# Using black soldier fly larvae reared on fruits and vegetables waste as a sustainable dietary source of provitamin A carotenoids

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

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**Abstract**

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

## 1) Introduction

The term vitamin A describes a group of fat-soluble molecules that can be obtained from the diet as preformed vitamin A (mainly retinyl esters) and from provitamin A carotenoids. The former is found in foods of animal origin while the latter are found mainly in vegetables and fruits. There are three main provitamin A carotenoids in the human diet: β-carotene, α-carotene and β-cryptoxanthin. Their capacity to be converted into vitamin A, i.e. their provitamin A activity, is intrinsically different because it depends on the number of β-ionone rings they possess, i.e. 2 for β-carotene and 1 for α-carotene and β-cryptoxanthin. It also depends on a diet-responsive regulatory network that controls their intestinal uptake and their conversion into vitamin A through a negative feedback regulation of *SCARB1* (scavenger receptor class B member 1) and *BCO1* (beta-carotene oxygenase 1) expression via ISX (intestine specific homeobox) in the intestine (Lobo, Amengual, Baus, Shivdasani, Taylor, & von Lintig, 2013). It finally depends on their bioavailability, i.e. on the fraction that is absorbed and available for use by the body, which is highly dependent on the food matrix they are incorporated in (Desmarchelier & Borel, 2017). Although preformed vitamin A or provitamin A carotenoids are present in many foods, vitamin A deficiency is frequent, whether in humans (https://www.who.int/vmnis/database/vitamina/en/. Accessed 31.03.21) or animals, due to both low intakes of vitamin A food sources and to variations in provitamin A carotenoids bioavailability and conversion efficiency. Despite the implementation of numerous programs (Mason, Greiner, Shrimpton, Sanders, & Yukich, 2015), the prevalence of vitamin A deficiency in low income countries has remained high, calling for new and more sustainable strategies to tackle this public health issue. This is all the more relevant considering the context of increasing world population, leading to an increase in vitamin A needs, together with global warming, leading to a probable decrease in yields and area under cultivation. Moreover,

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

There are currently more than 1,900 insects classified as edible (https://www.wur.nl/upload_mm/8/a/6/0fdfc700-3929-4a74-8b69-f02fd35a1696_Worldwide%20list%20of%20edible%20insects%202017.pdf. Accessed 31.03.21). High levels of carotenoids, including provitamin A carotenoids, are found in various insect species and high concentrations of retinol and β-carotene were measured in two edible species, *Eublaberus distanti* and *Gromphadorhina portentosa* (Ruth Charrondiere, Stadlmayr, Rittenschober, Mouille, Nilsson, Medhammar, et al., 2013). Furthermore, some insect species were reported to be able to bioaccumulate different lipid molecules from food substrates they were reared on (Liland, Biancarosa, Araujo, Biemans, Bruckner, Waagbo, et al., 2017) and a proof of concept that insects can accumulate provitamin A carotenoids, which are very hydrophobic lipids, is provided by the fact that feeding migratory locusts with carrots increased their β-carotene content (Oonincx & Poel, 2011). We have thus hypothesized that some edible insect species could constitute significant dietary sources of vitamin A for feed and food, either by their natural vitamin A content or by the vitamin A they could accumulate. Although insects are not yet commonly eaten around the world and issues of health safety or cultural acceptance remain (Roma, Palmisano, & De Boni, 2020), they are already used as an complementary source of proteins in food and feed in several countries where vitamin A deficiency still prevails (Kenis, Koné, Chrysostome, Devic, Koko, Clottey, et al., 2014; Xu, Shan, Lin, Miao, Lou, Wang, et al., 2021). Thus, the identification of an insect species rich in vitamin A, either naturally or through enrichment following the choice of adequate rearing substrates, would provide these countries with an additional and sustainable approach to increase vitamin A intakes if used as a food, or even indirectly if used as a feed for livestock.

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

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

## 2) Material and methods

*Chemicals*

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

*Experimental foods*

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

*BSFL farming*

BSFL were reared either on fruits and vegetables rich in provitamin A carotenoids or on white mushrooms, which served as the control food substrate since they do not contain provitamin A carotenoids. The rearing protocol was implemented and carried out by BioMiMetiC (Avignon, France). The objective was to obtain approximately 200 g of larvae, weighing approximately 150 mg each, on each food substrate. As a result, around 1,300 larvae were deposited on each food substrate. This approximate number of larvae was obtained by sowing 35 ± 5 mg eggs on each substrate. Indeed, the egg mass is about 24 ± 1 µg (Tomberlin, 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 ± 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 ×
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 × 418 x 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 ± 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

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

Provitamin A carotenoid bioaccessibility, i.e. the relative amount of provitamin A carotenoids that is transferred to mixed micelles during digestion, was assessed by using an in vitro model (Reboul, Richelle, Perrot, Desmoulins-Malezet, Pirisi, & Borel, 2006), which provides similar estimates of carotenoid bioaccessibility for diverse foods compared to the Infogest model (Rodrigues, Chitchumroonchokchai, Mariutti, Mercadante, & Failla, 2017). In summary, 2 g of sample (BSFL or food substrates coarsely crushed with a mortar), or lipids extracted from 2 g of BSFL, were placed in a 100 mL Erlenmeyer flask and mixed with 6.7 g mashed potatoes, 1.2 g minced meat and 200 mg olive oil and ground for 30 s at 22,000 rpm (T18 basic Ultra-Turrax disperser, IKA, Staufen, Germany). The mixture was homogenized 10 min at 37°C in a rotating incubator (190 rpm). To mimic the oral phase of digestion, 2.5 mL artificial saliva were then added and the mixture was incubated 10 min at 37°C in a rotating incubator (190 rpm). To mimic the gastric phase of digestion, the pH was adjusted to 4 ± 0.02 with 1 M HCl and 2 mL pepsin were added to the mixture which was then incubated for 30 min at 37°C in a rotating incubator (190 rpm). To mimic the duodenal phase of digestion, the pH was adjusted to 6 ± 0.02 with 0.9 M $NaHCO_3$ buffer before addition of 9 mL of a pancreatin-bile extract solution and 4 mL of a bile solution. The mixture was then incubated for 30 min at 37°C in a rotating incubator (190 rpm). Aliquots of 4 mL of the mixture, thereafter called digestate aliquots, were then collected. The remaining mixture was centrifuged at 1,860 x *g* for 1 h 12 min at 10°C. The recovered supernatant was filtered through a 0.8 µm filter and then through a 0.22 µm filter (mixed cellulose esters; Millipore, Molsheim, France). The clear aqueous samples obtained, which contain the mixed micelles, were stored at -80°C until lipid extraction and quantification of provitamin A carotenoids by HPLC.

*Lipid extraction from BSFL*

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

*Provitamin A carotenoid extraction from BSFL and food substrates*

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

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

*Carotenoid quantification*

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

*Calculations and statistics*

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

## 3) Results

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

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

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

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

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

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

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

*Provitamin A carotenoid concentrations in larval lipids*

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

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

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

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

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

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

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

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

*Bioaccessibility of provitamin A carotenoids in larval lipid fractions*

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

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

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

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

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

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

## 4) Discussion

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

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

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

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

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

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

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

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

**Abbreviation:**

BSFL (black soldier fly larvae).

**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.

**Credit author statement:**

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

**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.

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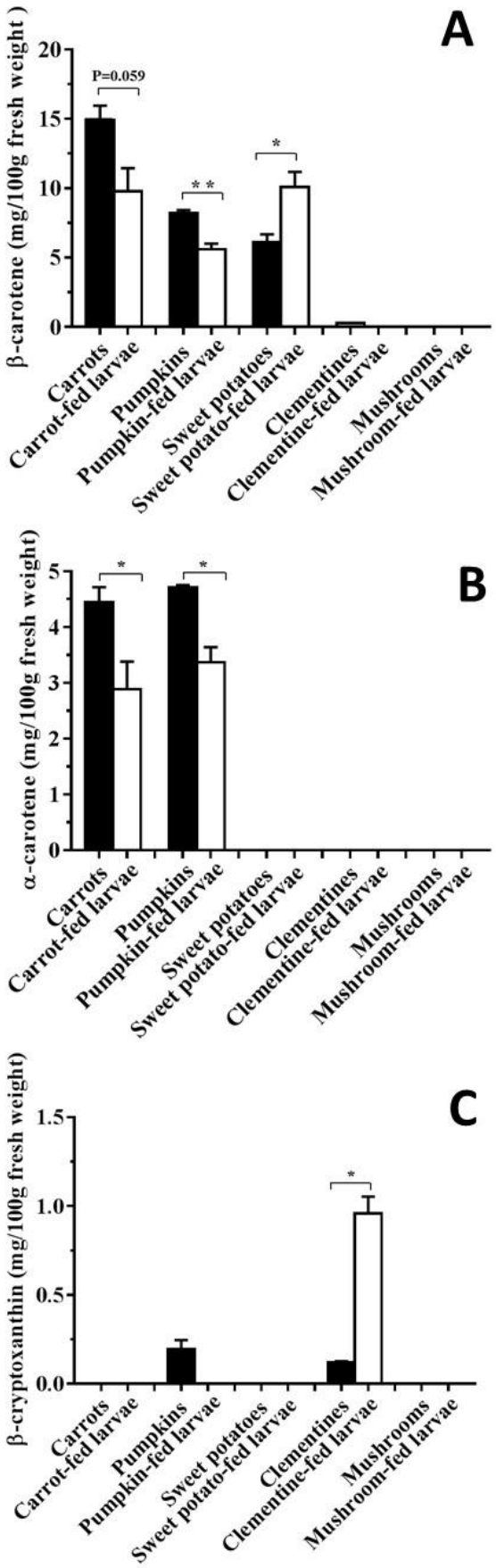
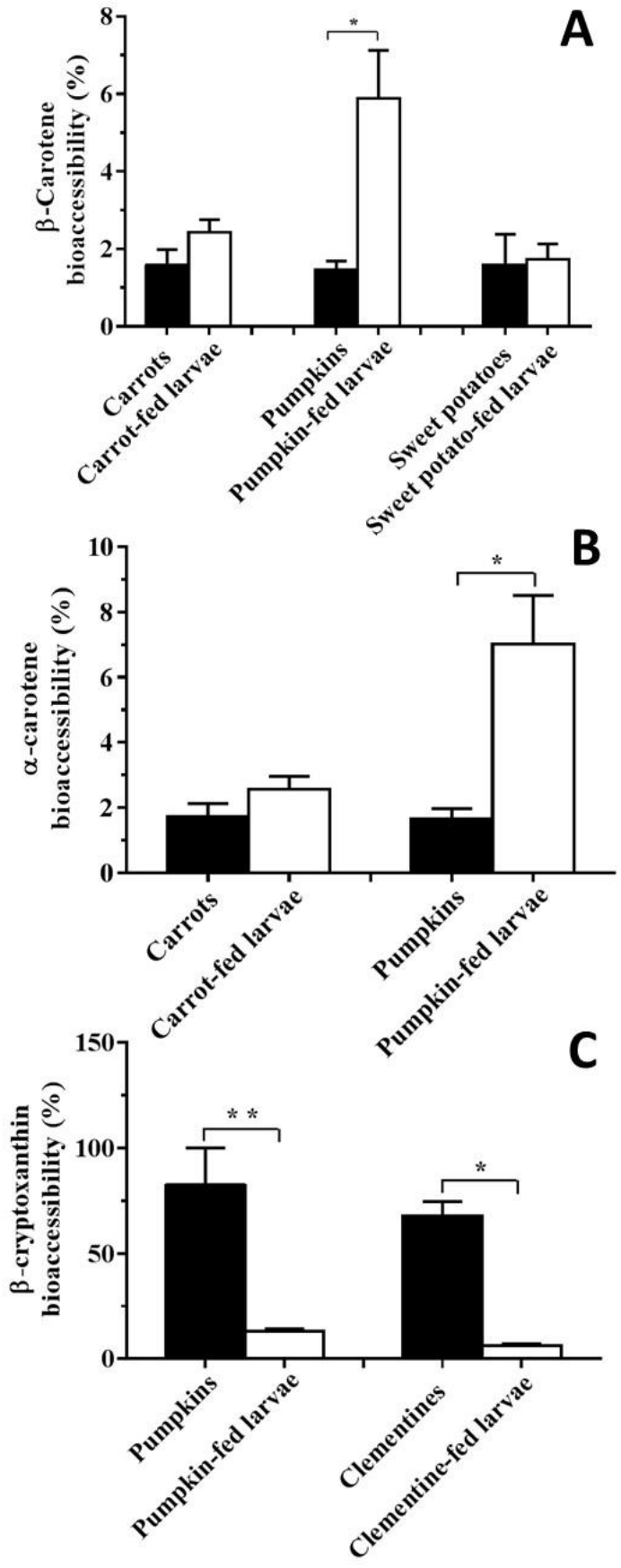

A
β-carotene (mg/100g fresh weight )
P=0.059
* *
*
Carrots
Carrot-fed larvae
Pumpkins
Pumpkin-fed larvae
Sweet potatoes
Sweet potato-fed larvae
Clementines
Clementine-fed larvae
Mushrooms
Mushroom-fed larvae
B
α-carotene (mg/100g fresh weight)
*
*
C
β-cryptoxanthin (mg/100g fresh weight)
*

654 **Figure 1**

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


A
β-Carotene bioaccessibility (%)
8
6
4
2
0
*
Carrots
Carrot-fed larvae
Pumpkins
Pumpkin-fed larvae
Sweet potatoes
Sweet potato-fed larvae
B
α-carotene bioaccessibility (%)
10
8
6
4
2
0
*
Carrots
Carrot-fed larvae
Pumpkins
Pumpkin-fed larvae
C
β-cryptoxanthin bioaccessibility (%)
150
100
50
0
* *
*
Pumpkins
Pumpkin-fed larvae
Clementines
Clementine-fed larvae

680 **Figure 2**

**Figure 2 legend: Provitamin A carotenoid bioaccessibility in BSFL reared on different food substrates and in their corresponding rearing substrates. Figure 2A:** β-carotene bioaccessibility. **Figure 2B:** α-carotene bioaccessibility. **Figure 2C:** β-cryptoxanthin bioaccessibility. Provitamin A carotenoid bioaccessibility, i.e. the quantity of provitamin A carotenoids that is transferred to mixed micelles during digestion, was estimated by using an in vitro digestion model (see Material & Methods). Bars represent mean ± SEM (n=4). For each couple (food substrate)/(food substrate-fed larvae), an asterisk indicates that bioaccessibility values are significantly different between the larvae and the food substrate ($p<0.05$; Student's *t*-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 | $7.5 \pm 0.8^{ab}$ |
| Pumpkins | 87.2 | $8.4 \pm 1.7^{a}$ |
| Sweet potatoes | 125.7 | $6.2 \pm 2.3^{b}$ |
| Clementines | 33.1 | $3.1 \pm 4.0^{c}$ |
| White mushrooms | 47.0 | $8.3 \pm 4.6^{a}$ |

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.**

695

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

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

**Table 3: Theoretical bioaccessible vitamin A amounts provided by the consumption of 100g of the food substrates compared to 100g of 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 | 59.8 ± 18.1 |
| | β-carotene | 14.9 ± 1.0 | 48.3 ± 15.0 | 1.6 ± 0.4 | 51.5 ± 16.0 | |
| Pumpkins | α-carotene | 4.7 ± 0.0 | 11.3 ± 2.5 | 1.6 ± 0.3 | 6.0 ± 1.3 | 27.6 ± 5.2 |
| | β-carotene | 8.2 ± 0.2 | 17.0 ± 2.6 | 1.5 ± 0.2 | 18.1 ± 2.8 | |
| | 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 | - | - | 4.9 ± 1.2 |
| | β-cryptoxanthin | 0.1 ± 0.0 | 9.3 ± 2.3 | 67.8 ± 6.9 | 4.9 ± 1.2 | |

[a]Measured in this study.

[b]Fresh weight.

[c]Measured in this study per g substrate following *in vitro* digestion and expressed for the consumption of 100g substrate.

[d]Calculated considering 1 molecule of β-carotene yields 2 molecules of retinol while 1 molecule of α-carotene or β-cryptoxanthin yield 1 molecule of retinol upon cleavage by β-carotene oxygenase 1.

[e]Calculated as the sum of the theoretical RAE provided by each provitamin A carotenoids.

Provitamin A carotenoids: provitamin A carotenoids.

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

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

**Table 3 continued**

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

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

709 [a]Measured in this study.

710 [b]fresh weight.

711 [c]Measured in this study per g substrate following *in vitro* digestion and expressed for the consumption of 100g substrate.

712 [d]Calculated 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 [e]Calculated 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 ± 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 (*Oncorhychus mykiss*) | 0.75 (Council, 2011) | Prepupae<br>Partially defatted larvae[e] | 50% (St-Hilaire, Sheppard, Tomberlin, Irving, Newton, McGuire, et al., 2007)<br>40% (Renna, Schiavone, Gai, Dabbou, Lussiana, Malfatto, et al., 2017) | 1.84<br>1.47 | 245%<br>196% |
| Atlantic salmon (*Salmo salar*) | 0.75 (Council, 2011) | | 100% (Lock, Arsiwalla, & Waagbø, 2015) | 3.67 | 489% |

| | | | | | |
|---|---|---|---|---|---|
| Pig<br>(*Sus scrofa domesticus*)<br>Weanling<br>Finishing | 0.66 (Council, 2012)<br>0.39 (Council, 2012) | Partially deffated larvae<br>Partially deffated larvae | 10-21% (piglets) (Biasato, et al., 2019; Neumann, Velten, & Liebert, 2018)<br>13% (growing pigs) (Neumann, Velten, & Liebert, 2018) | 0.37-0.77<br>0.48 | 56-117%<br>123% |
| Laying hen<br>(*Gallus gallus domesticus*) | 0.9 (Council, 1994) | Partially deffated larvae | 24% (Maurer, Holinger, Amsler, Früh, Wohlfahrt, Stamer, et al., 2016) | 0.88 | 98% |
| Broiler chicken<br>(*Gallus gallus domesticus*) | 0.45 (Council, 1994) | Partially deffated larvae<br>Highly deffated larvae<br>Prepupae | 25% (Schiavone, De Marco, Martínez, Dabbou, Renna, | 0.92<br>0.92<br>0.55 | 204%<br>204%<br>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** | | |
| Males | 900 µg (Medicine, 2001) | Prepupae | 245g | 0.90 | 100% |
| Females | 700 µg (Medicine, 2001) | Prepupae | 210g | 0.77 | 100% |

719 [a]Depending on national legislations.

720 [b]RAE: retinol activity equivalent.

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

722 [d]Calculated as the % of vitamin A requirements covered by BSFL incorporated at the maximum proportion allowed.

723 [e]Larvae are usually partly deffated 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 [f]For 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