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1 **USING BLACK SOLDIER FLY LARVAE REARED ON FRUITS AND VEGETABLES**
2 **WASTE AS A SUSTAINABLE DIETARY SOURCE OF PROVITAMIN A CAROTENOIDS.**

3

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10

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12 human food; entomo-conversion; *Hermetia illucens*.

13

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21

22

23 **Abstract**

24 We showed that black soldier fly larvae reared on fruits and vegetables rich in provitamin A
25 carotenoids can accumulate significant amounts of these vitamin A precursors. Using a
26 simulated gastro-intestinal digestion model, we demonstrated that α - and β -carotene from the
27 larvae are as bioaccessible as from the fruits and vegetables they were reared on. We calculated
28 that provitamin A carotenoid-rich larvae have the capacity to provide more vitamin A than
29 fruits and vegetables rich in these molecules. Remarkably, the incorporation of usual quantities
30 of these larvae in feed could cover the needs of several production animals for this vitamin.
31 Thus, our findings suggest that rearing black soldier fly larvae on by-products or waste rich in
32 provitamin A carotenoids could be a sustainable strategy to recycle a fraction of vitamin A
33 back into the food chain and could represent a new approach to fight against vitamin A
34 deficiency.

35

36 **1) Introduction**

37

38 The term vitamin A describes a group of fat-soluble molecules that can be obtained
39 from the diet as preformed vitamin A (mainly retinyl esters) and from provitamin A
40 carotenoids. The former is found in foods of animal origin while the latter are found mainly in
41 vegetables and fruits. There are three main provitamin A carotenoids in the human diet: β -
42 carotene, α -carotene and β -cryptoxanthin. Their capacity to be converted into vitamin A, i.e.
43 their provitamin A activity, is intrinsically different because it depends on the number of β -
44 ionone rings they possess, i.e. 2 for β -carotene and 1 for α -carotene and β -cryptoxanthin. It also
45 depends on a diet-responsive regulatory network that controls their intestinal uptake and their
46 conversion into vitamin A through a negative feedback regulation of *SCARBI* (scavenger
47 receptor class B member 1) and *BCOI* (beta-carotene oxygenase 1) expression via *ISX*
48 (intestine specific homeobox) in the intestine (Lobo, Amengual, Baus, Shivdasani, Taylor, &
49 von Lintig, 2013). It finally depends on their bioavailability, i.e. on the fraction that is absorbed
50 and available for use by the body, which is highly dependent on the food matrix they are
51 incorporated in (Desmarchelier & Borel, 2017). Although preformed vitamin A or provitamin
52 A carotenoids are present in many foods, vitamin A deficiency is frequent, whether in humans
53 (<https://www.who.int/vmnis/database/vitamina/en/>. Accessed 31.03.21) or animals, due to both
54 low intakes of vitamin A food sources and to variations in provitamin A carotenoids
55 bioavailability and conversion efficiency. Despite the implementation of numerous programs
56 (Mason, Greiner, Shrimpton, Sanders, & Yukich, 2015), the prevalence of vitamin A deficiency
57 in low income countries has remained high, calling for new and more sustainable strategies to
58 tackle this public health issue. This is all the more relevant considering the context of
59 increasing world population, leading to an increase in vitamin A needs, together with global
60 warming, leading to a probable decrease in yields and area under cultivation. Moreover,

61 breeders often have to add vitamin premixes to their animal feed to meet their nutritional needs
62 for optimal growth and health and many animal production systems would benefit from
63 cheaper and more sustainable vitamin A sources.

64 There are currently more than 1,900 insects classified as edible
65 (https://www.wur.nl/upload_mm/8/a/6/0fd35a1696-Worldwide%20list%20of%20edible%20insects%202017.pdf. Accessed
66 [31.03.21](https://www.wur.nl/upload_mm/8/a/6/0fd35a1696-Worldwide%20list%20of%20edible%20insects%202017.pdf)). High levels of carotenoids, including provitamin A carotenoids, are found in various
67 insect species and high concentrations of retinol and β -carotene were measured in two edible
68 species, *Eublabeus distanti* and *Gromphadorhina portentosa* (Ruth Charrondiere, Stadlmayr,
69 Rittenschober, Mouille, Nilsson, Medhammar, et al., 2013). Furthermore, some insect species
70 were reported to be able to bioaccumulate different lipid molecules from food substrates they
71 were reared on (Liland, Biancarosa, Araujo, Biemans, Bruckner, Waagbo, et al., 2017) and a
72 proof of concept that insects can accumulate provitamin A carotenoids, which are very
73 hydrophobic lipids, is provided by the fact that feeding migratory locusts with carrots increased
74 their β -carotene content (Oonincx & Poel, 2011). We have thus hypothesized that some edible
75 insect species could constitute significant dietary sources of vitamin A for feed and food, either
76 by their natural vitamin A content or by the vitamin A they could accumulate. Although insects
77 are not yet commonly eaten around the world and issues of health safety or cultural acceptance
78 remain (Roma, Palmisano, & De Boni, 2020), they are already used as an complementary
79 source of proteins in food and feed in several countries where vitamin A deficiency still
80 prevails (Kenis, Koné, Chrysostome, Devic, Koko, Clottey, et al., 2014; Xu, Shan, Lin, Miao,
81 Lou, Wang, et al., 2021). Thus, the identification of an insect species rich in vitamin A, either
82 naturally or through enrichment following the choice of adequate rearing substrates, would
83 provide these countries with an additional and sustainable approach to increase vitamin A
84 intakes if used as a food, or even indirectly if used as a feed for livestock.
85

86 We used the black soldier fly (*Hermetia illucens*) as a candidate to investigate whether
87 insects can constitute a significant dietary source of vitamin A for four main reasons. Firstly,
88 this species, which is native to the American continent, is now distributed globally throughout
89 the tropic and temperate zones, likely due to human-mediated transport. Thus, it can be bred in
90 many countries without risk of becoming an invasive species. Secondly, it is the only insect
91 species that has been approved for use as an animal feed in the US (for salmonid fish) and
92 Canada (for salmonid fish and broiler poultry). Therefore, we already have guarantees on the
93 health security of feeding this insect species to certain animals and we can reasonably assume
94 that this edible insect species will also be safe for the diet of other animal species and probably
95 for humans. Thirdly, this insect species is already used to convert many types of organic
96 substrates, e.g. fruits and vegetables or crop wastes, into valuable nutrients, e.g. proteins and
97 lipids, which are already reinjected in the food chain by providing them to livestock (Biasato,
98 Renna, Gai, Dabbou, Meneguz, Perona, et al., 2019) (feed) or potentially to humans (food)
99 (Wang & Shelomi, 2017). Therefore, we can take advantage of the exceptional capacity of this
100 edible insect species to put vitamin A back into the food chain, which is usually lost in plant
101 waste. The fourth reason to study the potential of black soldier fly larvae (BSFL) to accumulate
102 provitamin A carotenoids from fruits and vegetables by-products is the observation that their
103 nutritional composition partly depends on the composition of their feeding substrate (Liland, et
104 al., 2017). Finally, the fact that the fat content of BSFL is high, ranging between about 8% to
105 about 28-29% on a dry mass basis (X. Liu, Chen, Wang, Yang, Ur Rehman, Li, et al., 2017),
106 together with the observation that BSFL can accumulate lipids, e.g. monounsaturated and
107 omega-3 fatty acids as well as vitamin E (Barroso, Sanchez-Muros, Segura, Morote, Torres,
108 Ramos, et al., 2017; Liland, et al., 2017), make it a very good candidate to study its ability to
109 accumulate significant concentrations of provitamin A carotenoids.

110 On the basis of the rationale and assumptions set out above, this study first aimed at
111 assessing whether BSFL can accumulate significant amounts of provitamin A carotenoids when
112 they are reared on food substrates rich in these vitamin A precursors. It also aimed to verify
113 whether the provitamin A carotenoids accumulated in the larvae are bioaccessible, using an *in*
114 *vitro* model of digestion (Berthelsen, Klitgaard, Rades, & Müllertz, 2019; Dupont, Alric,
115 Blanquet-Diot, Bornhorst, Cueva, Deglaire, et al., 2019). The bioaccessibility of a fat-soluble
116 micronutrient, e.g. fat-soluble vitamins, carotenoids, is the proportion of this compound that is
117 incorporated in mixed micelles during digestion and is acknowledged as a good estimate of its
118 bioavailability (Bohn, Carriere, Day, Deglaire, Egger, Freitas, et al., 2018).

119 **2) Material and methods**

120

121 *Chemicals*

122 Ethanol, *n*-hexane and HPLC grade dichloromethane, methanol, methyl tert-butyl ether
123 and water were purchased from Carlo Erba reagents (Peypin, France). Carotenoid standards
124 (HPLC purity > 96%), i.e. α -, β -carotene, β -cryptoxanthin and echinenone, were from
125 Carotenature GmbH (Müdingen, Switzerland). All other chemicals and enzymes were from
126 Sigma-Aldrich (Saint-Quentin-Fallavier, France).

127

128 *Experimental foods*

129 Foods used as substrates for BSFL rearing, i.e. white mushrooms and fruits and
130 vegetables rich in provitamin A carotenoids, namely orange carrots (Hammaz, Charles, Kopec,
131 Halimi, Fgaier, Aarrouf, et al., 2021), Cinderella pumpkins, sweet potatoes and clementines, as
132 well as foods used in the in vitro digestion experiments, i.e. potatoes, minced beef meat (with
133 5% of fat) and olive oil, were purchased from local supermarkets.

134

135 *BSFL farming*

136 BSFL were reared either on fruits and vegetables rich in provitamin A carotenoids or on
137 white mushrooms, which served as the control food substrate since they do not contain
138 provitamin A carotenoids. The rearing protocol was implemented and carried out by
139 BioMiMetiC (Avignon, France). The objective was to obtain approximately 200 g of larvae,
140 weighing approximately 150 mg each, on each food substrate. As a result, around 1,300 larvae
141 were deposited on each food substrate. This approximate number of larvae was obtained by
142 sowing 35 ± 5 mg eggs on each substrate. Indeed, the egg mass is about 24 ± 1 μ g (Tomberlin,
143 Sheppard, & Joyce, 2002). The amount of each food substrate to add in the rearing boxes was

144 calculated in order to provide enough food to the larvae all along the growth period (i.e. 14
145 days) and to reach an individual larval mass of around 150 mg. This was calculated both by
146 considering that 340 mg of food substrate on a dry matter basis are needed to obtain
147 approximately an individual larval mass of 150 mg (Diener, Zurbrügg, & Tockner, 2009), and
148 by considering the relative humidity of the food substrates. The rearing procedure was as
149 follows: first, after collecting eggs from an oviposition support, they were placed in a rearing
150 room settled on a temperature of 29 ± 1 °C and a relative humidity of $65 \pm 5\%$. Twenty-four
151 hours later, whole fresh fruits, vegetables and mushrooms recently bought in a local
152 supermarket were cut into small pieces, placed in the rearing trays (clear plastic boxes $267 \times$
153 380×150 mm high) and coarsely ground using a hand blender. Once the food substrates were
154 prepared, hatching devices were placed on each substrate, allowing eggs to hatch about 3 cm
155 above the food substrates. Eggs were from black soldier fly colony maintained by BioMiMetiC
156 and were laid less than 24 hours before being used in the experiment. Each growing tray was
157 placed in a larger plastic box ($350 \times 418 \times 180$ mm high). Boxes were closed with mosquito net
158 and placed in a rearing room. Eggs and larvae from eggs were raised for 14 days at 29 ± 1 °C
159 and $65 \pm 5\%$ relative humidity, under 14:10 artificial light/dark cycles. At the end of the rearing
160 period, larvae were separated from their food substrates by sieving (2 x 2 mm). They were then
161 washed using tap water and put in a temperate water bath. They were finally filtered again
162 through a sieve (2 x 2 mm) and transferred to a plastic box containing only absorbent paper in
163 order to remove excess moisture. Larvae were kept for 5 days in this bare box in order to empty
164 their digestive tract from any remaining substrate. After this period, larvae were separated from
165 their exuviae and droppings, and washed again using tap water and a sieve to eliminate any
166 remaining organic particles. They were finally dried using absorbent paper before being frozen
167 at -20°C.

168

169 *Measurement of the bioaccessibility of provitamin A carotenoids from BSFL and their rearing*
170 *food substrates*

171 Provitamin A carotenoid bioaccessibility, i.e. the relative amount of provitamin A
172 carotenoids that is transferred to mixed micelles during digestion, was assessed by using an in
173 vitro model (Reboul, Richelle, Perrot, Desmoulins-Malezet, Pirisi, & Borel, 2006), which
174 provides similar estimates of carotenoid bioaccessibility for diverse foods compared to the
175 Infogest model (Rodrigues, Chitchumroonchokchai, Mariutti, Mercadante, & Failla, 2017). In
176 summary, 2 g of sample (BSFL or food substrates coarsely crushed with a mortar), or lipids
177 extracted from 2 g of BSFL, were placed in a 100 mL Erlenmeyer flask and mixed with 6.7 g
178 mashed potatoes, 1.2 g minced meat and 200 mg olive oil and ground for 30 s at 22,000 rpm
179 (T18 basic Ultra-Turrax disperser, IKA, Staufen, Germany). The mixture was homogenized
180 10 min at 37°C in a rotating incubator (190 rpm). To mimic the oral phase of digestion, 2.5 mL
181 artificial saliva were then added and the mixture was incubated 10 min at 37°C in a rotating
182 incubator (190 rpm). To mimic the gastric phase of digestion, the pH was adjusted to 4 ± 0.02
183 with 1 M HCl and 2 mL pepsin were added to the mixture which was then incubated for 30 min
184 at 37°C in a rotating incubator (190 rpm). To mimic the duodenal phase of digestion, the pH
185 was adjusted to 6 ± 0.02 with 0.9 M NaHCO₃ buffer before addition of 9 mL of a pancreatin-
186 bile extract solution and 4 mL of a bile solution. The mixture was then incubated for 30 min at
187 37°C in a rotating incubator (190 rpm). Aliquots of 4 mL of the mixture, thereafter called
188 digestate aliquots, were then collected. The remaining mixture was centrifuged at 1,860 x g for
189 1 h 12 min at 10°C. The recovered supernatant was filtered through a 0.8 µm filter and then
190 through a 0.22 µm filter (mixed cellulose esters; Millipore, Molsheim, France). The clear
191 aqueous samples obtained, which contain the mixed micelles, were stored at -80°C until lipid
192 extraction and quantification of provitamin A carotenoids by HPLC.

193

194 *Lipid extraction from BSFL*

195 Lipid extraction was carried out according to Bligh and Dyer with slight modifications.
196 BSFL were first crushed with liquid nitrogen as follows: about 3 g of whole larvae were ground
197 for 30 s under liquid nitrogen in a mortar grinder (Pulverisette 2, FRITSCH GmbH, Idar-
198 Oberstein, Germany) and the larval powder obtained was stored at -80°C until further analysis.
199 A sample (1 g) of the larval powder was homogenized with 3.75 mL of a solution of
200 trichloromethane/methanol (1:2) that contained 50 µl of 0.05% butylated hydroxytoluene
201 (antioxidant to protect the carotenoids from degradation). The mixture was then vortexed for
202 2 min before addition of 3.75 mL of trichloromethane. The mixture was vortexed again for 2
203 min before addition of 3.75 mL of a NaCl solution (9 g/L). After 2 min of vortex, it was
204 centrifuged for 15 min at 1,257 x g. The lower fraction of the two liquid phases was gently
205 removed with a Pasteur pipette and transferred directly into a pre-weighed tube. A second
206 extraction of the upper fraction was carried out in the same way by adding 3.75 mL of
207 trichloromethane. After 2 min of vortexing and 15 min of centrifugation at 1,257 x g, the lower
208 fraction was added to the first one in the pre-weighed tube. The pooled lower fractions were
209 then evaporated to dryness under nitrogen at 40°C. Tubes were weighed again to determine the
210 amount of dried lipids (**Table 1**).

211

212 *Provitamin A carotenoid extraction from BSFL and food substrates*

213 For BSFL and food substrates, 2 g of samples finely ground with a mortar were first
214 homogenized in 50 mL of distilled water. Then, a volume of 500 µl was taken, to which 500 µl
215 of ethanol containing echinenone (as internal standard) was added. For in vitro digestion
216 samples, i.e. digestates and mixed micelle-rich aqueous fractions, 2 mL were extracted to
217 which the same volume of ethanol, containing the internal standard, was added. A double
218 extraction with hexane was carried out on all samples. After centrifugation at 1,257 x g during

219 10 min at 4 °C, the hexane phases were recovered and then evaporated under nitrogen until a
220 dry film was obtained, which was then solubilized in 200 µL of methanol/dichloromethane
221 (65/35, v/v).

222

223 *Carotenoid quantification*

224 Injection volumes of 50 µL (BSFL and food substrate samples) and 150 µL (mixed
225 micelle samples and digestate samples) were used for HPLC analysis. Carotenoids were
226 separated as previously described (Hammaz, et al., 2021) and quantified at 450 nm. HPLC
227 analyses were carried out in gradient mode on a YMC-Pack YMC C30 column (250 x 4.6 mm;
228 5 µm) (Crawford Scientific Ltd, Strathaven, UK) preceded by a pre-column (10 x 4 mm; 5 µm)
229 set at 35°C. The HPLC system comprised a Dionex separation module (P680 HPLC pump and
230 ASI-100 Automated Sample Injector; Dionex, Aix-en-Provence, France), and a Dionex
231 UVD340U photodiode array detector. Carotenoid peaks were identified in the HPLC
232 chromatograms by comparing them to retention times and absorption spectrum of external
233 standards.

234

235 *Calculations and statistics*

236 Data were expressed as means ± SEM. Differences between means were tested using
237 either Student's *t*-test or ANOVA. Prior to Student's *t*-test or ANOVA, homogeneity of
238 variances was checked by Levene's test. In case of inhomogeneous variances, Welch's
239 correction was applied to Student's *t*-test while data were log-transformed for ANOVA. For
240 ANOVA, when Fisher's test was significant, Tukey-Kramer's test, which maintains the family-
241 wise error rate at $\alpha=0.05$, was used as a post hoc test for pairwise comparisons. For all tests, the
242 bilateral alpha risk was $\alpha=0.05$. Statistical comparisons were performed using StatView
243 software, version 5.0 (SAS Institute Inc., Cary, NC, USA).

244 **3) Results**

245

246 *Effect of the food substrate type on larval growth efficiency and larval lipid mass*

247 This study was not dedicated to compare the effect of the different food substrates on
248 larval growth efficiency, therefore we did not run biological replicates and hence we cannot
249 compute statistical tests. However, the wide-ranging masses of larvae obtained at the end of the
250 rearing period (**Table 1**), i.e. from 33.1 g with the clementine substrate to 125.7 g with the
251 sweet potato substrate, suggest that BSFL did not develop and grow with the same efficiency
252 on the different food substrates. Furthermore, they did not grow as efficiently on these
253 experimental substrates, which were constituted of only one species of fruits, vegetables or
254 mushrooms, as on the standard food substrate used by BioMiMetiC (complete feed for poultry,
255 Moulin des Hauts Rochers, Saint Grégoire, France). Indeed, this substrate usually provides
256 about 200 g larvae in the same rearing conditions (BioMiMetiC internal data). Moreover, the
257 fat masses of the larvae reared on the different experimental substrates (**Table 1**) were
258 markedly lower than that of larvae reared on their standard food substrate, i.e. about 16 g/100 g
259 (BioMiMetiC internal data). More precisely, they ranged between 20% (clementines) to 54%
260 (pumpkins) of this fat mass of reference. In summary, the experimental food substrates yielded
261 lower larva masses, which had less fat than larvae reared on their standard food substrate.

262

263 *Effect of the food substrate type on BSFL provitamin A carotenoid content*

264 β -Carotene concentrations in BSFL reared on the different food substrates are shown in
265 **Figure 1A**. The first noteworthy observation is that there was no detectable β -carotene in
266 larvae reared on the control food substrate, i.e. mushrooms, which do not contain this
267 provitamin A carotenoid. Conversely, when β -carotene was present in a food substrate, it was
268 found in the larvae reared on this substrate. More precisely, β -carotene concentrations were

269 44% and 32% lower in carrot and pumpkin-fed larvae, respectively, and 66% higher in sweet
270 potato-fed larvae than those measured in their respective substrates ($p=0.059$, $p=0.005$, and
271 $p=0.03$, respectively).

272 **Figure 1B** shows α -carotene concentrations in the food substrates and in the larvae fed
273 with these substrates. As observed for β -carotene, α -carotene was detected only in larvae reared
274 on food substrates that contained this provitamin A carotenoid, i.e. carrots and pumpkins. α -
275 Carotene concentrations in whole larvae were lower than those found in their rearing food
276 substrate, by 35% and 28% for carrot and pumpkin-fed larvae, respectively ($p=0.05$ and 0.036 ,
277 respectively).

278 β -Cryptoxanthin concentration in the larvae reared on the different food substrates is
279 shown in **figure 1C**. As observed for the two other provitamin A carotenoids, when no β -
280 cryptoxanthin was present in the food substrate, i.e. carrots, sweet potatoes and mushrooms, no
281 β -cryptoxanthin was recovered in the larvae. While there was no detectable β -cryptoxanthin in
282 pumpkin-fed larvae, the concentration of β -cryptoxanthin in clementine-fed larvae was 8 times
283 higher than that in the clementines ($p=0.01$).

284

285 *Provitamin A carotenoid concentrations in larval lipids*

286 Provitamin A carotenoid concentrations in larval lipids were systematically higher than
287 those measured in whole larvae (**Table 2**). They ranged from 3 times higher (β -cryptoxanthin
288 from clementine-fed larvae) to 5 times higher for α -carotene in carrot- and pumpkin-fed larvae.
289 However, these differences were not statistically significant due to the fact that the quantities of
290 lipids extracted from the larvae were very low and only 2 measurements could be made for this
291 fraction. This is due to the fact that the measurements of the concentration of provitamin A
292 carotenoids in larval lipids were not foreseen in our experimental plan. We must therefore
293 consider these data as exploratory.

294 Knowing the percentage of lipids of the larvae, the concentrations of provitamin A
295 carotenoids in the whole larvae, and the concentrations of provitamin A carotenoids in the
296 lipids of the larvae, we calculated the proportion of each provitamin A carotenoids found in the
297 lipid fraction of the larvae. These calculations show that the proportions of β -carotene, α -
298 carotene and β -cryptoxanthin were approximately 41%, 32% and 11% respectively (data not
299 shown).

300

301 *Effect of the food substrate type on BSFL preformed vitamin A content*

302 Both retinol and retinyl palmitate were found in larvae reared on the different food
303 substrates. Retinol concentration ranged between 8 and 12 $\mu\text{g}/100$ g fresh weight and retinyl
304 palmitate ranged between 140 and 200 $\mu\text{g}/100$ g. Therefore, retinyl palmitate represented about
305 95% of the vitamin A mass. Furthermore, there was no significant difference between the
306 concentrations of retinol and retinyl palmitate in the different groups (data not shown).

307

308 *Bioaccessibility of provitamin A carotenoids in BSFL and food substrates*

309 **Figure 2A** shows the bioaccessibility of β -carotene from BSFL and from their
310 corresponding food substrates following *in vitro* digestions. β -Carotene bioaccessibility did not
311 differ in carrot-fed and sweet potato-fed larvae and in their corresponding rearing substrates
312 ($p=0.153$ and $p=0.885$, respectively). Conversely, it was significantly higher ($p=0.013$) in
313 pumpkin-fed larvae than in pumpkins.

314 The bioaccessibility of α -carotene in carrot-fed larvae did not differ from that measured
315 in carrots ($p=0.197$) (**Figure 2B**). Concerning pumpkins, α -carotene bioaccessibility was
316 significantly higher when originating from larvae than from the rearing food substrate
317 ($p=0.033$).

318 **Figure 2C** shows the bioaccessibility of β -cryptoxanthin from the food substrates that
319 contained this carotenoid, i.e. pumpkins and clementines, and from the larvae reared on these
320 substrates. It is noteworthy that β -cryptoxanthin bioaccessibility was high in the substrates, i.e.
321 $82 \pm 18\%$ in pumpkins and $68 \pm 7\%$ in clementines. Conversely, β -Cryptoxanthin
322 bioaccessibility in pumpkin- and clementine-fed larvae was dramatically lower, about 84% and
323 91%, than that in pumpkins and clementines respectively ($p=0.008$ and $p=0.035$, respectively).

324

325 *Bioaccessibility of provitamin A carotenoids in larval lipid fractions*

326 Lipids extracted from the larvae were subjected to in vitro digestion to measure the
327 bioaccessibility of provitamin A carotenoids located in this fraction, which can be added in the
328 diet of certain livestock (**Table 2**). The bioaccessibility of provitamin A carotenoids from larval
329 lipids seemed to be higher compared to that from whole larvae for BSFL reared on carrots and
330 clementines, whereas it seemed lower in larval lipids than in whole larvae for BSFL reared on
331 pumpkins. The differences were not significant because of the very low number of repetitions
332 due to the low quantity of lipids. As explained above, this is due to the fact that the
333 measurements of provitamin A carotenoids in larval lipids were not foreseen in our
334 experimental plan. We must therefore consider these data as exploratory.

335

336 *Theoretical bioaccessible vitamin A amounts provided by provitamin A carotenoid-rich larvae* 337 *as compared to the substrates they were reared on*

338 In order to estimate the potential of the larvae as a significant dietary source of vitamin
339 A, we calculated the theoretical quantities of bioaccessible vitamin A provided by the larvae
340 reared on the various substrates rich in provitamin A carotenoids, and we compared these with
341 those theoretically brought by the substrates which they were reared on (**Table 3**). The results
342 of these calculations show that the larvae enriched with provitamin A carotenoids can

343 theoretically provide more bioaccessible vitamin A per unit of fresh weight than the substrates
344 rich in vitamin A which they were reared on. In fact, if we calculate the ratio between the
345 quantity theoretically provided by the larvae and the quantity theoretically provided by the
346 substrates which they were reared on, we observe that the larvae can provide 2 to 12 times
347 more vitamin A than the substrates they were reared on.

348

349 *Proportions of vitamin A requirements provided by the consumption of provitamin A*
350 *carotenoid-rich BSFL*

351 We then investigated whether incorporating these BSFL, or fractions thereof, into the
352 diet of different production animals could provide significant amounts of vitamin A to these
353 animals (**Table 4**). Our calculations show that the incorporation of usual quantities of BSFL, or
354 certain fractions thereof, in the diet of several production animal species, could largely meet
355 their vitamin A requirements. With regard to humans, we have calculated the daily quantities of
356 larvae necessary to meet the recommended dietary allowances in vitamin A. Although these
357 quantities seem relatively large (210 g/d for females and 245 g/d for males), a few tens of g/d
358 would nonetheless provide a significant part of the daily intake of vitamin A.

359 **4) Discussion**

360

361 The first objective of this study was to assess whether BSFL can accumulate significant
362 amounts of provitamin A carotenoids when reared on different food substrate rich in these
363 vitamin A precursors. We chose orange carrots and pumpkins as sources of both α - and β -
364 carotene, and clementines as a source of β -cryptoxanthin. White mushrooms were chosen as the
365 control food substrate since they do not contain carotenoids. Food substrates were provided
366 singly in order to measure their intrinsic capacity to allow the accumulation of provitamin A
367 carotenoids by BSFL. As expected, the growth of larvae provided these nutritionally
368 unbalanced food substrates was less efficient than that of larvae provided a standard food
369 substrate, but this did not affect the study objectives.

370 Our results clearly show that BSFL reared on food substrates rich in provitamin A
371 carotenoids can accumulate significant amounts of these compounds. To the best of our
372 knowledge, this constitutes the first demonstration thereof (Ruth Charrondiere, et al., 2013).
373 We put forward that BSFL do not synthesize provitamin A carotenoids and solely obtain them
374 from their food substrate. This hypothesis is supported by two arguments. Firstly, we did not
375 detect any provitamin A carotenoids in larvae that were reared on white mushrooms, which do
376 not contain provitamin A carotenoids. Secondly, provitamin A carotenoid species found in
377 larvae were only those present in the substrates they were reared on. Moreover, although these
378 substrates provided very different amounts of provitamin A carotenoids, the concentration of
379 preformed vitamin A, i.e. retinol and retinyl palmitate, which can be converted from provitamin
380 A carotenoids by many animal species (Borel & Desmarchelier, 2017), was not significantly
381 different between BSFL reared on the different food substrates. This suggests that BSFL cannot
382 convert provitamin A carotenoids into vitamin A, i.e. they likely do not possess any active
383 carotene oxygenase (Lobo, Amengual, Palczewski, Babino, & von Lintig, 2012; von Lintig &

384 Vogt, 2004). Interestingly, the concentrations of the accumulated provitamin A carotenoids
385 were fairly high, reaching the same order of magnitude as those in fruits and vegetables known
386 to be rich in these compounds, and even giving a light orange color to the carrot-fed larvae.
387 These concentrations were at least two thirds of those measured in the food substrates and they
388 were sometimes even higher, i.e. β -carotene in sweet potato-fed larvae and β -cryptoxanthin in
389 clementine-fed larvae. This can be partly explained by the fact that larvae are not able to
390 convert provitamin A carotenoids to vitamin A or other metabolites, thus leading to a
391 bioaccumulation of these compounds. Composition of the larval diet, e.g. its lipid content, may
392 also modulate carotenoid bioavailability, and thus bioaccumulation, but further studies are
393 needed to determine the optimal nutrient composition of the diet to maximize carotenoid
394 bioaccumulation.

395 The higher concentrations of provitamin A carotenoids found in larval lipids as
396 compared to whole larvae, together with the fact that these molecules are very hydrophobic,
397 suggested at first that most provitamin A carotenoids were located in the lipid fraction of the
398 larvae. However, we calculated that only 11% of β -cryptoxanthin and around 40-43% of α - and
399 β -carotene were present in the lipid extracts. Carotenoids, although very hydrophobic, are
400 actually relatively poorly soluble in triglycerides (Borel, Grolier, Armand, Partier, Lafont,
401 Lairon, et al., 1996; Goupy, Genot, Hammaz, Halimi, Caris-Veyrat, & Borel, 2020). In
402 addition, they can associate with proteins (Goupy, Genot, Hammaz, Halimi, Caris-Veyrat, &
403 Borel, 2020; Mensi, Borel, Goncalves, Nowicki, Gleize, Roi, et al., 2014) as well as with
404 phospholipids (Borel, et al., 1996; Goupy, Genot, Hammaz, Halimi, Caris-Veyrat, & Borel,
405 2020). Whatever their location in the non-lipid fraction of the larvae, these molecules are
406 distributed in the two fractions of the larvae that are currently used in feed (see examples in
407 **Table 4**) and that could be used in food according to present legislation in different countries.

408

409 Since carotenoids are usually not efficiently absorbed (Desmarchelier & Borel, 2017)
410 and since many dietary factors can impair their absorption (Desmarchelier & Borel, 2017), the
411 second part of this study was dedicated to comparing the bioaccessibility of the provitamin A
412 carotenoids recovered in the larvae, or in their lipid extract, with that of carotenoids present in
413 the fruits and vegetables they were reared on. Indeed, we hypothesized that some compounds
414 present in insects, such as chitin, which is found in BSFL and which could reduce triglyceride
415 absorption (Zacour, Silva, Cecon, Bambirra, & Vieira, 1992), might impair provitamin A
416 carotenoid bioaccessibility. The results obtained suggest that the effects of the larval
417 components on carotenoid bioaccessibility depend on the carotenoid species. Indeed, while the
418 bioaccessibility of α - and β -carotene from larvae were not different, or even significantly
419 higher, compared to that from their food substrates, the bioaccessibility of β -cryptoxanthin
420 from the pumpkin and the clementine-fed larvae was dramatically lower than that from their
421 substrates. β -Cryptoxanthin was mainly located in the non-lipid fraction, i.e. about 89% (see
422 the result paragraph on this topic), possibly because it is less apolar than α - and β -carotene. We
423 hypothesize some compounds from this fraction, e.g. proteins, could have impaired its
424 bioaccessibility (Iddir, Dingo, Porras Yaruro, Hammaz, Borel, Schlee, et al., 2020). Finally,
425 note that the results of the exploratory experiments on larval lipids suggest that the provitamin
426 A carotenoids located in this fraction are also readily bioaccessible, which was expected as
427 carotenoids solubilized in triglycerides are usually more bioaccessible than carotenoids located
428 in food matrices (Goupy, Genot, Hammaz, Halimi, Caris-Veyrat, & Borel, 2020).

429 The amount of vitamin A that could be supplied to farm animals or humans by
430 provitamin A carotenoid-rich larvae depends not only on their provitamin A carotenoid content
431 but also on their bioaccessibility and vitamin A conversion efficiency. We therefore calculated
432 the total bioaccessible retinol activity equivalents 100g of fresh larvae reared on the different
433 food substrates could provide (**Table 3**). These calculations suggest that provitamin A

434 carotenoid-rich larvae have the capacity to provide more retinol activity equivalents per unit of
435 fresh weight than the fruits and vegetables they were reared on. More precisely, the retinol
436 activity equivalent (RAE) enrichment in larvae versus their respective substrate ranged from
437 2.1 to 11.7, which makes these larvae significant dietary vitamin A sources for human and
438 animal species that are able to convert provitamin A carotenoids to vitamin A, i.e. most tame
439 animals except cats. If the quantities of larvae currently used in the diet of certain production
440 animals (mostly as a protein source) were replaced by equivalent quantities of larvae enriched
441 with provitamin A carotenoids, their vitamin A requirements would be met (**Table 4**). With
442 regard to humans, we calculated that the consumption of about 100 g of larvae per day could
443 provide 50% of female vitamin A RDA, which could be particularly relevant in the fight
444 against vitamin A deficiency (McLean, Klemm, Subramaniam, & Greig, 2020). Importantly, in
445 most countries where vitamin A deficiency is still prevalent, insects have been consumed for a
446 very long time and their consumption thus does not pose socio-cultural problems (Baiano,
447 2020). The use of edible insects to increase vitamin A intakes therefore seems a feasible and
448 acceptable strategy, as illustrated by the facts edible insects have already been used to fight
449 against iron deficiency in some of these countries (Bauserman, Lokangaka, Gado, Close,
450 Wallace, Kodondi, et al., 2015) and is an acceptable practice for pregnant women in rural
451 Liberia (Coley, Perosky, Nyanplu, Kofa, Anankware, Moyer, et al., 2020).

452 In summary, the results of this study allow us to suggest that BSFL reared on food
453 substrates rich in provitamin A carotenoids could be used as a source of vitamin A, either in
454 feed or food (Hawkey, Lopez-Viso, Brameld, Parr, & Salter, 2021). The fact that BSFL do not
455 seem to cleave/convert provitamin A carotenoids into vitamin A is not an issue because humans
456 and most production animals are able to convert provitamin A carotenoids into vitamin A
457 (Amengual, Widjaja-Adhi, Rodriguez-Santiago, Hessel, Golczak, Palczewski, et al., 2013; von
458 Lintig & Vogt, 2004). Finally, since BSFL readily grow on fruits and vegetables wastes (da

459 Silva & Hesselberg, 2020; C. Liu, Wang, & Yao, 2019), we believe that food industry by-
460 products with well controlled food safety could constitute a way to recover provitamin A
461 carotenoids in products intended for disposal. This would also make it possible to add value to
462 plant by-products produced by the food industry (Ojha, Bussler, & Schluter, 2020) and would
463 reduce waste production, which would be beneficial for the environment. Therefore,
464 enrichment of BSFL with provitamin A carotenoids from by-products could be a sustainable
465 strategy to put a fraction of vitamin A back into the food cycle, which would otherwise be
466 irretrievably lost.

467 **Abbreviation:**

468 BSFL (black soldier fly larvae).

469

470 **Supporting information:**

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

473

474 **Credit author statement:**

475 **Patrick Borel:** had the idea of the research and has primary responsibility for final manuscript
476 content; designed the protocol, analyzed and interpreted data, project coordination, acquisition
477 of funding, drafting of the manuscript. **Faiza Hammaz:** carotenoid analysis, in vitro digestions,
478 analyzed and interpreted data, drafting material and methods and drawing the figures. **Lisa**
479 **Morand-Laffargue:** review & editing of the manuscript. **Charlotte Halimi:** carotenoid
480 analysis supervision. **Benjamin Creton:** larvae rearing on the different substrates and larvae
481 collection. **Damien Sabatier:** co-designed the protocol, acquisition of funding, Review &
482 Editing of the manuscript. **Charles Desmarchelier:** statistical analysis, analyzed and
483 interpreted data, Review & Editing of the manuscript.

484

485 **Conflicts of interest:**

486 BC and DS work in the BioMiMetiC company. This company conducts research and
487 development activities aimed at enhancing the value of entomo-conversion on a wide variety of
488 organic materials generated in the area at all levels of the food value chain.

489

490

491 **5) References**

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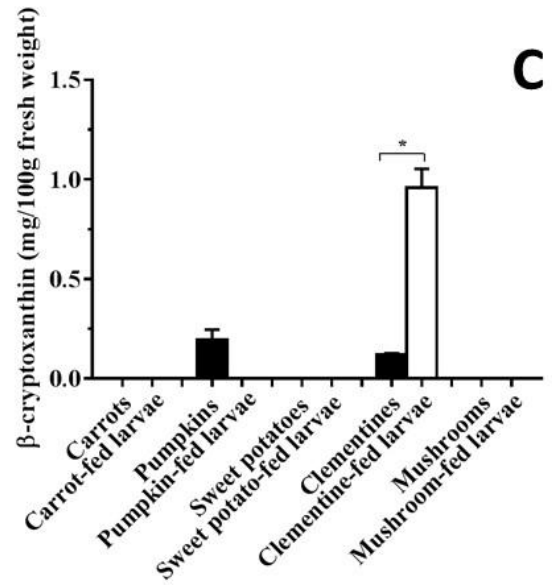
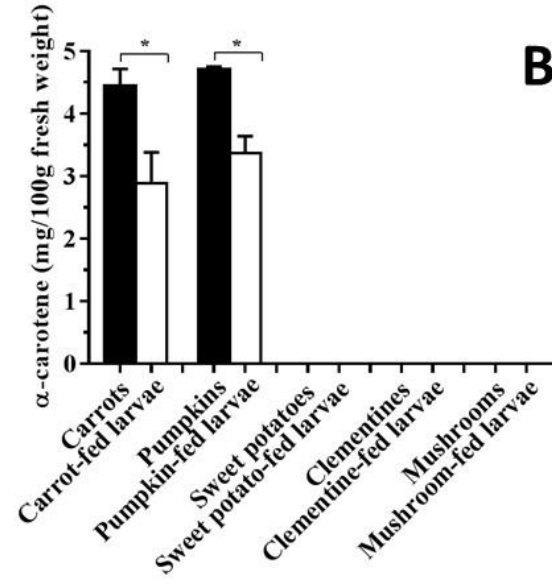
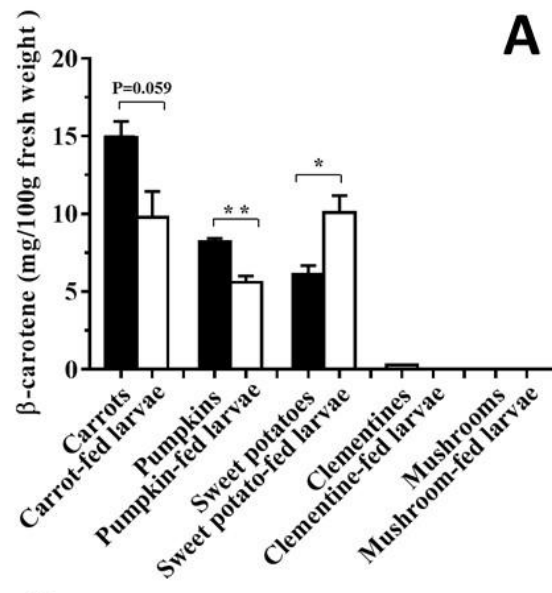
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653



654 **Figure 1**

655 **Figure 1 legend: Provitamin A carotenoid concentrations in BSFL reared on different**
656 **food substrates and in their corresponding rearing substrates. Figure 1A: β -carotene**
657 **concentrations. Figure 1B: α -carotene concentrations. Figure 1C: β -cryptoxanthin**
658 **concentrations. Bars represent means \pm SEM (n=3). For each couple (food substrate)/(food**
659 **substrate-fed larvae), an asterisk indicates that concentrations are significantly different**
660 **between the larvae and the food substrate (*: $p < 0.05$; **: $p < 0.005$, Student's *t*-test).**

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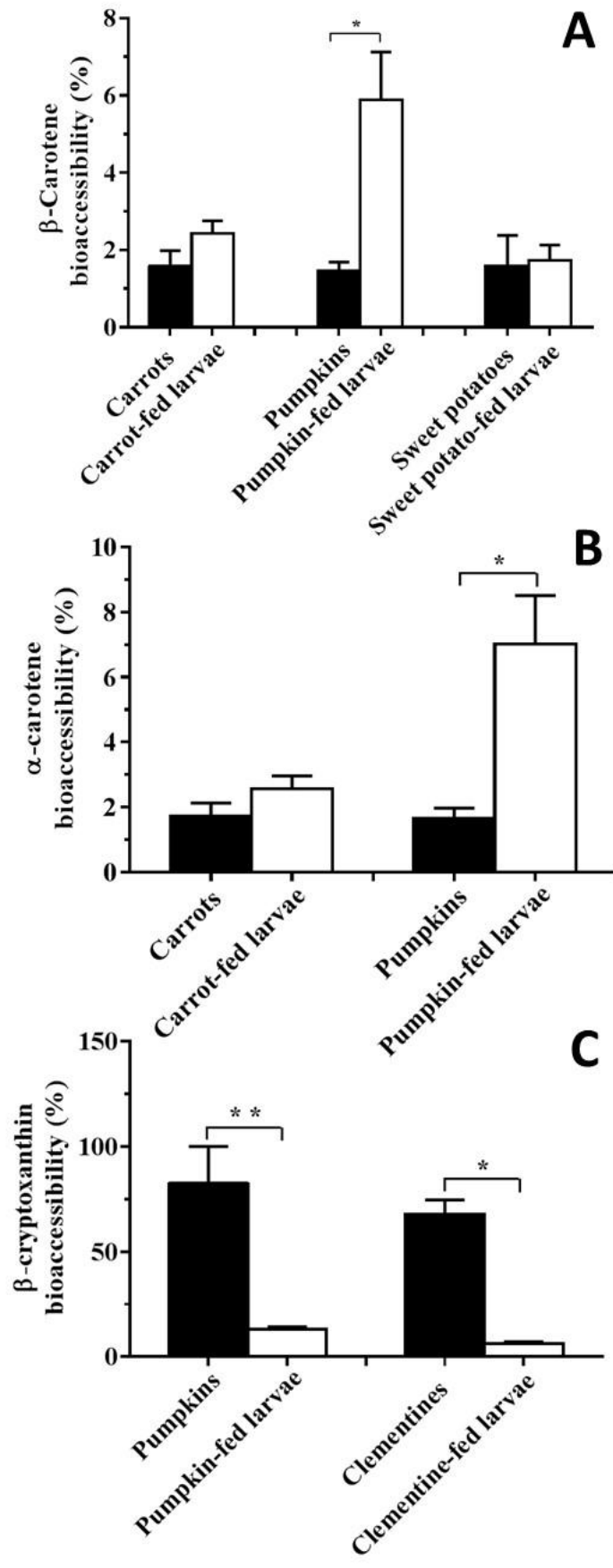
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680 Figure 2

681 **Figure 2 legend: Provitamin A carotenoid bioaccessibility in BSFL reared on different**
682 **food substrates and in their corresponding rearing substrates. Figure 2A: β -carotene**
683 **bioaccessibility. Figure 2B: α -carotene bioaccessibility. Figure 2C: β -cryptoxanthin**
684 **bioaccessibility. Provitamin A carotenoid bioaccessibility, i.e. the quantity of provitamin A**
685 **carotenoids that is transferred to mixed micelles during digestion, was estimated by using an in**
686 **vitro digestion model (see Material & Methods). Bars represent mean \pm SEM (n=4). For each**
687 **couple (food substrate)/(food substrate-fed larvae), an asterisk indicates that bioaccessibility**
688 **values are significantly different between the larvae and the food substrate ($p < 0.05$; Student's *t*-**
689 **test).**

690 **Table 1: Characteristics of the larvae collected at the end of the rearing period.**

Food substrate	Fresh weight (g)	Lipid mass (mg/g fresh weight)
Orange carrots	98.6	7.5 ± 0.8 ^{ab}
Pumpkins	87.2	8.4 ± 1.7 ^a
Sweet potatoes	125.7	6.2 ± 2.3 ^b
Clementines	33.1	3.1 ± 4.0 ^c
White mushrooms	47.0	8.3 ± 4.6 ^a

691 Values of lipid masses represent means ± SEM of 3 measurements. Means bearing different
692 superscript letters were significantly different (ANOVA followed by Tukey-Kramer's test,
693 p<0.05).

694 **Table 2: Provitamin A carotenoid concentration and bioaccessibility in whole larvae and in larval lipid fractions.**

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Food substrate provided to the BSFL	ProVA CAR	Provitamin A carotenoid concentration (mg/100g fresh weight or lipids)		Provitamin A carotenoid bioaccessibility (%)	
		<u>Whole larvae</u>	<u>Larval lipids</u>	<u>Whole larvae</u>	<u>Larval lipids</u>
Orange carrots	α -carotene	2.9 \pm 0.5	14.4 \pm 6.1	2.6 \pm 0.4	44.5 \pm 21.8
	β -carotene	9.8 \pm 1.7	43.4 \pm 19.9	2.4 \pm 0.3	24.3 \pm 9.0
Pumpkins	α -carotene	3.4 \pm 0.3	17.8 \pm 2.1	7.0 \pm 1.5	1.9 \pm 0.3
	β -carotene	5.6 \pm 0.4	24.4 \pm 6.4	5.9 \pm 1.2	1.5 \pm 1.2
	β -cryptoxanthin	< LoQ	0.8 \pm 0.4	13.2 \pm 1.1	< LoQ
Sweet potatoes	β -carotene	10.1 \pm 1.1	40.8 \pm 17.2	1.7 \pm 0.4	0.7 \pm 0.3
Clementines	β -carotene	< LoQ	0.3 \pm 0.2	< LoQ	< LoQ
	β -cryptoxanthin	1.0 \pm 0.9	3.3 \pm 1.7	6.4 \pm 0.8	20.0 \pm 4.5

696 Values represent means \pm SEM of 3 or 4 values for whole larvae and of 2 values for larval lipids. LoQ: limit of quantification.

697 **Table 3: Theoretical bioaccessible vitamin A amounts provided by the consumption of 100g of the food substrates compared to 100g of**
 698 **larvae reared on these substrates.**

Food substrate	Provitamin A CAR	Provitamin A carotenoid concentration ^a (mg/100g substrate) ^b	Provitamin A carotenoid amount in the micellar fraction (µg/100g substrate) ^c	Bioaccessibility (%) ^a	Theoretical RAE amount in the micellar fraction (µg/100g substrate) ^d	Theoretical total RAE amount in the micellar fraction (µg/100g substrate) ^e
Carrots	α-carotene	4.4 ± 0.3	15.5 ± 4.0	1.7 ± 0.4	8.3 ± 2.1	
	β-carotene	14.9 ± 1.0	48.3 ± 15.0	1.6 ± 0.4	51.5 ± 16.0	59.8 ± 18.1
Pumpkins	α-carotene	4.7 ± 0.0	11.3 ± 2.5	1.6 ± 0.3	6.0 ± 1.3	
	β-carotene	8.2 ± 0.2	17.0 ± 2.6	1.5 ± 0.2	18.1 ± 2.8	27.6 ± 5.2
	β-cryptoxanthin	0.2 ± 0.1	10.7 ± 1.3	82.4 ± 17.6	5.7 ± 0.7	
Sweet potatoes	β-carotene	6.1 ± 0.6	22.5 ± 9.9	1.6 ± 0.8	24.0 ± 10.6	24.0 ± 10.6
Clementines	β-carotene	0.3 ± 0.0	< LoQ	-	-	
	β-cryptoxanthin	0.1 ± 0.0	9.3 ± 2.3	67.8 ± 6.9	4.9 ± 1.2	4.9 ± 1.2

699 ^aMeasured in this study.

700 ^bFresh weight.

701 ^cMeasured in this study per g substrate following *in vitro* digestion and expressed for the consumption of 100g substrate.

702 ^dCalculated considering 1 molecule of β -carotene yields 2 molecules of retinol while 1 molecule of α -carotene or β -cryptoxanthin yield 1
 703 molecule of retinol upon cleavage by β -carotene oxygenase 1.

704 ^eCalculated as the sum of the theoretical RAE provided by each provitamin A carotenoids.

705 Provitamin A carotenoids: provitamin A carotenoids.

706 RAE: retinol activity equivalent. LoQ: limit of quantification.

707 Values are means \pm SEM (n=3 for provitamin A carotenoid concentrations in substrates and n=4 for bioaccessibility measurements).

708 **Table 3 continued**

Larvae	Provitamin A carotenoids	Provitamin A carotenoid concentration ^a (mg/100g substrate ^b)	Provitamin A carotenoid amount in the micellar fraction (μ g/100g larvae) ^c	Bioaccessibility (%) ^a	Theoretical RAE amount in the micellar fraction (μ g/100g larvae) ^d	Theoretical total RAE amount in the micellar fraction (μ g/100g larvae) ^e	Enrichment in larvae vs respective substrate
Carrot-fed	α -carotene	2.9 \pm 0.5	30.3 \pm 1.0	2.6 \pm 0.4	16.2 \pm 0.5		
	β -carotene	9.8 \pm 1.7	99.8 \pm 5.5	2.4 \pm 0.3	106.4 \pm 5.9	122.6 \pm 5.9	2.1
Pumpkin-fed	α -carotene	3.4 \pm 0.3	174.6 \pm 56.9	7.0 \pm 1.5	93.1 \pm 30.4		
	β -carotene	5.6 \pm 0.4	251.5 \pm 80.1	5.9 \pm 1.2	268.4 \pm 85.5	367.4 \pm 116.1	13.3

	β -cryptoxanthin	< LoQ	11.0 \pm 0.8	13.1 \pm 1.1	5.9 \pm 0.4		
Sweet potato-fed	β -carotene	10.1 \pm 1.1	120.5 \pm 38.9	1.7 \pm 0.4	128.6 \pm 41.5	128.6 \pm 41.5	5.4
Clementine-fed	β -carotene	< LoQ	< LoQ	-	-		
	β -cryptoxanthin	1.0 \pm 0.1	21.8 \pm 3.4	6.4 \pm 0.8	11.6 \pm 1.8	11.6 \pm 1.8	2.4

709 ^aMeasured in this study.

710 ^bfresh weight.

711 ^cMeasured in this study per g substrate following *in vitro* digestion and expressed for the consumption of 100g substrate.

712 ^dCalculated considering 1 molecule of β -carotene yields 2 molecules of retinol while 1 molecule of α -carotene or β -cryptoxanthin yield 1
713 molecule of retinol upon cleavage by β -carotene oxygenase 1 in the human body.

714 ^eCalculated as the sum of the theoretical RAE provided by each provitamin A carotenoid.

715 RAE: retinol activity equivalent. LoQ: limit of quantification.

716 Values are means \pm SEM (n=3 for provitamin A carotenoid concentrations in substrates and n=4 for bioaccessibility measurements).

717

718 **Table 4. Vitamin A amounts provided by the incorporation of provitamin A carotenoid-rich larvae in the diet of livestock and humans.**

Species	Vitamin A requirements (mg/kg of diet)	BSFL fraction provided in the diet^a	Maximum proportion of the BSFL fraction allowed in the diet (weight%)	mg RAE^b provided by BSFL incorporated at the maximum proportion^c	Vitamin A adequacy^d
Rainbow trout (<i>Oncorhynchus mykiss</i>)	0.75 (Council, 2011)	Prepupae Partially defatted larvae ^e	50% (St-Hilaire, Sheppard, Tomberlin, Irving, Newton, McGuire, et al., 2007) 40% (Renna, Schiavone, Gai, Dabbou, Lussiana, Malfatto, et al., 2017)	1.84 1.47	245% 196%
Atlantic salmon (<i>Salmo salar</i>)	0.75 (Council, 2011)		100% (Lock, Arsiwalla, & Waagbø, 2015)	3.67	489%

Pig			10-21% (piglets)		
<i>(Sus scrofa domesticus)</i>	0.66 (Council,		(Biasato, et al., 2019;		
Weanling	2012)	Partially deffated larvae	Neumann, Velten, &	0.37-0.77	56-117%
Finishing	0.39 (Council,	Partially deffated larvae	Liebert, 2018)	0.48	123%
	2012)		13% (growing pigs)		
			(Neumann, Velten, &		
			Liebert, 2018)		
Laying hen	0.9 (Council,		24% (Maurer,		
<i>(Gallus gallus domesticus)</i>	1994)	Partially deffated larvae	Holinger, Amsler,	0.88	98%
			Früh, Wohlfahrt,		
			Stamer, et al., 2016)		
Broiler chicken	0.45 (Council,	Partially deffated larvae	25% (Schiafone, De	0.92	204%
<i>(Gallus gallus domesticus)</i>	1994)	Highly deffated larvae	Marco, Martínez,	0.92	204%
		Prepupae	Dabbou, Renna,	0.55	122%

Madrid, et al., 2017)
 25% (Schiavone, et al.,
 2017)
 15% (Pieterse,
 Erasmus, Uushona, &
 Hoffman, 2019)

Human	RDA	Hypothetical BSFL fraction transformed and incorporated in the diet ^f	Daily amount needed to provide 100% of RDA		
	900 µg				
Males	(Medicine, 2001)	Prepupae	245g	0.90	100%
	700 µg				
Females	(Medicine, 2001)	Prepupae	210g	0.77	100%

719 ^aDepending on national legislations.

720 ^bRAE: retinol activity equivalent.

721 ^cThese values were calculated by using the larvae that had the higher RAE concentration in this study, i.e. the pumpkin-fed ones.

722 ^dCalculated as the % of vitamin A requirements covered by BSFL incorporated at the maximum proportion allowed.

723 ^eLarvae are usually partly defatted by pressing with an oil press. The crude fat concentration decreases from about 26.5 to 11.0 g/100 g fresh
724 matter (Maurer, et al., 2016).

725 ^fFor humans, it is only theoretical as there is not yet authorization to incorporate this or that fraction of BSFL in food. Therefore, we
726 hypothesized that prepuae could be incorporated in food after after verifying their food safety and organoleptic constraints. Furthermore, we have
727 made a theoretical calculation on the quantity of larvae rich in provitamin A carotenoids which would be necessary to supply 100% of the
728 recommended dietary allowances (RDA) in vitamin A