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Concentrations of fat-soluble vitamins and carotenoids in black soldier fly larvae (*Hermetia Illucens*) fed with fermented authorized and unauthorized biowaste in Europe

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ABSTRACT

The nutritional and market values of black soldier fly larvae (BSFL) can be improved by enriching them with compounds of interest. It has recently been shown that these larvae can bioaccumulate fat-soluble vitamins and carotenoids under optimal rearing conditions. Based on these preliminary results, the aim was to determine whether BSFL can be significantly enriched in these compounds when raised under conditions usually used by breeders such as preliminary substrate fermentation. BSFL were raised on EU-authorized substrates, *i.e.* plant biowastes, and EU-unauthorized biowastes from school canteens and supermarkets. Vitamins A, E and D, as well as 10 carotenoids were quantified by HPLC-DAD in the substrates and larvae. Although BSFL mass were lower in the apricot (85 ± 4 mg) group compared to the control group (121 ± 8 mg), the dry weight, total protein and lipid content of all the groups were not different. Most compounds present in the fermented substrates were found in the larvae, but in lower concentrations than those observed under the optimal rearing conditions, *e.g.* β-carotene in larvae reared on carrots 9.6 ± 0.4 mg/kg FW *vs*98 $\pm$ 17 mg/kg, respectively. Regarding unauthorized substrates, they allowed larvae to bioaccumulate wider variety of micronutrients than other substrates, although in smaller quantities. Thus, raising BSFL under standard industrial breeding conditions makes it possible to obtain larvae enriched in micronutrients, without impacting their total protein and lipid content. However, the rearing conditions must be optimized if higher concentrations of these micronutrients in the larvae are wanted and further studies are necessary to confirm the results of this exploratory study.

Introduction

Insect breeding for animal or human nutrition is strongly encouraged by the WHO and the FAO because it presents numerous environmental and socio-economic advantages (van Huis et al., 2021). The black soldier fly (*Hermetia illucens*) is one of the most widely used insects for animal feed because its larvae can be fed on a very wide range of organic substrates such as biowaste. Currently in the EU, and in many other parts of the world, breeders cannot entirely exploit this capacity because substrates containing fish and meat wastes are forbidden for now. Nevertheless, it would be very interesting to use them, both from an economical and environmental point of view, thanks to the competitive costs of these biowaste compared to those currently used, and that it would contribute to their recycling.

Black soldier fly larvae (BSFL) are used in particular as an alternative source of proteins of high nutritional quality, but they are also very rich in lipids and contain many micronutrients, whether vitamins, minerals or trace elements (Spranghers et al., 2017). Unlike usual livestock, *e.g.* cattle, sheep, poultry, the nutritional composition of insects can be significantly modified by the nature of the substrate on which they are raised (Chia

Abbreviations: BSFL, black soldier fly larvae; WB, wheat bran; SC, school canteen; SMKT, supermarket; F, fermented; NF, not-fermented; FW, fresh weight; DW, dry weight; AS, authorized substrate; NAS, not authorized substrate.

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et al., 2020; Pamintuan et al., 2020). For example, it has been shown that BSFL can be enriched with calcium (Finke, 2003) and fatty acids from the omega-3 family (Barroso et al., 2017; El-Dakar et al., 2020; Erbland et al., 2020), which is a very interesting asset for increasing both their nutritional and market values. Fat-soluble micronutrients such as vitamins A, E and carotenoids are of the utmost importance for both human and animal nutrition and it has been precisely shown that BSFL can bioaccumulate high concentrations of these compounds when raised under conditions that were assumed optimal for maximizing their bioaccumulation (Borel et al., 2021; Morand-Laffargue et al., 2023abc). However, these conditions are not those applied by BSFL breeders for several reasons. First of all, it is common for the breeder to let the substrates ferment for a few days for various reasons including facilitating substrate digestion by the larvae (Campenhout, 2021; Mohd-Noor et al., 2017). This fermentation, which is generally carried out in a closed enclosure which becomes depleted of oxygen over time, leads to the establishment of different types of fermentation which can coexist, namely lactic, anaerobic, alcoholic and butyric, depending on the composition of the substrates and the micro-environments present (more or less aerated, humid, sugar- or nitrogen-rich areas). It is nevertheless accepted that lactic fermentation is the dominant one. This fermentation stage allows bacteria and fungi to predigest the dietary fibers of plants, which facilitates subsequent digestion by the larvae, and therefore their growth. Consequently, it increases the lipid and protein content of BSFL (Kuttiyatveetil et al., 2019; Li et al., 2023; Mohd-Noor et al., 2017). Lactic fermentation also kills and prevents the development of potentially pathogenic bacteria, and therefore their subsequent contact with the larvae (Gao et al., 2019). Then the breeder does not give the fermented substrates as is to the larvae, but generally mixes them with wheat bran (WB) so that the humidity level of the mixture approaches 70 % (Bekker et al., 2021; Laksanawimol et al., 2024). It is also customary not to deposit fly eggs on the substrate, but rather to deposit juvenile larvae, that is to say larvae which are approximately 3–9 days old and which have previously been raised on a substrate optimized for the growth of juvenile larvae (Meneguz et al., 2018; Sheppard et al., 2002). We hypothesized that fermenting the substrates, mixing them with WB, and rearing the larvae on micronutrient-rich substrate for a much shorter duration than that used under optimal conditions, could significantly impair the bioaccumulation of micronutrients in the larvae. The question here is about the magnitude of these differences and the factors responsible for it. The comprehension of the phenomenon at stake would allow us to propose improvements of breeder rearing conditions in order to optimize the bioaccumulation of fat-soluble vitamins and carotenoids in the larvae.

To verify our hypothesis, BSFL were fed with different substrates containing fat-soluble vitamins and carotenoids, under standard industrial breeding conditions. These substrates were either authorized in the EU (AS), such as wheat bran (WB), carrots (C), apricots (A) and salad (S), or not authorized in the EU (NAS), among other things because they may contain meat or fish waste, such as school canteen (SC) and supermarket (SMKT) wastes. Then the concentrations of micronutrients in fermented (F) and non-fermented (NF) substrates were measured in order to evaluate the effect of the fermentation process on the concentrations of micronutrients in the substrates. And finally, the micronutrient concentrations in the larvae were measured and compared with the concentrations that were previously measured under conditions theoretically allowing optimal bioaccumulation of these micronutrients. The nutritional interest to use NAS will be discussed.

Material and methods

Chemicals

Solvents used *i.e.* ethanol, n-hexane, methyl tert-butyl ether, methanol, water, chloroform and dichloromethane were purchased from Carlo Erba (Val de Reuil, France). The sodium hydroxide pellets were purchased from Prolabo (Paris, France). Standards *i.e.* retinol, retinyl palmitate, α-tocopherol, γ-tocopherol, lutein, β-cryptoxanthin, zeaxanthin, astaxanthin, phytoene, lycopene, ergocalciferol, cholecalciferol, retinyl acetate and retinyl palmitate were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Phytofluene standard was purchased from Biosynth (Bratislava, Slovakia). Alpha and β-carotene were generously provided by DSM Nutritional Products (Basel, Switzerland). The Pierce BCA Protein Assay kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

BSFL rearing

Preparation of rearing substrates

Wheat brans were furnished by Moulins Soufflet (Corbeil-Essonnes, France), apricots by Terre de Crau (Mouriès, France) and salads and carrots by Agrial (Normandie, France). Their macronutrient composition is detailed in Table 1. A total of 712 kg of SC biowaste were collected on July 1st 2022 among 4 SCs in France and 1335 kg of SMKT biowaste were collected in 3 SMKTs in France on the same day. Those NAS were treated by Paprec (Paris, France) the 4th July 2022 as follows. Packaging was removed and the biowaste was crushed and mixed to obtain a biowaste soup. At BioMiMetiC, carrots, apricots, salads, SMKT and SC waste were minced and then mixed with water to reach a water content of 95 %. Then they were fermented during 7 d (15 d for the salad) in a fermentation bucket (Auer, 25 L), without any starter culture. The fermented substrates were mixed with WB (70 % fermented substrate - 30 % wheat bran FW) to obtain a water content of 70 % in the food given to the larvae, which is required for optimum digestibility by the larvae (Bekker et al., 2021; Laksanawimol et al., 2024). The water contents were measured using desiccators. WB, when given as such to the larvae, was hydrated to obtain similar water content in the substrate. The standard food was a mix of 70 % apple (Mesfruits, Cavaillon, France), excluded from the market, and 30 % WB commonly used at BioMiMetiC to feed the BSFL after their juvenile period and served as a reference substrate. Seven kg of each substrate were put in each growing tray (clear plastic boxes 600 × 400 mm).

Establishment of the larvae on the substrates and growth conditions

The rearing procedure was performed as previously described (Borel et al., 2021). Briefly, eggs were placed in a hatchery in a dark incubation room at 28 ± 1 °C, humidity ratio: 65–70 %. After hatching, larvae were fed with the standard BioMiMetiC juvenile food, a bio-poultry feed. After 8.5 d of growth, larvae were separated from the frass by sieving (2 × 2 mm) and rinsed using tap water. Aliquots of around 7000 larvae were prepared (estimated with the mean number of larvae in 6 aliquots of 1 g) and placed on the growing trays with the substrates. Two trays for each diet were prepared. These were placed in the incubation room at 28 ± 1 °C, 70 % humidity ratio for 7 d in darkness. The larvae were collected at the end of the rearing period (15.5 d) and frozen to death at −20 °C. All samples were stored at this temperature before analysis.

Weight, dry matter, and lipid and protein content of the larvae

First and foremost, twenty fresh larvae of each group were weighed

Table 1
Macronutrient content of apricot, carrot, salad and wheat bran. Data extracted from the Ciqual tables (https://ciqual.anses.fr/).

	Apricot	Carrot	Salad	Wheat bran
Energie, EU N° 1169/2011 (kcal/100 g)	45.9	40.2	14.3	279
Water (g/100 g)	86.2	87.5	94.9	13.0
Proteins, N x 6.25 (g/100 g)	0.81	0.63	1.13	15.2
Carbohydrates (g/100 g)	9.01	7.59	1.03	23.6
Lipids (g/100 g)	< 0.5	< 0.5	< 0.5	4.35
Dietary fibers (g/100 g)	1.7	2.7	1.8	42

at the end of the rearing period to compare their mass. Then, the dry matter was determined with four larvae dried at 70 °C during 5 d We chose these numbers of larvae because we had to share the larvae with other laboratories that had to do other analyses, and because we believed that they were representative of the larval populations in each group. This appears to be the case given the reasonable variability of the vast majority of the measurements we made. We are nevertheless aware that these numbers may have been too small to have sufficiently representative samples for some variables and we have recalled this when interpreting the results in the discussion, as well as in the conclusion. Following this, larval lipid contents were measured gravimetrically on the dry larvae following a modified Bligh & Dyer method (Bligh and Dyer, 1959). Four dry larvae per group were mixed with 1 mL of a chloroform/methanol solution (1:2) and crushed with a MM301 ball mill (Retsch, Eragny sur Oise, France) using three stainless steel balls (30 Hz, 10 min). The mixture was then transferred into glass tubes and 2.75 mL of chloroform/methanol (1:2) was added. The mixture was then vortexed 10 s before addition of 3.75 mL of chloroform. The mixture was vortexed again before adding 3.75 mL of distilled water. Tubes were agitated 10 min and centrifuged (15 min, 1257 × *g* at 4 °C). The lower fraction was collected with a Pasteur pipette and transferred into a pre-weighed tube. A second extraction of the upper fraction was performed the same way by adding 3.75 mL of chloroform. The pooled lower fractions were then evaporated to dryness under nitrogen. Tubes were weighed again to determine the amount of extracted lipids. The upper fraction, which was obtained during the extraction of lipids, contained the proteins and was evaporated to dryness under nitrogen overnight. Finally, the protein extraction was carried out following a procedure previously described (Cuff et al., 2021) with minor modifications. Briefly, 10–20 mg of the powders previously obtained were weighed. Then, 1 mL of 0.1 M NaOH was added to each tube. The tubes were placed in a Thermo-shaker at 80 °C, shook at 300 RPM for 30 min and then left at room temperature overnight. Afterwards, they were centrifuged for 10 min at 13,000 × *g* at 10 °C. Six hundred µL of supernatant were diluted (1/10) in the adequate volume of 0.1 M NaOH, so as the protein concentration was within range of the BCA Protein kit (ThermoFisher Scientific, Waltham, USA). When using this kit, absorbance was measured at 540 nm after 10 min of incubation.

Fat-soluble vitamin and carotenoid content of substrates and larvae

Extraction of fat-soluble vitamins and carotenoids was carried out as previously described (Borel et al., 2021). Firstly, 2 g of substrate or 10 larvae per group were crushed using a mortar with liquid nitrogen and stored at −20 °C before extraction. All extractions were performed under penumbra at ambient temperature. Secondly, substrates or larvae powders were mixed with 500 µL of distilled water. Then 500 µL of an ethanolic solution of retinyl acetate (used as internal standard) and 2 mL of hexane were added. Mixtures were agitated 10 min and centrifuged 10 min at 4 °C and 2500 × *g*. The upper phase was collected and a second extraction of the lower phase was carried out. The pooled upper phases were evaporated to dryness under nitrogen at room temperature. Lastly, the molecules of interest were resolubilized with 200 to 1000 µL of methanol:dichloromethane (65 %:35 %) in order to be later injected in an HPLC system. Molecule quantification was done according to Gleize et al. (2012). Briefly, molecule separation was carried out in gradient mode on a YMC Carotenoid S-5 µm column (250 × 4.6 mm; 5 µm) preceded by a pre-column (10 × 4 mm; 5 µm) set at 35 °C. The HPLC system was the Ultimate 3000 Thermo module (LPG-3400 SD HPLC pump) with a Thermo DAD 3000 photodiode array detector. All molecules were identified by their retention times and absorption spectra coincident with authentic standards. Vitamin D2 and D3 were identified at 264 nm, tocopherols and phytoene were quantified at 290 nm, phytofluene, retinol and retinyl palmitate at 325 nm, carotenoids except lycopene at 450 nm and lycopene at 472 nm.

Calculations

Measurements of fat-soluble micronutrients were realized on raw and fermented substrates. Only the standard food and carrot substrates were measured as given to larvae. The other substrates (apricots, salad, SMKT and SC) were not available as given to the larvae so the real concentration of a molecule *x* in the substrate *y* given to larvae needed was estimated using Eq. (1):

$$\begin{aligned} &\textit{Concentration of } x \textit{ in rearing substrate } y \\ &= 70\% \times \textit{concentration of } x \textit{ measured in fermented substrate } y \\ &\quad + 30\% \times \textit{mean concentration of } x \textit{ in wheat bran} \end{aligned} \tag{1}$$

Similarly, when wheat bran was used as a substrate as such, it was humidified to reach a ratio of 70 % of humidity, meaning that the real concentration of a molecule x in wheat bran given to larvae needed to be calculated using Eq. (2):

$$\begin{aligned} &\textit{Concentration of } x \textit{ in wheat bran} = 33\% \\ &\times \textit{concentration of } x \textit{ measured in wheat bran} \end{aligned} \tag{2}$$

In order to better compare the concentrations of micronutrients in the rearing substrate *vs* in the larvae, the graphs of the bioaccumulation part were constructed using these concentrations. Note that only the figures expressed in DW are presented in the manuscript for the fermentation part, the figures expressed in FW are presented in supplementary material (**Figures S1 to S6**). This choice was made because the objective was to determine if fermentation could degrade some of the compounds studied which are very labile. However, when the concentrations are expressed in FW they are dependent on the water content of the substrate, and this water content increased during fermentation (74 %−94 % to 95 %−99 % for our substrates).

Statistical analysis

Results are expressed as means ± SEM. Differences between groups for the BSFL weight, lipid and protein concentrations were tested using one-way ANOVA. Normality of residues was checked using Shapiro-Wilk test and, in case of abnormality, data were log-transformed. Homogeneity of variances was tested using a Brown-Forsythe test and, in case of inhomogeneity, a Welch's correction was applied. When a significant effect was detected by the ANOVA, a post-hoc Tukey test was used to compare the means. Results from the fermentation and bioaccumulation sections were treated as follows: first of all, outliers were checked using the ROUT method (Motulsky and Brown, 2006) and then removed for the rest of the calculation. Then two-ways ANOVA were performed using a full model. Normality of residues was tested using Shapiro-Wilk test and, in case of abnormality, data were log-transformed. Heteroscedasticity was tested using the Spearman's rank correlation test. When a significant effect was detected, a Bonferroni post-hoc test was performed to compare the means. If a molecule was not detected in the substrate or the corresponding BSFL, the non-parametric test of Kolmogorov-Smirnov was used to compare the means of this group. Unpaired *t*-test were performed when a molecule was found in only one group. Normality of residues was tested using Shapiro-Wilk test and, in case of abnormality, data were log-transformed. In case of inhomogeneity of variances, the Welch's correction was applied. Values of $p < 0.05$ were considered significant. All statistical analyses were carried out using GraphPad Prism version 10.0.3 for Windows.

Results

Effect of fermentation on the fat-soluble vitamin and carotenoid content of the substrates

Figs. 1 **and** 2 present the concentrations of vitamins and carotenoids in fermented (F) *vs* in not-fermented substrates (NF). It is first important to mention that the molecules of cholecalciferol (vitamin D3), ergocalciferol (vitamin D2), retinol (vitamin A), retinyl acetate (vitamin A), retinyl palmitate (vitamin A) and astaxanthin (a carotenoid mainly present in certain fish and seafood) were not detected, either in the crude substrates or in the fermented ones.

Vitamin E concentration in the aforementioned substrates before and after fermentation was assessed with α and γ-tocopherol molecules (Fig. 1 **and Figure S1**). These compounds were only found in SC and SMKT waste. Overall, γ-tocopherol concentration (Fig. 1**B**) was higher than α-tocopherol concentration in both substrates (Fig. 1**A**), independently of the fermentation process. Moreover, SC waste was richer in vitamin E than the SMKT one by a factor of around 2.5. Alpha and γ-tocopherol concentrations were not affected by the fermentation of the SC and SMKT wastes. However, all the carotenoids' concentrations were differently affected by the fermentation depending on the substrate and the molecule observed.

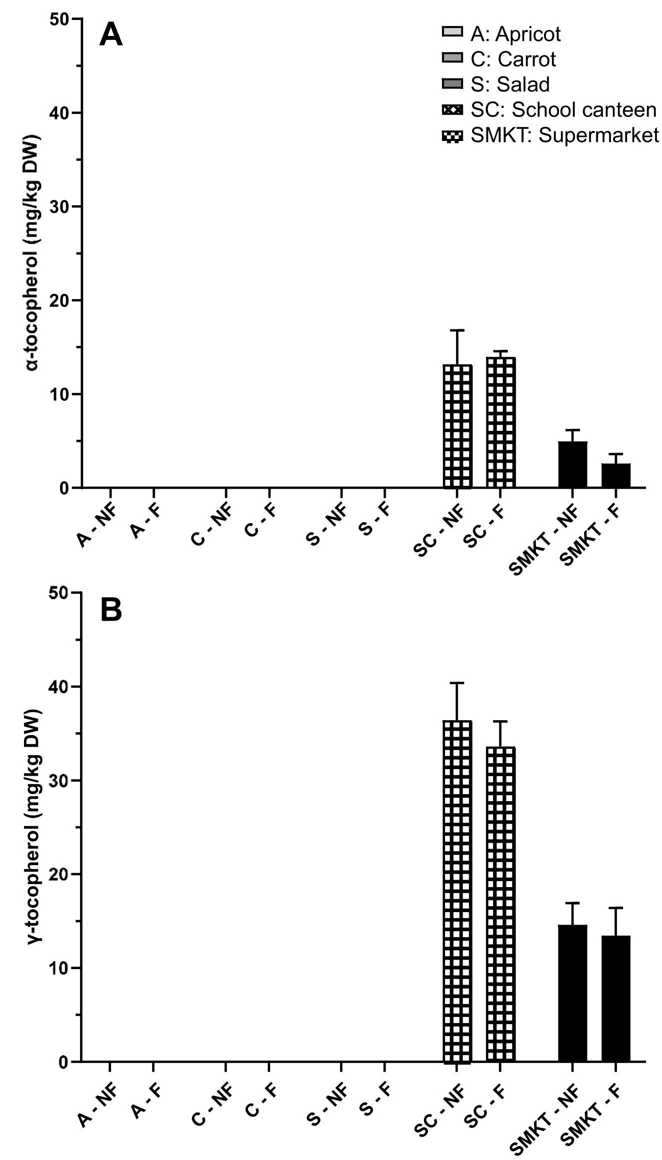


Fig. 1. α-tocopherol (A) and γ-tocopherol (B) concentrations in non-fermented and fermented substrates.
NF: Non-fermented substrate; F: Fermented substrate. Bars represent means ± SEM ($n = 4$). No significant difference between the means of the NF and F substrates were observed (Two-ways ANOVA, with substrate and fermentation as the factors).

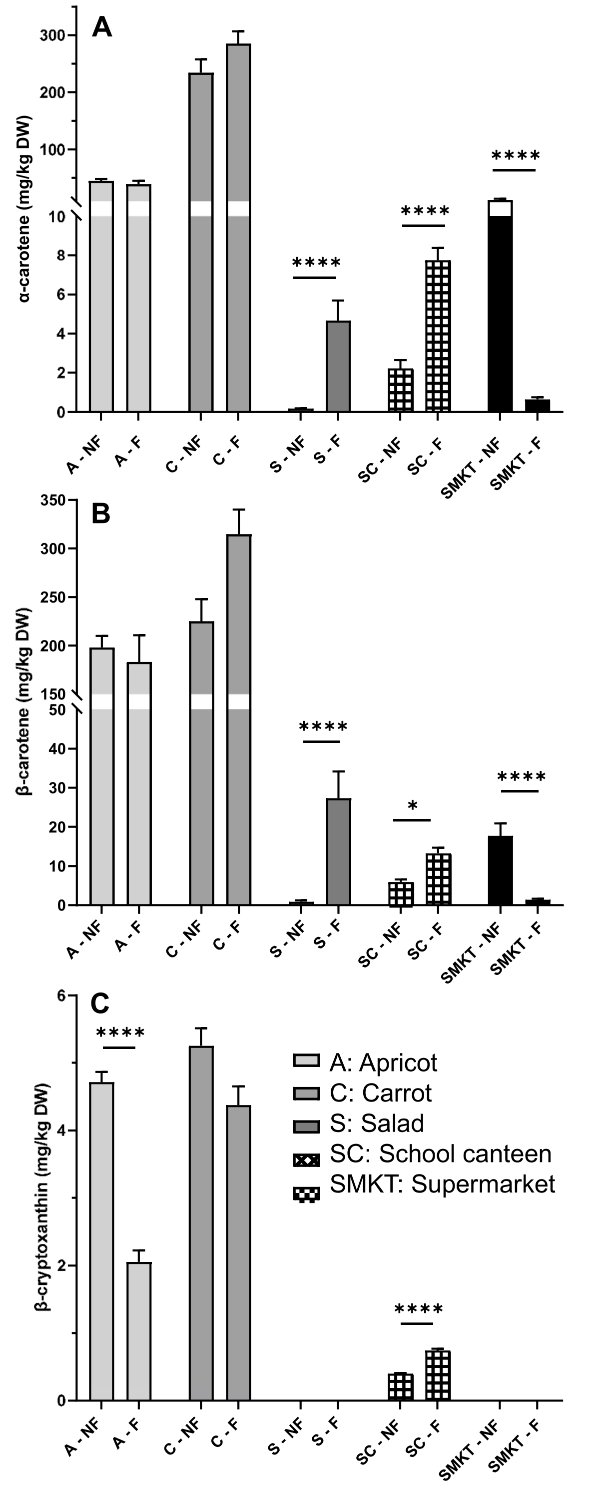


Fig. 2. Provitamin A carotenoid concentrations in non-fermented and fermented substrates.
NF: Non-fermented substrate; F: Fermented substrate. Bars represent means ± SEM ($n = 4$). Asterisks indicate a significant difference between the means of the NF and F substrates (*: $p < 0.05$; ****: $p < 0.0001$, two-ways ANOVA, with substrate and fermentation as the factors, followed by Bonferroni comparisons).

The concentrations of the three provitamin A carotenoids, *i.e.* α- and β-carotene and β-cryptoxanthin, in the different substrates are shown in Fig. 2 and **Figure S2**. While α (Fig. 2**A**) and β-carotene (Fig. 2**B**) were found in all studied substrates, β-cryptoxanthin (Fig. 2**C**) was only detected in apricot, carrot and SC waste. The effect of fermentation on the concentrations of α and β-carotene in the different substrates was equivalent: the concentrations of these two compounds in apricots and carrots remained non-significantly different, in SMKT waste they plummeted while in salad and SC waste they increased. Concerning β-cryptoxanthin, its concentration in apricots dropped while in SC waste it increased.

The concentrations of the carotenes which are not provitamin A, *i.e.* lycopene, phytoene and phytofluene are presented in **Figure S3 and S4**. Fermentation had no significant effect on the concentration of these carotenes in apricots, while it reduced their concentrations in SMKT waste and reduced the concentrations of phytofluene in carrots and salad. Surprisingly, fermentation increased the concentration of phytoene in carrots and SC waste and it also increased, but not significantly, that of lycopene in this latter substrate. Concerning the non-provitamin A xanthophylls, *i.e.* lutein and zeaxanthin (**Figure S5** and **Figure S6**), lutein was found in all the studied substrates (**Figure S5A**). After fermentation, lutein concentration either remained stable in apricot, carrots, SC and SMKT wastes or increased in salad. Zeaxanthin concentration, either fell, in apricots and SMKT waste or increased in salad and SC waste.

The fermentation effect on the concentrations of micronutrients in the different substrates, when expressed as FW or DW, are presented in **Table S1**.

Fresh weight and lipid and protein content of the larvae

BSFL FW are presented in Fig. 3**A**. The standard food (70 % apple and 30 % WB), commonly used by the breeder, serves as a reference value here. Larvae reared on WB, carrot, salad and SC had comparable masses to the standard ones (120.5 mg). Therefore, only larvae reared on apricots and SMKT waste had lower mass than the ones reared on the standard food. The study of the evolution of BSFL weights of the different groups from day 3 to day 7 (**Figure S7**) after deposit on these substrates showed that only BSFL from the salad group had a mass increase. BSFL from other groups had a weight gain period until day 5 or 6 and then a weight loss period. However, the dry weight of all BSFL groups at the end of the rearing period were not different (**Figure S8A**). Concerning the protein and lipid content of the larvae, they were not affected by the rearing substrate, either when they were expressed in FW (126.5 ± 5.2 and 70.8 ± 3.5 mg/g on average respectively, Fig. 3**B and C**) or in DW (432.6 ± 11.7 and 241.6 ± 9.3 mg/g on average respectively, **Figure S8**).

Fat-soluble vitamin and carotenoid content of the larvae

First of all, as for the substrates, the molecules of cholecalciferol (vitamin D3), ergocalciferol (vitamin D2), retinol (vitamin A), retinyl acetate (vitamin A), retinyl palmitate (vitamin A) and astaxanthin were not detected in all BSFL tested. The data are presented on a FW basis in this part because the main objective was to measure the concentration of micronutrients in native larvae, but results expressed on a DW basis are available in supplements (**Figures S9 to S12**).

Juvenile larvae

The vitamin and carotenoid contents in the juvenile food and in the juvenile larvae (8.5 d) reared on this substrate are presented in Table 2. Neither α-carotene nor phytofluene were detected here. Surprisingly, α-tocopherol and phytoene were also not detected in the juvenile food, while they were found in the juvenile larvae. On the contrary, β-cryptoxanthin was only detected in the juvenile food. The other molecules, γ-tocopherol, β-carotene, lutein, lycopene and zeaxanthin, were all present in higher concentrations in the juvenile food than in the juvenile larvae.

Larvae

Figs. 4**-**7 present the concentrations in vitamins or carotenoids in each BSFL group in comparison with the concentration in their rearing substrate (after mixing with WB in the case of apricots, carrots, salad, SC and SMKT waste).

In Fig. 4, the levels of the two vitamin E molecules assessed, α and γ-tocopherol, are shown. Contrary to the juvenile BSFL (Table 2), the 15.5 d larvae did not contain any α-tocopherol (Fig. 4**A**). Γ-tocopherol (Fig. 4**B**) however, was detected in BSFL reared on the standard food, WB and salad. In two groups (standard and WB), the γ-tocopherol concentration was higher than in the rearing substrate. Regarding the salad group, the γ-tocopherol concentration was lower in BSFL than in the rearing substrate.

Fig. 5 displays the concentrations in provitamin A carotenoids, *i.e.* α-carotene (Fig. 5**A**), β-carotene (Fig. 5**B**) and β-cryptoxanthin (Fig. 5**C**). A-carotene was found in apricots, carrots, salad, SC and SMKT fermented substrates, as well as in the larvae which have consumed these fermented substrates. For the apricot and salad groups, the concentrations were lower in the BSFL than in their corresponding rearing substrates. For the SMKT group, the opposite phenomenon was observed, in fact the concentration was higher in the BSFL than in the substrate. Concerning the SC group, the concentrations in the BSFL and in the substrate were non significantly different. As shown in Fig. 5**B**, β-carotene was found in all BSFL groups except the WB one. For the apricot and carrot groups, concentrations in the larvae and in the substrates were not different. However, larvae from the standard group were less rich than their substrate, while for the NAS groups and salad, larvae were richer in β-carotene than their substrate. Concerning β-cryptoxanthin (Fig. 5**C**), what is obvious is that the concentrations in the larvae were always higher than those measured in the rearing substrates. Moreover, β-cryptoxanthin was not detected in many substrates. Note nevertheless that this carotenoid was present in the juvenile food.

Fig. 6 shows the concentrations in lycopene (Fig. 6**A**), phytoene (Fig. 6**B**) and phytofluene (Fig. 6**C**) in BSFL and in their rearing substrates. Lycopene was found in all BSFL groups except carrots and WB. It was quantified in BSFL reared on standard food and salad while it was not detected in their respective rearing substrates. However, note again that it was present in the juvenile food. Lycopene concentration was lower in BSFL than in their substrate for the BSFL reared on apricots and SC waste. BSFL reared on SMKT waste nevertheless, highlighted higher lycopene concentration than in the substrate. Phytoene (Fig. 6**B**) was present in the apricot, carrot, SC and SMKT BSFL groups. It was therefore found in all larvae which consumed substrates which contained this carotenoid and not in those which consumed substrates which did not contain it. Note that it was not present in the juvenile food as well. Phytofluene (Fig. 6**C**) was only found in the apricot BSFL group. Only two substrates contained quantifiable concentrations of phytofluene, apricots and carrots, and only larvae reared on apricots had a quantifiable concentration of this colorless carotenoid.

The concentrations in lutein and zeaxanthin in the rearing fermented substrates and in the BSFL raised on these substrates are presented in Fig. 7**A and B** These two xanthophylls were found in all BSFL groups and in all fermented substrates with the exception of zeaxanthin in the carrot substrate. The highest concentrations in these xanthophylls were recorded in BSFL reared on salad (2.81 ± 0.25 mg/kg for lutein and 0.78 ± 0.08 mg/kg for zeaxanthin). Overall, the concentrations of these two xanthophylls were generally higher in the larvae than in their corresponding substrate, with the exception of lutein and zeaxanthin from SC waste and lutein from salad.

The overall concentration in vitamins of the different BSFL are displayed on an heatmap (**Figure S13**). Most of the micronutrients are present in concentrations lower than 0.5 mg/kg. Only γ-tocopherol, α and β-carotene, phytoene and lutein present concentrations higher than 1 mg/kg.

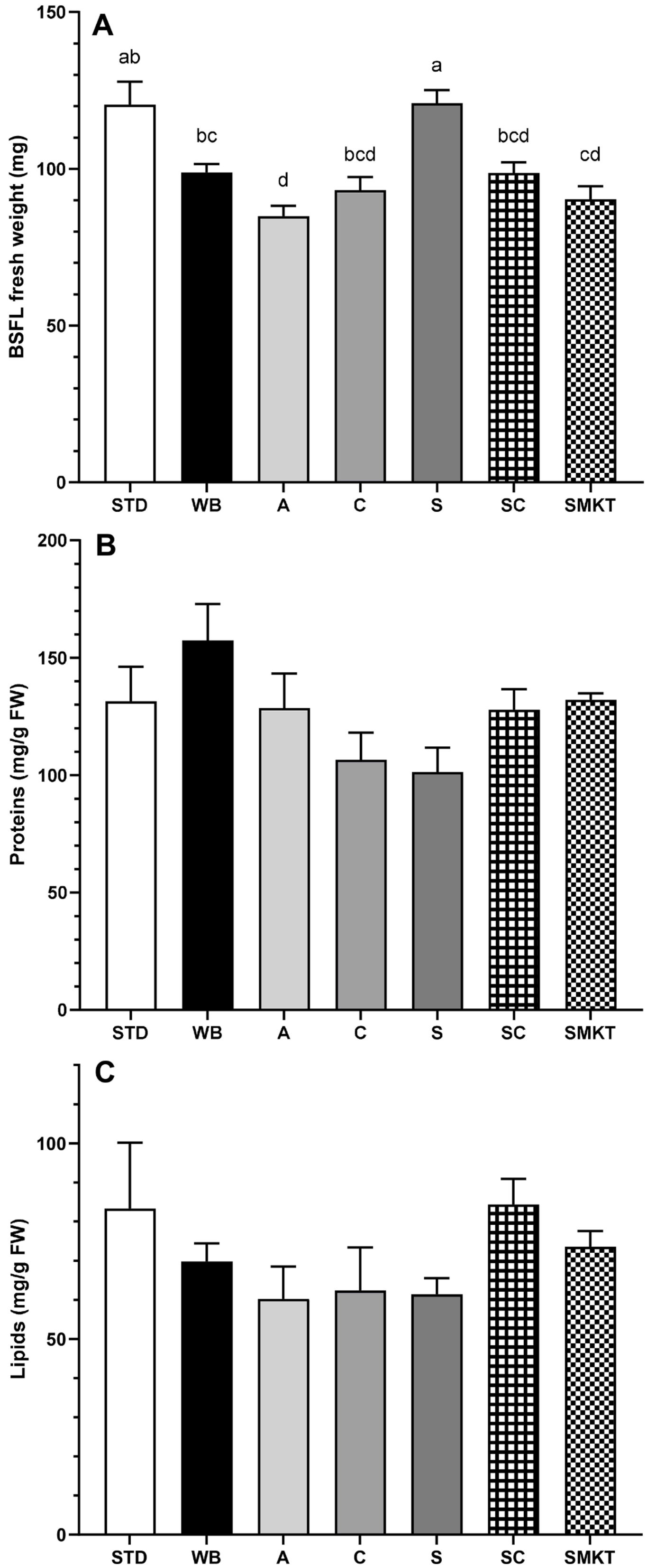


Fig. 3. Fresh weight (A) and protein (B) and lipid (C) concentrations of BSFL reared on different substrates.
All the substrates, except the standard and the WB ones, were fermented and mixed with WB (see material and methods). Bars represent means ± SEM ($17 > n > 31$ for A and $n = 4$ for B and C). In Figure A, different letters mean that the means of the groups are significantly different ($p < 0.05$; One-way ANOVA followed by Tukey's post-hoc test). In Figures B and C the ANOVA were not significant.

Table 2
Vitamin E and carotenoid content (mg/kg fresh weight) of juvenile larvae and their food.

	Juvenile BSFL[1] food	Juvenile BSFL[1]
α-tocopherol	ND	2.09
γ-tocopherol	15.35	2.22
α-carotene	ND	ND
β-carotene	0.20	0.05
β-cryptoxanthin	0.26	ND
Lycopene	0.23	0.08
Phytoene	ND	0.10
Phytofluene	ND	ND
Lutein	1.25	0.69
Zeaxanthin	1.21	0.43

[1] BSFL = Black soldier fly larvae
ND: not detected, *i.e.* below the detection limit.

Discussion

Vitamin and carotenoid concentrations in raw substrates

Measuring the concentration of the studied compounds in the raw, *i.e.* unfermented, substrates, and more particularly in SC and SMKT for which no data were found in the literature, was an essential prerequisite for evaluating the effect of fermentation on the concentrations of these compounds in the substrates. The concentrations of vitamin E and carotenoids observed in WB, apricots, carrots and salad agreed with what was expected according to the French reference food table (https://ciqual.anses.fr/) and literature (Grassmann et al., 2007; Kan et al., 2014; Reif et al., 2013; Shemesh et al., 2017; Suchowilska et al., 2020; Surles et al., 2004; Zhou et al., 2005). Regarding SC and SMKT, it was expected to find all the micronutrients present in usually consumed food in France, including dairy, eggs, fruits and vegetables, meat and fish. Interestingly, these biowaste contained most of the micronutrients found in commonly consumed fruits and vegetables, except β-cryptoxanthin, zeaxanthin and phytofluene for SMKT. It is also interesting to point out that vitamin E was only found in these biowaste. Conversely, the concentrations in α and β-carotene was 12 to 110 times lower in these biowaste than in carrots. This certainly depends on the foods that made up these biowaste and their relative proportions, as well as the difference in lability of the different compounds during the treatment/storage of these biowaste. Moreover, the proportion of nutriments in these biowaste is prone to seasonal variations, food eating habits or sampling places for instance. However, micronutrients of animal origin such as preformed vitamin A, *i.e.* retinol and retinyl esters (retinyl palmitate), which is particularly present in liver and dairy products, vitamin D3, *i.e.* cholecalciferol, which is particularly present in fish and fish products and astaxanthin, which is notably present in shellfish, were not detected. This can be explained both by the low proportion of foods from animal origin in these biowastes, by their intrinsic low concentrations in food, and by the fact that they could have been degraded by the processing and storage of these biowaste between the time of their collection and their receipt by the BSFL breeder.

Effect of fermentation on micronutrient content of the substrates

As explained in the material and methods section, the substrates were fermented before being fed to the larvae. Knowing that most fat-soluble vitamins and carotenoids are sensitive to oxidation, acidic pH and temperature (Boon et al., 2010), and that the bacteria that develop during fermentation can metabolize some of these compounds (Bartkiene et al., 2013; Lee et al., 2018; Oloo et al., 2014), their concentrations in the fermented substrates were measured. The apparent effect of fermentation on the concentrations of micronutrients was very different for each molecule and type of waste when expressed in FW or in DW. This was also reported in the literature for different vegetables species (Kiczorowski et al., 2022) and even for different variety of the same tomato species (Bartkiene et al., 2013). Four situations of concentration variations following fermentation were observed (**Table S1**). In the first situation, the concentration in micronutrients after fermentation was equal to the concentration before fermentation in DW but decreased in FW. This was the case for 46 % of the couple molecule/substrate such as α and γ-tocopherol in SC and SMKT waste for example. Considering that fermentation led to a huge increase in the water content of the substrate (from 75 % to 99 % for SMKT for example), we explain this result by the fact that micronutrients were only diluted and that fermentation had no significant effect on them. Riciputi et al. (2016) have also found that tocopherols were not affected by the fermentation process of tofu. Carciochi et al. (2016), on the contrary saw a decrease of all tocopherol isomers during fermentation of quinoa seeds. In the second situation, the concentrations in micronutrients after fermentation were lower than those measured before fermentation (27 % of the couple molecule/substrate) whether the concentrations were expressed in FW or in DW. The hypothesis here is that fermentation led to a loss of these compounds. This was the case for phytofluene in carrots, salad and SMKT waste. These losses were also reported in the literature. Odongo et al. (2017) observed that the total carotenoid content of fermented Ethiopian kale dropped by 75 %, with the largest losses being for lutein (98 %). Kun et al. (2008) and Oloo et al. (2014) also reported losses of α and β-carotene after fermentation of sweet potatoes (6 % loss of β-carotene) and carrot juice (between 15 and 45 % loss). According to Lee et al. (2018), loss in carotenoid content can happen due to the formation of volatile carotenoid cleavage derivatives during fermentation. In the third situation, representing 11 % of the couple molecule/substrate, the concentrations in micronutrients increased after fermentation both in FW and DW. This case gathers the highest increase observed in micronutrient content. This was only observed in salad, which is the only substrate whose humidity level did not increase enormously during fermentation (only 1 %). In the fourth situation, representing 16 % of the couple molecule/substrate, the concentration in micronutrient after fermentation increased in DW but decreased in FW, which was the case for α and β-carotene from SC for example. In that situation, fermentation also led to an increase in micronutrient concentration, counterbalanced by the increase in the water content of the substrate during fermentation. In the two last cases, fermentation might have either led to an easier extractability of these micronutrients or to their synthesis. Carotenogenesis, *i.e.* the production of carotenoids during fermentation processes has been reported with several microorganisms described to be able to produce β-carotene, γ-carotene and lycopene for instance (Cutzu et al., 2013; Hu et al., 2013; Mapelli-Brahm et al., 2020; Nanou and Roukas, 2016; Szutowska et al., 2021). Szutowska et al. (2021) investigated the fermentation of kale juices and saw losses of zeaxanthin, β-cryptoxanthin, β-carotene and all-trans lutein while 9-*cis* lutein increased. This study showed that fermentation led to different outcomes depending on the isomer considered. In this present study, isomers were not separated which could explain the differences depending of the matrix. Bartkiene et al. (2013) studied the effect of different fermentations on lycopene content in tomato pulp from two different varieties of tomatoes. They observed an increase in lycopene content in one variety and a decrease in the other one after spontaneous fermentation.

It is important to emphasize that the fermentation of SC was the only fermentation which did not lead to significant degradation of micronutrients. Indeed, their DW concentrations were either not affected by fermentation (α and γ-tocopherol, lycopene, lutein) or increased after fermentation (provitamin A carotenoids, phytoene, zeaxanthin). Our hypothesis to explain this surprising result is that the mixture of fruits and vegetables which certainly constituted the majority of SC provided a greater diversity of antioxidant compounds than the single substrates, and very varied antioxidants are more effective in protecting each other from the oxidation than isolated antioxidants (Wang et al., 2011). Concerning the SMKT, which also contains a mixture of different fruits

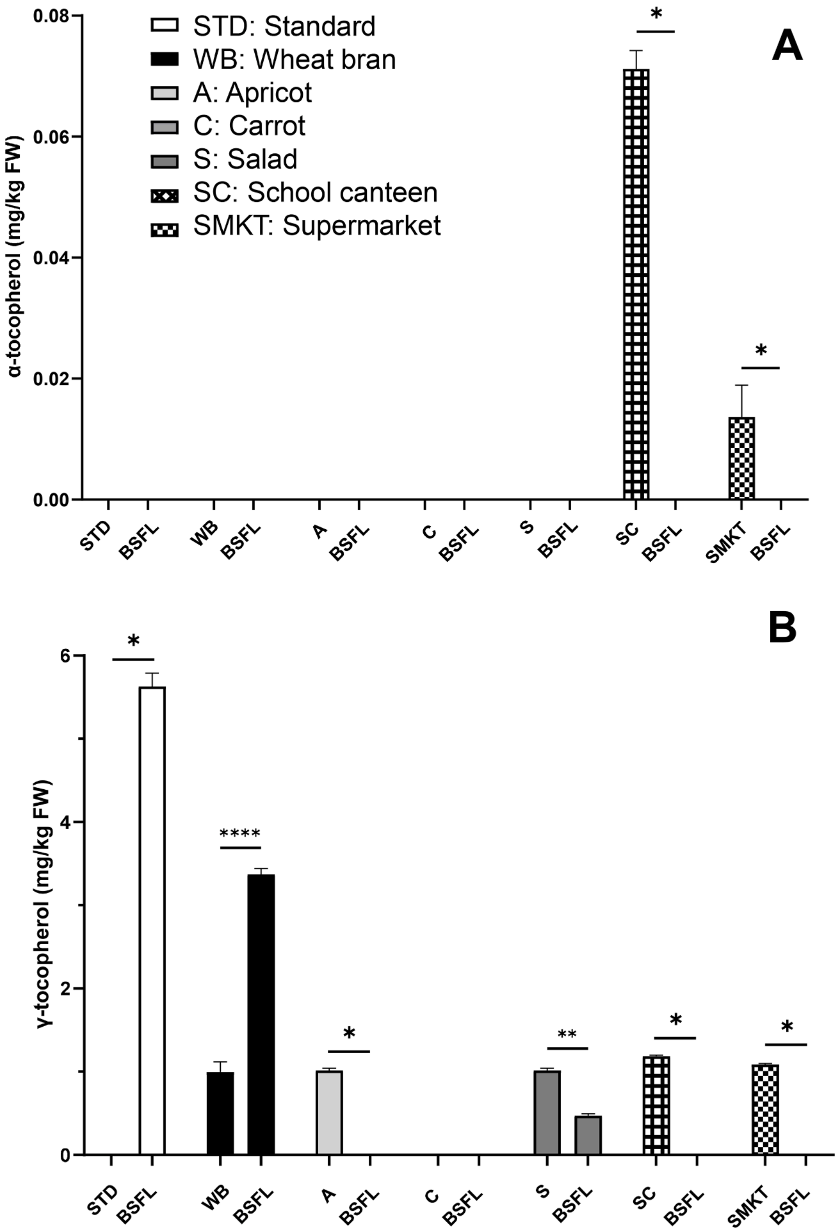


Fig. 4. α-tocopherol (A) and γ-tocopherol (B) concentrations in the fermented substrates and in BSFL reared on the corresponding fermented substrates.
All the substrates, except the standard and the WB ones, were fermented and mixed with WB (see material and methods). For each substrate group "BSFL" indicates larvae that were reared on the corresponding substrate. Bars represent means ± SEM ($n = 4$). Asterisks indicate a significant difference between the mean concentration measured in the BSFL and in the corresponding substrate (*: $p < 0.05$; **: $p < 0.005$; ****: $p < 0.0001$, two-ways ANOVA, with matrix and group as the factors, followed by Bonferroni comparisons, or Kolmogorov-Smirnov test when a molecule was not detected).

and vegetables, a surprising negative effect of fermentation on the micronutrient content was observed. This biowaste might contain prooxidant compounds, *e.g.* iron from meat, which are not present in SC biowaste, or present in much lower quantities. Therefore, factors such as the type of micronutrient, the isomer, the initial matrix, the variety and the fermentation conditions should be taken carefully into account for the fermentation process. It might be therefore important for a BSFL breeder to carefully choose the matrix with the micronutrient of interest for him not according to food tables but rather according to the impact of fermentation.

Lipid and protein content of the larvae

The lipid and protein content of BSFL are among the most important criteria to look after when choosing a substrate. No significant differences were observed in this study. This means that even if the larval weights were different, the lipid and protein contents of the larvae were not significantly affected by the type of substrate. Moreover, the lipid content in dry weight, ranging from 20.4 % to 27.5 %, and the protein content in larvae in dry weight, between 38.5 % and 45.9 %, are coherent with the literature (Lu et al., 2022; Spranghers et al., 2017; Wang and Shelomi, 2017). All tested substrates can therefore ensure BSFL a correct total protein and lipid content in the end. We are nevertheless aware that, since only a relatively small number of larvae per group were analysed, further analyses with a larger number of larvae per group are necessary to confirm these results. Further analysis of the amino acid composition and fatty acid profile could further reveal differences in the quality of proteins and lipids present in the different groups of larvae (Chia et al., 2020; Meneguz et al., 2018). Finally, further studies including analysis of the chitin and other carbohydrate

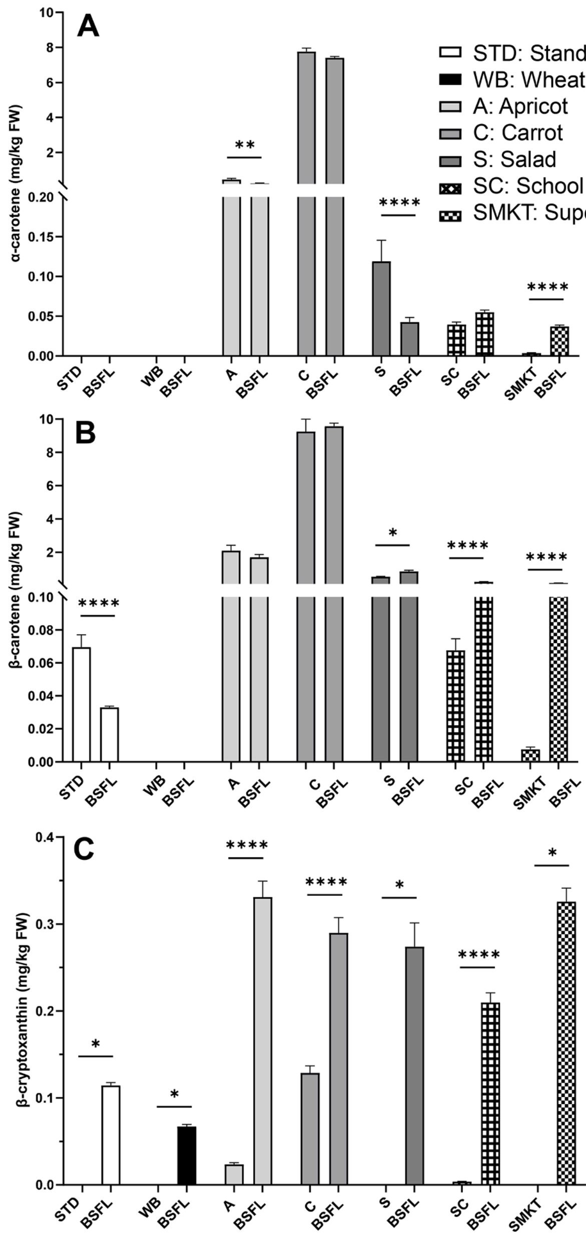


Fig. 5. α-carotene (A), β-carotene (B) and β-cryptoxanthin (C) concentrations in the substrates and in BSFL reared on the corresponding substrates.
All the substrates, except the standard and the WB ones, were fermented and mixed with WB (see material and methods). For each substrate group "BSFL" indicates larvae that were reared on the corresponding substrate. Bars represent means ± SEM (n = 4). Asterisks indicate a significant difference between the mean concentration measured in the BSFL and in the corresponding substrate (*: $p < 0.05$; **: $p < 0.005$; ****: $p < 0.0001$, two-ways ANOVA, with matrix and group as the factors, followed by Bonferroni comparisons or Kolmogorov-Smirnov test when a molecule was not detected in the fermented substrate).

content of the larvae could finally make it possible to know whether the differences in the weight of the larvae were due to a variation in their water content and/or their content in these other compounds.

Vitamin and carotenoid concentrations in larvae

The main objective of this study was to determine whether, by rearing larvae following a usual BSFL breeder procedure, a significant bioaccumulation of vitamin E and carotenoids could happen. It was previously demonstrated that BSFL cannot synthesize vitamin E, α and

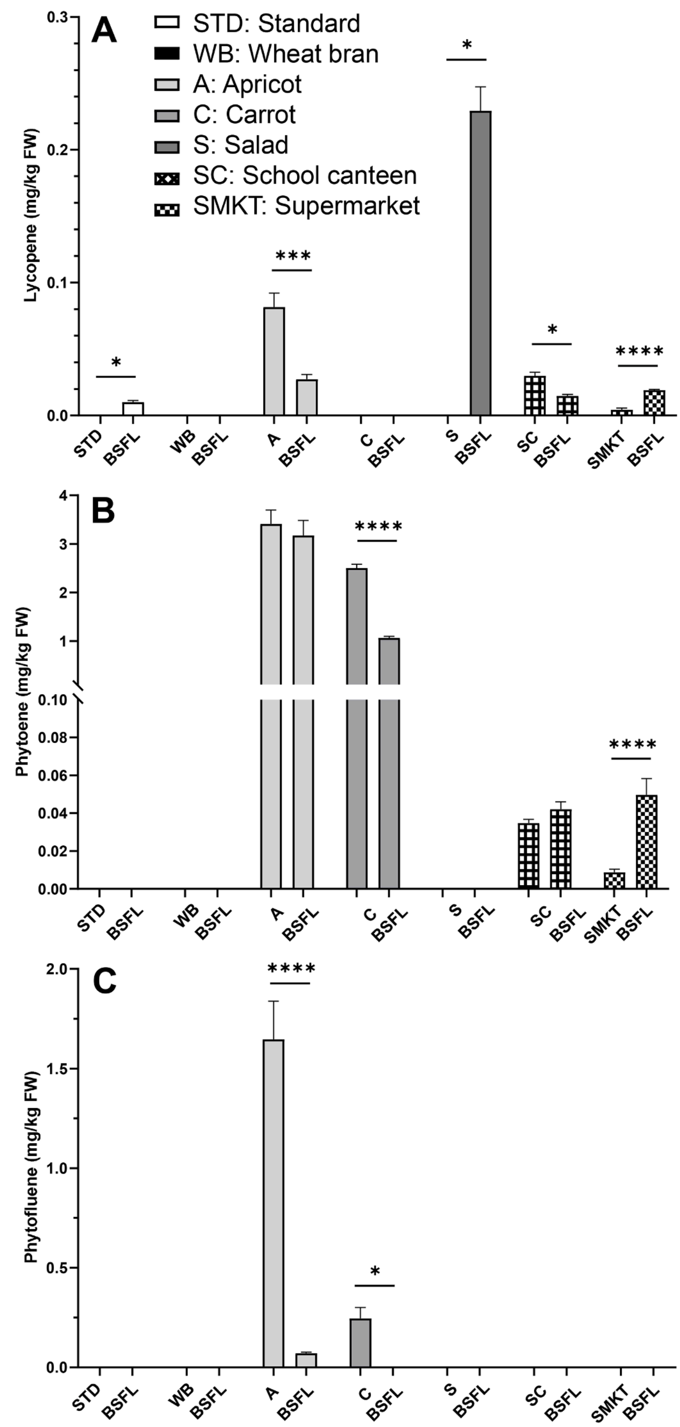
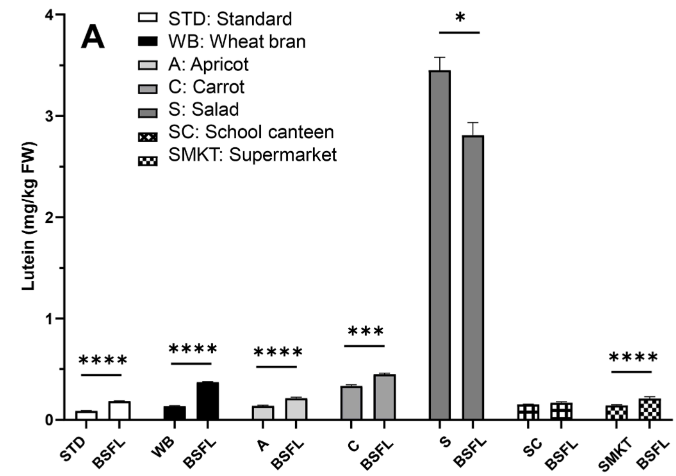


Fig. 6. Lycopene (A), phytoene (B) and phytofluene (C) concentrations in the substrates and in BSFL reared on the corresponding substrates.
All the substrates, except the standard and the WB ones, were fermented and mixed with WB (see material and methods). For each substrate group "BSFL" indicates larvae that were reared on the corresponding substrate. Bars represent means ± SEM (n = 4). Asterisks indicate a significant difference between the mean concentration measured in the BSFL and in the corresponding substrate (*: $p < 0.05$; ***: $p < 0.0005$; ****: $p < 0.0001$). Lycopene and phytoene: Two-ways ANOVA, with matrix and group as the factors, followed by Bonferroni comparisons or Kolmogorov-Smirnov test when a molecule was not detected in the substrate. Phytofluene: unpaired t-test or Kolmogorov-Smirnov test when the molecule was not detected in BSFL.



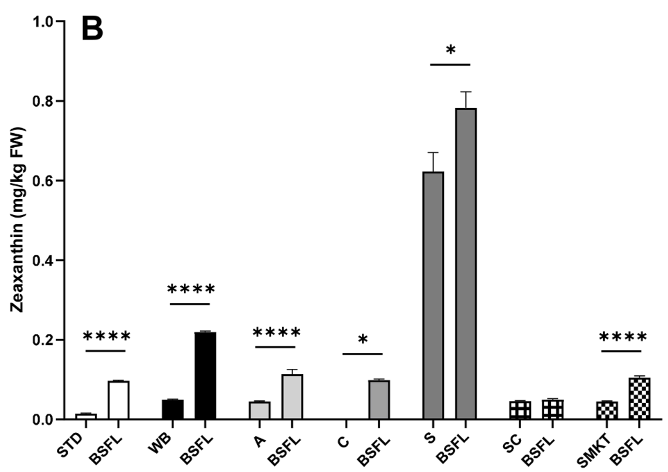


Fig. 7. Lutein (A) and zeaxanthin (B) concentrations in the substrates and in BSFL reared on the corresponding substrates.
All the substrates, except the standard and the WB ones, were fermented and mixed with WB (see material and methods). For each substrate group "BSFL" indicates larvae that were reared on the corresponding substrate. Bars represent means ± SEM ($n = 4$). Asterisks indicate a significant difference between the mean concentration measured in the BSFL and in the corresponding substrate (*: $p < 0.05$; ***: $p < 0.0005$; ****: $p < 0.0001$, two-ways ANOVA, with matrix and group as the factors, followed by Bonferroni comparisons or Kolmogorov-Smirnov test when a molecule was not detected in the fermented substrate).

β-carotene, β-cryptoxanthin and lutein (Borel et al., 2021; Morand--Laffargue, et al., 2023; Morand-Laffargue et al., 2023). And in this study, it was demonstrated that lycopene, phytoene and phytofluene cannot be synthesized as well by BSFL, since they were not found in BSFL reared on substrates not containing these micronutrients. Then, γ-tocopherol and carotenoids found in the larvae were transferred from their rearing substrates. Among the molecules quantified in larvae, higher concentrations in larvae than in their rearing substrate were found in 65 % of the cases, meaning that larvae bioaccumulated these molecules. For example, γ-tocopherol from WB and α-carotene from SC and SMKT were bioaccumulated in BSFL. Liland et al. (2017) also demonstrated that with increasing inclusions of brown algae in feeding media, the vitamin E concentrations in larvae increased. However, in this study, bioaccumulation of fat-soluble vitamins and carotenoids was really dependent of the substrate.

Surprisingly, some micronutrients such as ɣ-tocopherol (Fig. 4B), β-cryptoxanthin (Fig. 5C) and lycopene (Fig. 6A), were not present in the studied substrates but were found in the larvae (in STD, WB, S and SMKT groups for β-cryptoxanthin and STD and S for γ-tocopherol and lycopene). As the larvae do not synthesize these compounds, different hypotheses to explain their presence in the larvae can be proposed. First of all, as the larvae were raised during their first 8 d of life on the substrate for juvenile larvae, which contained these micronutrients (Table 2), it is likely that the larvae have begun to bioaccumulate these micronutrients before 7 d old and that these micronutrients were still present in the larvae more than 7 d after having been deprived of this substrate. If this hypothesis is correct, it supports our optimal breeding method to bioaccumulate a maximum of micronutrients by the larvae (Borel et al., 2021), which consists of hatching the eggs directly on the substrate rich in micronutrients to maximize the time during which the larvae consume this substrate. Another hypothesis could be that these compounds were synthesized by bacteria or fungi that developed on the substrate during the rearing period, or that were present in the microbiota or mycobiota of the larvae. Indeed, many microorganisms are able to synthesize micronutrients (Cutzu et al., 2013; Hu et al., 2013; Mapelli-Brahm et al., 2020; Nanou and Roukas, 2016; Szutowska et al., 2021). Among the micronutrients present in the larvae but not in the substrate, ɣ-tocopherol is the one that raises the most questions. It was indeed present in greater quantities in the larvae raised on the standard substrate, which apparently did not contain this molecule, than in the larvae raised on WB, which contained ɣ-tocopherol (Fig. 4**B**). Our hypothesis is that there was indeed ɣ-tocopherol in the standard substrate, because this substrate was a mixture of apples and WB, but that its concentration was too low to be detected by our measurement technique. On the other hand, we suppose that it must have been much more bioavailable in this mixture of apples and WB than in WB alone because its bioavailability may have been improved by the apple polyphenols (Jia et al., 1998).

The comparisons of the concentrations of ɣ-tocopherol, α and β-carotene, β-cryptoxanthin and lutein, show that they were about 1.3, 4, 10, 30 and 3 times lower, respectively, in the breeder's usual breeding conditions than in optimal conditions (Borel et al., 2021; Morand--Laffargue et al., 2023a, 2023c). The effects of procedures diverging between the breeder and the optimal conditions can be objectively compared using α and β-carotene which were also provided by carrots in previous studies under optimal conditions. The difference in concentrations did not come from the bioaccumulation rate, which was close to 1 in the present study under breeder's conditions and which was even slightly lower in previous study under optimal conditions, around 0.6–0.7. It comes from the FW concentrations of these two provitamin A carotenoids in their respective rearing substrate which were approximately 6 times lower for α-carotene and 16 times lower for β-carotene in the present study compared to the concentrations in the non-fermented carrots used in the previous studies under optimal conditions. The fermentation and the mixing with WB has considerably lowered the quantity of α and β-carotene (from 31 to 29 mg/kg fresh weight to 8 and 9 mg/kg, respectively) in the rearing substrate of breeder's conditions. Therefore, the first factor which influences the final concentration of these micronutrients in the larvae is the concentration of these micronutrients in the substrate given to the larvae. It would therefore be interesting to modify the breeding conditions, while respecting the constraints of the breeders, to improve the micronutrient concentrations in the rearing substrate.

Interest of using yet unauthorized biowaste in the EU

A priori, unauthorized biowaste are very inexpensive and available throughout the entire year and therefore are very interesting from an economic point of view, to raise BSFL. There was no difference with the larvae raised on these biowaste and on the other substrates regarding the protein and lipid content of the larvae obtained at the end of the rearing period. These results differ from previous studies which indicate a substrate effect on these indicators (Chia et al., 2020; Shumo et al., 2019; Spranghers et al., 2017) but this is perhaps because our study was not sufficiently statistically powered to find significant differences on these parameters. However, the larvae raised on NAS had lower weight than their standard counterparts (25 % less for SMKT and 20 % less for SC) which is difficult to explain because, although we did not have the macronutrient and therefore energy composition of these substrates, we thought, and we still think, that they were richer in energy than the authorized substrates because, a priori, they must have contained, in addition to plants, waste from vegetable oils, meat and fish which are a good source of energy. It is nevertheless possible that the energy density of the mixtures of WB and NAS was lower than that of the AS, because the breeder did not adjust the mixtures to have the same final protein content, but to have the same final moisture content. However, the NAS were relatively liquid and were therefore mixed in a smaller proportion with WB. Regarding micronutrients, these substrates, along with apricots, led to BSFL with the greatest variety of bioaccumulated carotenoids: all the main provitamin A carotenoids plus lycopene, phytoene, lutein and zeaxanthin. They either contained approximately the same concentrations of micronutrients as larvae raised on the STD substrate, such as lutein and zeaxanthin, or higher concentrations than larvae raised on the STD substrate, case of provitamin A carotenoids, lycopene and phytoene. Shumo et al. (2019) also showed that BSFL reared on kitchen waste contained α-tocopherol, γ-tocopherol and even provitamin D3. This means that, if these biowaste do not present toxicological and microbiological risks, it would be more interesting to raise BSFL on these substrates than on STD substrate if micronutrient content is a sought-after quality criterion. Nevertheless, it is important to keep in mind that these biowaste are very different from one another, prone to the type of meal eaten by children at the canteen that day or the food left that day by SMKTs. Therefore, the results obtained here need to be validated with more samples, collected at different time points and areas.

Conclusion

It was first demonstrated that rearing BSFL under usual industrial rearing conditions, allows the larvae to bioaccumulate vitamin E and carotenoids. Nevertheless, concentrations of micronutrients in the larvae were significantly lower than those observed under laboratory rearing conditions. Further research is therefore needed, particularly to optimize the fermentation process which results in a significant loss of the studied micronutrients. It has also been shown that heterogeneous biowaste, composed of a mixture of plant and animal waste and which are therefore not yet authorized for the breeding of edible insects in Europe, contain a greater variety of the studied micronutrients than biowaste consisting solely of plants. Finally, it was shown that the larvae bioaccumulate the micronutrients present in these NAS while having the same protein and lipid contents as the larvae raised on AS. From a nutritional point of view, this makes NAS interesting, provided that they are deemed safe, both on a microbiological and chemical safety point of view. More thorough studies, with a greater number of larvae per group, regarding the precise composition of substrates in lipid, protein, carbohydrates, fibers and other micronutrients are necessary to confirm the results of this exploratory study and fully understand the observed differences in micronutrient bioaccumulation.

Ethical statement

All animal experiments were carried out in accordance with ARRIVE guidelines, Guidance on the operation of the Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63 for the protection of animals used for scientific purposes.

CRediT authorship contribution statement

Marie Papin: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Charlotte Sabran:** Methodology, Investigation. **Lisa Morand-Laffargue:** Writing – review & editing, Methodology. **Damien Sabatier:** Resources. **Ayoub Sefah:** Resources, Investigation. **Erwan Engel:** Writing – review & editing, Funding acquisition, Conceptualization. **Christelle Planche:** Writing – review & editing, Funding acquisition, Conceptualization. **Patrick Borel:** Writing – review & editing, Writing – original draft, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Some supplies were provided by BioMiMetiC. Ayoub SEFAH and Damien SABATIER work for BioMiMetiC company. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2025.100614.

Data availability

Data will be made available on request.

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