulverisette 2, FRITSCH GmbH, Idar-Oberstein, Germany) and 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). Then, 2.5 mL of artificial saliva were added 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, which was incubated for 30 min at 37 °C. 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 at 37 °C 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 lutein by HPLC.

Micelle size and zeta potential analysis

The size, measured by dynamic light scattering, and charge (ζ-potential) of particles in the micellar fraction obtained at the end of *in vitro* digestions were measured using a Zetasizer (NanoZS, Malvern Instruments Ltd, Worcestershire, UK). Just before the measurements, the micellar fractions were filtered with a 0.22 µm filter, to eliminate possible agglomerates. Measurements were conducted at 25 °C with a wavelength of 633 nm.

Caco-2 cell culture and lutein uptake efficiency

Caco-2 cells (clone TC7) were a kind gift from Dr. M. Rousset (U178 INSERM, Villejuif, France). Cell culture was adapted from (Desmarchelier *et al.*, 2017). Briefly, 1 mL of complete medium (i.e., DMEM supplemented with 16% FBS, 1% non-essential amino acids and 1% antibiotics) was added on the cells every day during the first 7 days and every other day during the next 7 days to obtain confluent differentiated cell monolayers. The day before the experiment, the medium was replaced by a serum-free complete medium. To measure lutein uptake efficiency, cell monolayers were washed twice with 1 mL of PBS. Then, the micellar fractions obtained at the end of the *in vitro* digestion were diluted 5 times with DMEM and 1.5 mL thereof were added on the cells. The micellar solutions were left in contact with the cells for 4 hours (Liu *et al.*, 2004) at 37 °C. After incubation, the medium was recovered. The cells were then washed twice with PBS and were finally collected in 500 µL PBS and stored at -80 °C prior to lutein extraction and HPLC analysis.

Lutein extraction

Lutein was extracted as described previously for other carotenoids (Borel *et al.*, 2021), using a sample volume of 500 µL. For food substrate and BSFL samples, 1 g was finely crushed with liquid nitrogen and the powders thus obtained were diluted and homogenized in 20 mL distilled water.

Lutein quantification

The lutein-containing extracts, which were previously evaporated and solubilized in methanol/dichloromethane (65/35, v/v), were injected into the HPLC system. Injection volumes were determined after an initial injection and adjusted to obtain signals within the calibration range. Thus, 5 to 50 µL BSFL and substrate samples, 100 to 150 µL micellar fractions, 25 to 150 µL digestate samples and 180 µL cell samples were injected into the HPLC system. The apparatus and method used, i.e. column, mobile phase, flow rate and detection method, were the same as previously described (Borel *et al.*, 2021).

Statistics

Results are expressed as means ± SEM. The homogeneity of variances ($p > 0.05$) was assessed by Levene's test. If the variances were inhomogeneous, data were log-transformed. To assess the normality of the data, Q-Q plots of standardized residuals were used. Differences between groups were tested using several two-way ANOVA. When a significant effect of the matrix in which the lutein was incorporated, i.e. plant or insect, was highlighted by the ANOVA, the comparison of the means between the plant matrix and the insect matrix was carried out by pairwise comparisons using bilateral t-tests, with a Bonferroni adjustment. 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).

3) Results

Lutein concentration in larvae reared on lutein-rich substrates and in the corresponding substrates.

Figure 1 represents the lutein concentration in larvae reared on lutein-rich substrates and in these substrates. Neither mushrooms nor the larvae reared on these mushrooms contained any detectable amount of lutein while larvae reared on other substrates all contained lutein. What is most striking in **Figure 1A** (results expressed on a fresh weight basis) is that lutein concentration in larvae was always higher than lutein concentration in the corresponding rearing substrate. Moreover, larvae reared on kale contained 160.2 ± 3.4 mg/kg of lutein while kale contained 23.0 ± 3.5 mg/kg of lutein. Thus, lutein concentration in these larvae was about 7 times higher ($p < 0.001$) than in the corresponding substrate. Note that lutein concentration was still more than 3 times higher ($p < 0.001$) in these larvae than in kale on a dry weight basis (**Figure 1B**). The larvae reared on parsley contained more than twice as much lutein as the substrate itself on a fresh weight basis (69.8 ± 5.7 mg/kg vs 29.2 ± 1.4 mg/kg; $p < 0.001$). On a dry weight basis however, there was no significant difference ($p = 0.75$). Lutein concentration in endive was 0.2 ± 0.02 mg/kg and the larvae fed with endives contained 15 times more lutein (3.0 ± 0.2 mg/kg) on a fresh weight basis ($p < 0.001$), and still about 3 times more on a dry weight basis ($p < 0.001$). There was a significant difference in lutein concentration in broccoli and in larvae reared on broccoli (1.3 ± 0.07 and 2.4 ± 0.3 mg/kg on a fresh weight basis ($p < 0.05$) and 11.4 ± 0.6 and 7.6 ± 1.1 mg/kg on a dry weight basis ($p < 0.05$), for broccoli and larvae respectively).

Figure 1. Lutein concentration (mg/kg) in rearing substrates and in larvae.

Lutein bioaccessibility in larvae reared on lutein-rich substrates and in the corresponding substrates.

Bioaccessibility values are shown in **Table 1**. For kale and broccoli, lutein bioaccessibility in the substrates and in the larvae was not significantly different. Conversely, lutein bioaccessibility was about 2 times lower ($p < 0.01$) in larvae reared on endive and parsley as compared to the corresponding substrates. Lutein bioaccessibility was not significantly different between the substrates while lutein bioaccessibility in the larvae exhibited some variability according to the substrates, namely it was approximately twice as high in larvae reared on broccoli and kale compared to larvae reared on endive and parsley (means around 14% for the former and 8% for the latter).

	Rearing substrate	**Larvae**	**Substrate vs larvae comparison** *****p*-value**
Broccoli	12.1 ± 0.5^{a}	13.7 ± 1.2^{a}	0.26
Endive	16.9 ± 1.3^{a}	8.1 ± 0.9^{b}	<0.01
Kale	16.2 ± 2.8^{a}	14.8 ± 1.2^{a}	0.67
Parsley	14.5 ± 1.1^{a}	7.8 ± 0.5^{b}	<0.01

Table 1. Bioaccessibility of lutein* from larvae and their corresponding rearing substrates.

*Percent of lutein recovered in the micelle fraction relative to that 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, followed by pairwise comparisons using bilateral t-tests.

^{a,b} In each column, means that bear different superscript letters are significantly different ($p < 0.05$; ANOVA followed by Tukey's HSD test).

Lutein amount in mixed micelles from either digestion of larvae fed with lutein-rich substrates or from digestion of their corresponding substrates.

Figure 2 shows the lutein amount in the micelle-rich fractions obtained after *in vitro* digestion of either larvae fed lutein-rich substrates or their corresponding substrates. This amount depends both on lutein concentration in the matrix (larvae or substrates) and on lutein bioaccessibility in the matrix. The amount of lutein in micelles corresponds to the quantity of lutein that is presented to the intestinal cell for its absorption. On a fresh weight basis (**Figure 2A**), the mixed micelles obtained after digestion of larvae fed on kale contained about 4 times more lutein than those obtained after digestion of kale (21.9 ± 2.5 and 5.4 ± 1.0 mg/kg, respectively; $p < 0.001$). On a dry weight basis (**Figure 2B**), they still contained more than twice as much lutein as those obtained after kale digestion (72.9 ± 8.5 and 31.7 ± 5.8 mg/kg, respectively; $p < 0.01$). For the other substrates, on a fresh weight basis, no significant difference was observed between larvae and their corresponding substrates. Conversely, on a dry weight basis, the quantity of lutein in micelles that came from the digestion of larvae compared to those that came from the digestion of their corresponding substrates differed significantly for parsley ($p < 0.001$), endive ($p < 0.01$) and broccoli ($p < 0.01$).

Figure 2. Lutein quantity in mixed micelles obtained after *in vitro* digestions of rearing substrates and of larvae.

Zeta potential of mixed micelles from in vitro digestions

Table 2 shows the zeta potential of the mixed micelles obtained after *in vitro* digestion of larvae fed on lutein-rich substrates and on their corresponding substrates. The zeta potential of micelles from larva digestion was approximately 2 to 4 times higher than that of their substrates ($p < 0.05$). Note that only one population of particles was detected in the mixed micelle rich fractions. Its size was around 9.0 ± 0.4 nm and there was no significant difference between the size of mixed micelles obtained after digestion of larvae and the size of those obtained after digestion of substrates (data not shown).

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Table 2. Zeta potential of micelles from *in vitro* digestion of larvae and substrates.

	Zeta potential (mV)		Substrate vs larvae comparison *p-value
	Micellar fraction from rearing substrate digestion	Micellar fraction from larva digestion	
Broccoli	-8.4 ± 0.8	-4.6 ± 1.0	0.039
Endive	-21.0 ± 1.3	-5.6 ± 2.3	<0.01
Kale	-15.4 ± 1.6	-7.0 ± 1.2	<0.01
Parsley	-14.2 ± 1.3	-5.6 ± 0.1	<0.01

Values are means ± SEM (n=4). *p-value of a two-way ANOVA followed by pairwise comparisons using bilateral t-tests.

Uptake efficiency of micellarized lutein by Caco-2 cells

Table 3 shows lutein uptake efficiency from the micellar fraction of digestions of larvae reared on kale and parsley compared to that from the micellar fraction of digestions of the substrates themselves. Note that the results with the other substrates are not shown because the amounts of lutein in the micelles were too low to be able to be correctly measured in the cells (below the quantification limit of 0.02 µg/mL). Lutein in the mixed micelle-rich fraction obtained after digestion of larvae reared on kale was approximately twice as

efficiently taken up compared to lutein from the fraction obtained after digestion of kale ($p < 0.001$). For parsley, cellular uptake was approximately 2 times greater when lutein was supplied by the micellar fraction from larva digestion than by the micellar fraction from substrate digestion ($p < 0.001$).

Table 3. Lutein uptake efficiency by Caco-2 cells. *

	Micellar fraction from rearing substrate digestion	Micellar fraction from larva digestion	Substrate vs larvae comparison **p-value
Kale	9.1 ± 0.3	23.6 ± 0.9	<0.001
Parsley	8.4 ± 0.8	21.0 ± 0.8	<0.001

*Percentage of lutein that was recovered in the Caco-2 cells relative to the quantity of lutein in the medium before incubation of the cell monolayers with mixed micelle-rich fractions for 4 hours at 37 °C. Values are means ± SEM (n=4). **p-value of a two-way ANOVA followed by pairwise comparisons using bilateral t-tests.

Figure 3 shows the amount of lutein taken up by Caco-2 cells. This value is very interesting because it depends both on the concentration of lutein in the matrix (plant substrate or larvae), on the bioaccessibility of lutein in this matrix, and on the efficiency of absorption of micellarized lutein by the intestinal cell. It therefore makes it possible to compare the quantity of lutein absorbed by the cells, i.e. bioavailable, for the same quantity of digested matrix. The cells incubated with micelles from the digestion of larvae reared on kale contained around 7 (when normalized to dry weight) to around 13 (when normalized to fresh weight) more lutein than the cells incubated with micelles from the digestion of kale, i.e. 40.2 ± 3.6 vs 5.4 ± 1.1 mg/kg respectively (dry weight; $p < 0.001$), and 12.1 ± 1.1 vs 0.9 ± 0.2 mg/kg respectively (fresh weight; $p < 0.001$). Regarding parsley, there was a significant difference ($p = 0.012$) between the cells incubated with micelles from the digestion of larvae reared on parsley (1.9 ± 0.3 mg/kg) and those incubated with micelles from the digestion of parsley (0.9 ± 0.1 mg/kg) on a fresh weight basis, but not on a dry weight basis ($p = 0.3$)

Figure 3. Lutein quantity taken up by Caco-2 cells incubated with micelles obtained after *in vitro* digestions of rearing substrates or larvae.

4) Discussion

First of all, the results obtained with the white mushrooms (control substrate that does not contain any detectable amount of lutein) allow us to conclude that BSFL do not naturally contain lutein, just as they do not contain provitamin carotenoids (Borel *et al.*, 2021). Secondly, BSFL bioaccumulate lutein to concentrations more or less proportional to those present in the vegetables on which they were reared. Normalizing the results to dry matter weight attenuated the difference in concentration between larvae and vegetables likely because substrates have a higher water content compared to larvae, but nonetheless, larvae reared on kale still displayed a much higher lutein concentration compared to kale. It is therefore evident that the larvae bioaccumulate lutein. This is probably due to the fact that, unlike vegetables, they are very rich in lipids, which can solubilize lutein, a fat-soluble molecule. Whether this bioaccumulation is due to a specific mechanism allowing the larva to store this carotenoid for future use, for example in adulthood, or whether it is simply due to a "reservoir effect" in which lutein is deposited, remains to be determined. However, the fact that BSFL can also bioaccumulate another xanthophyll, zeaxanthin (data not shown), which was present in the studied vegetables but at much lower concentration than lutein, as well as

provitamin A carotenoids (Borel *et al.*, 2021; Leni *et al.*, 2022) and vitamin E (Shumo *et al.*, 2019) suggests that they act as a fat tank having the ability to store various fat-soluble molecules by solubilizing them (Borel *et al.*, 1996; Goupy *et al.*, 2020).

After having shown that BSFL can bioaccumulate high concentrations of lutein, we compared the bioaccessibility of lutein in larvae and in their corresponding rearing substrates. It could not be ruled out that certain molecules, known or unknown, and present in the insect matrix, could not interfere with bioaccessibility. The results obtained show that either there was no difference in lutein bioaccessibility between the larvae and the substrates (broccoli and kale), or the bioaccessibility of lutein from larvae was half as much as that measured from the substrates (endive and parsley). In our previous study (Borel *et al.*, 2021), we found that the bioaccessibility of another xanthophyll, β-cryptoxanthin, was dramatically lower in clementine-fed larvae (6.4 %) and in pumpkin-fed larvae (13.1 %) as compared to their corresponding substrates (respectively 67.8 and 82.4 %). The synthesis of these results allows us to suggest that the bioaccessibility of xanthophylls is generally lower in larvae than in plant substrates. We hypothesize that this is due to xanthophyll complexing with insect matrix compounds that inhibit their release from this matrix and their transfer to mixed micelles. These compounds could be chitin, or proteins with a particular affinity for xanthophylls. Nevertheless, to explain why the bioaccessibility of lutein from the larvae reared on kale and broccoli was not lower to that observed for the corresponding substrates we hypothesize that kale and broccoli contain one, or several, compound(s) which is/are specific to these vegetables and which improve the bioaccessibility of xanthophylls from larvae. These compounds could be isothiocyanates. Indeed, it has been shown that they cause partial Z-isomerization of carotenoids (Honda et al., 2020a,2020b), and it has also been shown that xanthophyll Z-isomers are more bioaccessible than corresponding E-isomers (Coral-Hinostroza *et al.*, 2004; Yang *et al.*, 2017). However, this hypothesis remains to be verified in a future study.

Bioaccessibility measures the transfer efficiency of lipid phytochemicals from food matrices to micelles during digestion, which is a key step for bioavailability. Nevertheless, what is important in terms of animal or human nutrition is the absolute quantity of the compound of interest, here lutein, that reaches the intestinal cells in an absorbable form, i.e. micellarized lutein. This quantity depends both on the concentration of lutein in the food matrix and on its bioaccessibility in this matrix, i.e. quantity in the micelles = quantity provided by the matrix multiplied by bioaccessibility. The micellarized lutein quantity measured (**Figure 2A**) show that the larvae reared on parsley, endive, and broccoli did not provide intestinal cells with significantly different lutein quantities compared with their respective substrates. In other words, consuming 100 g of these substrates or 100 g of larvae having consumed these substrates does not bring different lutein quantity to intestinal cells. However, this figure shows that consuming 100 g of larvae reared on kale provided intestinal cells with about 4 times more lutein than 100 g of kale (**Figure 2A**). Note that when these data are expressed in dry weight (**Figure 2AB**), the larvae reared on parsley, endive and broccoli provide significantly less micellarized lutein than their respective substrates. On the other hand, the larvae reared on kale still provide significantly more micellarized lutein than their substrate. And it is also these kale larvae which provide more micellarized lutein than all the other substrates or larvae studied.

Together with bioaccessibility, another key step of lutein bioavailability is its intestinal uptake efficiency. Indeed, whether BSFL digestion releases one or more compounds that could modulate lutein uptake efficiency has never been studied. First of all, it is important to mention that the uptake efficiency of lutein from the micellar fraction of the digestion of the two plant matrices studied (parsley and kale), which was approximately 9%, was quite comparable to what was measured previously, i.e. 10 (Garrett *et al.*, 2000) and 15%

(Chitchumroonchokchai *et al.*, 2004), which supports the validity of our measurements. Secondly, we notice that micellarized lutein was taken up more efficiently, about 2 to 3 times, when it came from larvae than when it came from the corresponding substrates. This suggests that either the larvae do not contain one or more compounds present in the plant substrates which inhibit absorption, e.g. plant fibers (Mamatha and Baskaran, 2011), or, on the contrary, they contain one or several compounds that allow a better uptake of lutein by intestinal cells. One of these compounds could be the lipids brought by the larvae. Indeed, BSFL are very rich in lipids, triglycerides in particular, and it is likely that their hydrolysis by pancreatic lipase during *in vitro* digestion generated fatty acids and monoglycerides that changed the composition of mixed micelles. This enrichment of the micelles with triglyceride hydrolysis products can explain a better uptake of the micelles by the enterocyte by two mechanisms. The first is that these compounds, in particular fatty acids, allow a better interaction with the domains of the lipid rafts where SR-BI and CD36, which have been involved in carotenoid uptake (Reboul and Borel, 2011), are located (Goncalves *et al.*, 2013, 2015). The second is that this enrichment in hydrolysis products has reduced the negative charge of the micelles, which is what we observed, and this reduction has in turn reduced the electrostatic repulsion between the micelles and the apical membrane, which is also negatively charged.

Knowing the absolute quantity of micellarized lutein following the digestion of larvae relative to that following the digestion of the corresponding substrates, and knowing the cellular uptake efficiency of micellar lutein coming from larvae relative to that of micellar lutein coming from the substrates, it can be estimated that larva consumption would lead to a theoretical amount of absorbed lutein about 2 times (case of parsley) to about 13 times (case of kale) higher compared to that following the consumption of an equal quantity of their corresponding rearing substrate. This of course remains to be verified in an in vivo study but nevertheless, these promising results suggest that BSFL could constitute a nutritionally relevant source of lutein, what is more by recycling and valorizing lutein-rich vegetable waste.

To show the relevance of using BSFL enriched with lutein in animal nutrition, or even in human nutrition if they were to be authorized, we calculated the quantities of lutein that could be provided to different animal species if we included in their diet the quantity of larvae recommended for each species (**Table S1**). These theoretical calculations show in particular that lutein-rich larvae incorporated in the diet could provide one-third of the maximum recommended lutein intake in broiler chicken. More interestingly, their use could provide laying hens with up to 86% of the lutein amount that is usually added to the diet to obtain adequate yolk color.

In conclusion, this study shows that BSFL can be enriched in lutein at higher concentrations than some of the most lutein-rich vegetables, such as parsley and kale, when the concentrations are expressed in fresh weight. Moreover, the results of the *in vitro* digestion model coupled with Caco-2 cells suggest that that consumption of lutein-enriched larva would lead to higher amount of absorbed lutein compared to the consumption of an equal quantity of lutein-rich vegetables. Finally, our calculations show that the incorporation of lutein-enriched larvae in the feed of several species of farm animals would provide them with quantities of lutein which could sometimes be equivalent to the quantities necessary to satisfy the organoleptic quality criteria of their products. Furthermore, our study shows that the recycling of lutein from food waste containing lutein-rich plants by BSFL is theoretically possible. Indeed, the proteins and oils contained in BSFL are slowly being valorized by the food industry, but this study also shows that micronutrients such as lutein could also be valued from food waste streams, contributing to a circular economy and cleaner production. Finally, these results and those obtained in our previous study (Borel *et al.*, 2021) confirm that the enrichment of BSFL with vitamins or phytochemicals of nutritional and/or organoleptic

interest can lead to the production of BSFL with higher nutritional quality, thus improving most likely their market value.

Supporting information:

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

5) References

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