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1 Research article

2
3 **Black soldier fly larvae (*Hermetia Illucens*) as a sustainable and**
4 **concentrated source of bioavailable lutein for feed.**

5
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18
19 **Abstract**

20
21 Black soldier fly larvae (BSFL) are increasingly used to recycle and convert food
22 waste into feed. We attempted to assess whether they can bioaccumulate lutein, a xanthophyll
23 used as a food coloring, and whether it is then sufficiently bioavailable for an economically
24 relevant incorporation of BSFL into feed. Vegetables and larvae lutein concentrations were
25 measured by HPLC. Lutein bioaccessibility was estimated by *in vitro* digestion and lutein
26 absorption efficiency by Caco-2 cells. BSFL were at least as rich, and sometimes richer ($p <$
27 0.05), in lutein than the vegetables they were reared on. For example, the larvae reared on
28 kale contained 160.2 ± 3.4 mg/kg vs 23.0 ± 3.5 mg/kg of lutein, on a fresh weight basis, for
29 the kale substrate. For the same substrate, lutein bioaccessibility was not statistically different
30 between BSFL and the substrate (respectively 14.8 ± 1.2 % and 16.2 ± 2.8 %; $p = 0.7$).
31 Finally, by considering the lutein concentration in BSFL enriched in lutein and in lutein-rich
32 substrates, as well as the bioaccessibility and intestinal absorption efficiency of lutein
33 contained in these matrices, it was estimated that consumption of lutein-enriched larvae would
34 lead to a theoretical amount of absorbed lutein about 2 to 13 times higher compared to that
35 following the consumption of an equal quantity of lutein-rich vegetables. Thus, BSFL can be
36 used as a sustainable and concentrated source of bioavailable lutein for feed and, indirectly,
37 for food.

38
39
40 **Keywords:** edible insects, circular economy, nutritional quality, xanthophyll, bioaccessibility.

41
42 **Conflicts of interest:** BC and DS work in the BioMiMetiC company. This company conducts
43 research and development activities aimed at enhancing the value of entomo-conversion on a
44 wide variety of organic materials generated in the area at all levels of the food value chain.

45
46
47 **Abbreviation:**

48 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

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

101

102 **2) Material and Methods**

103

104 *Chemicals*

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

114

115 *Experimental foods*

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

124

125 *BSFL farming*

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

129

130 *Measurement of dry matter*

131 Larva dry matter was determined by measuring water loss after 72 h of freeze-drying.
132 For the substrates, dry matter values were taken from the CiquaI database
133 (<https://ciquaI.anses.fr/> Accessed 20.06.22).

134

135 *In vitro digestions to assess lutein bioaccessibility*

136 The *in vitro* digestion experiments used to measure lutein bioaccessibility were carried
137 out in quadruplicate as previously described (Borel *et al.*, 2021). Before the experiment, 1 g of
138 substrate or larva sample was ground with liquid nitrogen in a mortar grinder (Pulverisette 2,
139 FRITSCH GmbH, Idar-Oberstein, Germany) and mixed with 6.7 g mashed potato, 1.2 g
140 minced meat and 200 mg olive oil. The mixture was homogenized for 10 min at 37 °C in a
141 rotary incubator (190 rpm). Then, 2.5 mL of artificial saliva were added and the mixture was
142 once again incubated 10 min at 37 °C. After adjusting the pH to 4 ± 0.02, 2 mL pepsin were
143 added to the mixture, which was incubated for 30 min at 37 °C. Then, pH was adjusted to 6 ±
144 0.02 prior to the addition of 9 mL pancreatin-bile extract solution and 4 mL bile solution. The
145 mixture was then incubated for 30 minutes at 37 °C and the digestate aliquots were collected.
146 The remaining mixture was centrifuged at 1,860 × g for 1 h 12 min at 10 °C. Aliquots of
147 filtered supernatant, containing the mixed micelles were stored at - 80 °C until lipid extraction
148 and quantification of lutein by HPLC.

149

150 *Micelle size and zeta potential analysis*

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

156

157 *Caco-2 cell culture and lutein uptake efficiency*

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

170

171 *Lutein extraction*

172 Lutein was extracted as described previously for other carotenoids (Borel *et al.*, 2021),
173 using a sample volume of 500 μL . For food substrate and BSFL samples, 1 g was finely
174 crushed with liquid nitrogen and the powders thus obtained were diluted and homogenized in
175 20 mL distilled water.

176

177 *Lutein quantification*

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

185

186 *Statistics*

187 Results are expressed as means \pm SEM. The homogeneity of variances ($p > 0.05$) was
188 assessed by Levene's test. If the variances were inhomogeneous, data were log-transformed.
189 To assess the normality of the data, Q-Q plots of standardized residuals were used.
190 Differences between groups were tested using several two-way ANOVA. When a significant
191 effect of the matrix in which the lutein was incorporated, i.e. plant or insect, was highlighted
192 by the ANOVA, the comparison of the means between the plant matrix and the insect matrix
193 was carried out by pairwise comparisons using bilateral t-tests, with a Bonferroni adjustment.
194 Values of $p < 0.05$ were considered significant. All statistical analyses were performed using
195 R version 4.1.1 for Windows (R Core Team, 2021).

196

197 **3) Results**

198

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

201 **Figure 1** represents the lutein concentration in larvae reared on lutein-rich substrates
 202 and in these substrates. Neither mushrooms nor the larvae reared on these mushrooms
 203 contained any detectable amount of lutein while larvae reared on other substrates all contained
 204 lutein. What is most striking in **Figure 1A** (results expressed on a fresh weight basis) is that
 205 lutein concentration in larvae was always higher than lutein concentration in the
 206 corresponding rearing substrate. Moreover, larvae reared on kale contained 160.2 ± 3.4 mg/kg
 207 of lutein while kale contained 23.0 ± 3.5 mg/kg of lutein. Thus, lutein concentration in these
 208 larvae was about 7 times higher ($p < 0.001$) than in the corresponding substrate. Note that
 209 lutein concentration was still more than 3 times higher ($p < 0.001$) in these larvae than in kale
 210 on a dry weight basis (**Figure 1B**). The larvae reared on parsley contained more than twice as
 211 much lutein as the substrate itself on a fresh weight basis (69.8 ± 5.7 mg/kg vs 29.2 ± 1.4
 212 mg/kg; $p < 0.001$). On a dry weight basis however, there was no significant difference ($p =$
 213 0.75). Lutein concentration in endive was 0.2 ± 0.02 mg/kg and the larvae fed with endives
 214 contained 15 times more lutein (3.0 ± 0.2 mg/kg) on a fresh weight basis ($p < 0.001$), and still
 215 about 3 times more on a dry weight basis ($p < 0.001$). There was a significant difference in
 216 lutein concentration in broccoli and in larvae reared on broccoli (1.3 ± 0.07 and 2.4 ± 0.3
 217 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
 218 basis ($p < 0.05$), for broccoli and larvae respectively).

219
 220 **Figure 1. Lutein concentration (mg/kg) in rearing substrates and in larvae.**

221
 222 *Lutein bioaccessibility in larvae reared on lutein-rich substrates and in the corresponding*
 223 *substrates.*

224 Bioaccessibility values are shown in **Table 1**. For kale and broccoli, lutein
 225 bioaccessibility in the substrates and in the larvae was not significantly different. Conversely,

	Rearing substrate	Larvae	Substrate vs larvae comparison ** <i>p</i> -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

226 lutein bioaccessibility was about 2 times lower ($p < 0.01$) in larvae reared on endive and
 227 parsley as compared to the corresponding substrates. Lutein bioaccessibility was not
 228 significantly different between the substrates while lutein bioaccessibility in the larvae
 229 exhibited some variability according to the substrates, namely it was approximately twice as
 230 high in larvae reared on broccoli and kale compared to larvae reared on endive and parsley
 231 (means around 14% for the former and 8% for the latter).

232
 233 **Table 1. Bioaccessibility of lutein* from larvae and their corresponding rearing**
 234 **substrates.**

235 *Percent of lutein recovered in the micelle fraction relative to that found in the digestate at the
 236 end of the *in vitro* digestion. Values are means \pm SEM (n=4). ** *p*-value of a two-way
 237 ANOVA, followed by pairwise comparisons using bilateral t-tests.

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

240

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

243

244 **Figure 2** shows the lutein amount in the micelle-rich fractions obtained after *in vitro*
 245 digestion of either larvae fed lutein-rich substrates or their corresponding substrates. This
 246 amount depends both on lutein concentration in the matrix (larvae or substrates) and on lutein
 247 bioaccessibility in the matrix. The amount of lutein in micelles corresponds to the quantity of
 248 lutein that is presented to the intestinal cell for its absorption. On a fresh weight basis (**Figure**
 249 **2A**), the mixed micelles obtained after digestion of larvae fed on kale contained about 4 times
 250 more lutein than those obtained after digestion of kale (21.9 ± 2.5 and 5.4 ± 1.0 mg/kg,
 251 respectively; $p < 0.001$). On a dry weight basis (**Figure 2B**), they still contained more than
 252 twice as much lutein as those obtained after kale digestion (72.9 ± 8.5 and 31.7 ± 5.8 mg/kg,
 253 respectively; $p < 0.01$). For the other substrates, on a fresh weight basis, no significant
 254 difference was observed between larvae and their corresponding substrates. Conversely, on a
 255 dry weight basis, the quantity of lutein in micelles that came from the digestion of larvae
 256 compared to those that came from the digestion of their corresponding substrates differed
 257 significantly for parsley ($p < 0.001$), endive ($p < 0.01$) and broccoli ($p < 0.01$).

258

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

261

262 *Zeta potential of mixed micelles from in vitro digestions*

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

270

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

	Zeta potential (mV)		Substrate vs larvae comparison * <i>p</i> -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

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

274

275 *Uptake efficiency of micellized lutein by Caco-2 cells*

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

282 efficiently taken up compared to lutein from the fraction obtained after digestion of kale ($p <$
 283 0.001). For parsley, cellular uptake was approximately 2 times greater when lutein was
 284 supplied by the micellar fraction from larva digestion than by the micellar fraction from
 285 substrate digestion ($p < 0.001$).

286
 287

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

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

292

293 **Figure 3** shows the amount of lutein taken up by Caco-2 cells. This value is very
 294 interesting because it depends both on the concentration of lutein in the matrix (plant substrate
 295 or larvae), on the bioaccessibility of lutein in this matrix, and on the efficiency of absorption
 296 of micellized lutein by the intestinal cell. It therefore makes it possible to compare the
 297 quantity of lutein absorbed by the cells, i.e. bioavailable, for the same quantity of digested
 298 matrix. The cells incubated with micelles from the digestion of larvae reared on kale
 299 contained around 7 (when normalized to dry weight) to around 13 (when normalized to fresh
 300 weight) more lutein than the cells incubated with micelles from the digestion of kale, i.e. 40.2
 301 ± 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
 302 mg/kg respectively (fresh weight; $p < 0.001$). Regarding parsley, there was a significant
 303 difference ($p = 0.012$) between the cells incubated with micelles from the digestion of larvae
 304 reared on parsley (1.9 ± 0.3 mg/kg) and those incubated with micelles from the digestion of
 305 parsley (0.9 ± 0.1 mg/kg) on a fresh weight basis, but not on a dry weight basis ($p = 0.3$)

306

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

309

310 4) Discussion

311

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

327 provitamin A carotenoids (Borel *et al.*, 2021; Leni *et al.*, 2022) and vitamin E (Shumo *et al.*,
328 2019) suggests that they act as a fat tank having the ability to store various fat-soluble
329 molecules by solubilizing them (Borel *et al.*, 1996; Goupy *et al.*, 2020).

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

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

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

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

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

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

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

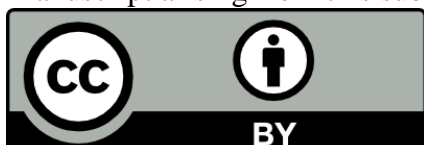
427 interest can lead to the production of BSFL with higher nutritional quality, thus improving
428 most likely their market value.

429

430 **Supporting information:**

431 The costs of this project were covered equally by the own budget of P. Borel's research team,
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435 document and will be applied to all subsequent versions up to the Author Accepted
436 Manuscript arising from this submission.



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439 **5) References**

440

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