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Sustainable strategies to increase the content of protein, unsaturated fatty acids and vitamins of *Tenebrio molitor* larvae flours by vegetable waste supplementation †

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Abstract: *Tenebrio molitor* larvae were fed with wheat bran and supplemented (1:1) with tomato or cucumber agricultural waste for 6 weeks. After supplementation, larvae were dried in a pilot-infrared oven (68°C for 4h) and ground to obtain the flours. The quality attributes and nutritional value of insect flours differed based on the supplemented diet. Unsaturated fatty acids, proteins, starch, and certain vitamins were enhanced in flours from supplemented larvae. Therefore, tomato and cucumber waste can be revalorized as supplements of *T. molitor* conventional diet to obtain insect flours and higher nutritional value with acceptable quality attributes.

Keywords: Insect farming; mealworm flour; alternative protein; linoleic acid; vitamins; quality attributes; colour; lipid oxidation; vegetable waste

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1. Introduction

Huge amount of vegetable waste is generated by agri-food industries, reaching 3-5% of annual production, which entails economic losses and high environmental impacts. Besides, in the next years, population growth will cause higher demand for food protein products, which is difficult to achieve if meat alternatives are not proposed. For this reason, the revalorization of generated waste is more and more necessary to reduce the carbon footprint, obtain economic profitability and develop novel and nutritive food products for future.

Insects are a profitable and environmentally friendly source, as they can transform low-value organic by-products into high-value food or feed [1]. Therefore, insect farming has been proposed as a potential solution for previous explained issues since they lead to lower emissions of ammonia and greenhouse gases than conventional farms due to their greater efficiency in the conversion of feed into protein, a reduced requirement of land area and water, and the lower investment in capital and equipment [2]. *Tenebrio molitor* is one of the most interesting insects to generate food and feed, due to its high protein content (~50 %), high quality fat (~30 %), and micronutrients such as vitamins or minerals. Furthermore, their ability to feed on any source makes them a perfect candidate for being fed with wastes. Food technologist and agri-food companies are interested in the production of these new sources of alternative proteins with the aim of developing insect meals that are suitable for animal and human nutrition while reducing environmental issues.

Vegetable wastes are an excellent source of bioactive compounds, which when consumed exerts biological activity: antioxidant, anti-inflammatory, hypoglycaemic, among others. Most common bioactive compounds in vegetables include phenolic compounds, terpenoids, vitamins, and sulphur compounds. Therefore, the consumption of vegetable wastes by *T. molitor* larvae could contribute to improve the nutritional composition, functional value, and quality of mealworm flours. The main aim of this study was evaluating the effect of supplementing the diet of *T. molitor* larvae with cucumber and tomato wastes on the quality attributes and nutritional profile of insect flours.

2. Material and Methods

2.1. Insect Farming

Insect farming was carried out at the Insectalia S.L. company facilities. *T. molitor* larvae were fed with wheat bran (W) as a control diet or supplemented (1:1) with agricultural wastes from tomato (T) or cucumber (C) cultivation. Water was added to the control diet to compensate the wastes moisture. Five trays containing 100 g of 40-day larvae were used for each studied diet condition (C+W, T+W and W). Larvae were grown in a controlled-chamber room in 12h light-dark cycles (27˚C and 50% humidity) and fed once per week for 6 weeks. After that, they were starved for 48h, collected and frozen at – 20°C until being processed.

2.2. Insect Processing

Frozen larvae (4 kg) corresponding to each treatment condition were blended in a Thermomix® TM6 to obtain a paste. Paste was extended in an oven tray and were dried in an infrared irradiation-oven at 68°C until reaching <5% humidity (~4 h). Thereafter, the dried paste was grinded to obtain the corresponding flour. All insect flours (C+W, T+W and W) were stored at room temperature until analyses were performed.

2.3. Nutritional Value

2.3.1. Fatty Acids Profile

Fatty acid methyl esters (FAMEs) were obtained following the AOAC official method 996.06 and analysed in an Agilent 7890B gas chromatograph coupled to a 7200 quadruple-time-of-flight mass spectrometer with electron impact ionization. FAMEs were separated in a HP-88 capillary column, using Helium as carrier gas. The oven temperature was increased from 80°C to 145°C (8°C/min), maintained for 26 min, then increased to 200°C (2°C/min), maintained for 1 min and finally up to 220°C (8°C/min). Injector and transfer line temperatures were maintained at 250 and 240°C, respectively. Detector's instrumental conditions were ionization source temperature, 230°C; ionization energy, 70 eV; mass range, 50 – 500 m/z; solvent delay, 2 min. Extraction and derivatization were done in triplicate. Tentatively identified FAMEs were confirmed by comparison with Supelco's mix of 37 FAMEs.

2.3.2. Vitamins Content

Vitamins content was outsourced and determined by HPLC-MS.

2.3.3. Protein Content

Total protein content was outsourced and determined by Kjeldahl method.

2.3.4. Available Starch Content

Total available starch was measured in the flours following the manufacture instructions described in the digestible and resistant starch assay kit (Megazyme;

K-DSTRS). Results were expressed as % (w/w) of fresh weight. Extraction and determination of starch in flour were made in duplicate.

2.4. Quality Attributes

2.4.1. Moisture

Flour (2 g) was dried in an oven (Memmert GmbH, Schwabach, Germany) at 95 ºC for 4 h and the final weight was measured. Flours were evaluated in triplicate.

2.4.2. Colour

The CIELab parameters (lightness, L*; green-red chromaticity, a*; and blue-yellow chromaticity, b*) were utilized to characterise the colour of insect flours by using a colorimeter (PCE-CSM 3, PCE Instruments UK Ltd., United Kingdom). Nine readings were made in flour by changing the position of the colorimeter in each measure.

2.4.3. Lipid Oxidation

Lipid oxidation of flours was evaluated through the spectrophotometric determination of 2-thiobarbituric acid-reactive substances (TBARS), using the lipid peroxidation (MDA) assay kit MAK085 (Sigma-Aldrich). Lipid oxidation was expressed as mg of malondialdehyde (MDA) per kg of flour. Extraction and determination in flours were made in duplicate.

2.5. Statistical Analysis

Statistical analyses were carried out using the Statgraphics Centurion XVI.I software (Statgraphics Technologies Inc., Virginia, USA). Results were reported as the mean ± standard deviation. Results were subjected to an analysis of variance (ANOVA) followed by Tukey post hoc test to establish statistical differences among mean values. The statistical significance level was set up at $p < 0.05$.

3. Results and Discussion

3.1. Nutritional Value

Table 1. Effect of different larvae diet (W: wheat bran diet; C+W: cucumber waste supplemented diet; T+W: tomato waste supplemented diet) on flour total protein and total available starch contents.

Insect Flour	Total Protein content (% w/w fresh weight)	Total Available Starch content (% w/w fresh weight)
C+W	49.73 ± 0.19 a	1.03 ± 0.10 a
T+W	49.10 ± 0.18 b	1.09 ± 0.13 a
W	45.58 ± 0.22 c	0.39 ± 0.02 b

Different letters indicate significant ($p < 0.05$) differences among flours.

Total protein content was significantly enhanced in flours obtained from larvae fed with T+W and C+W (Table 1). Similar results were reported by Ruschioni et al. [3] when supplementing diet with olive pomace (similar protein content to W diet, 11-15% DW). Controversial results have been reported about the influence of diet on the larvae protein content. Oonincx et al. [4] have hypothesized that high protein diets lead to larvae with a higher protein content. Conversely, Van Broekhoven et al. [5] demonstrated that the protein content in larvae was similar despite diets differed in their protein content up to 2 and 3 times. In our study, the main dietary source of protein was W, with around 15 % in fresh weight (21 and 14 times higher than in C and T, respectively). The total amount of protein given to the larvae in the diet to obtain 1 kg of flour was approximately 0.87 kg (C+W), 0.92 kg (T+W) and 1.2 kg (W), which shows that larvae supplemented with C+W diet were the most efficient in bioconverting dietary protein in their own protein. Further studies are necessary to elucidate the effects of diet composition on larvae metabolism, growth, and composition. Diet influences the amino acid composition, but there is no established correlation between protein levels in diet and in larvae, although it has been suggested that protein denaturation might play a role [6].

Starch content was higher in C+W and T+W flours (Table 1). As far as we know, there are no available studies in which starch content of *T. molitor* flours after supplementation is evaluated. However, some authors have reported that larvae adapt their metabolism to the provided diet, and a high level of starch can reduce their growth [7], as observed in our research since the growth of larvae fed with W was lower than that of supplemented larvae (data not shown). Differences are probably related to changes in larval metabolism, which is adapted to the provided nutrients, although further studies are necessary to understand such changes.

Table 2. Effect of different larvae diet (W: wheat bran diet; C+W: cucumber waste supplemented diet; T+W: tomato waste supplemented diet) on flour fatty acid profile.

Insect Flours	Fatty acids (%) *							
	Myristic acid C14:0	Palmitic acid C16:0	Stearic acid C18:0	Elaidic acid C18:1n9t	Linoleic acid C18:2	Saturated fatty acids	Monounsaturated fatty acids	Polyunsaturated fatty acids
C+W	11.81 ± 0.27 b	22.33 ± 0.10 c	5.29 ± 0.06 a	22.14 ± 0.05 c	33.23 ± 0.18 a	39.70 ± 0.11 c	24.73 ± 0.05 c	34.94 ± 0.17 a
T+W	10.95 ± 0.03 c	23.71 ± 0.02 b	5.17 ± 0.02 a	23.20 ± 0.25 b	32.23 ± 0.24 b	40.08 ± 0.07 b	25.68 ± 0.24 b	33.66 ± 0.19 b
W	16.94 ± 0.03 a	24.23 ± 0.46 a	4.72 ± 0.17 b	24.28 ± 0.10 a	24.24 ± 0.08 c	46.09 ± 0.32 a	27.94 ± 0.03 a	25.29 ± 0.05 c

* Fatty acids were quantified in a relative way, expressing the % concentration of each individual compound with respect to the total content of FAMEs identified in the samples; Different letters indicate significant ($p < 0.05$) differences among fatty acids content in flours.

Table 2 shows the main fatty acids found in *T. molitor* flour, with linoleic acid, elaidic acid and palmitic acid being the most abundant. The % of linoleic acid (referred to total fatty acid content) was higher in C+W and T+W flours (1.37 and 1.33 times, respectively) than in W flour, whereas the opposite trend was observed for myristic acid. Such modification led to an increase in the % of polyunsaturated fatty acids in supplemented flours. Therefore, the fatty acid composition in *T. molitor* flour strongly depends on diet, as previously described [5]. High oleic and linoleic acid contents have been found in insects fed with grains. However, the lipid profile and content in larvae is more affected by the non-fibrous carbohydrates, starch and protein contents [5, 10] than by polyunsaturated fatty acids in diets. Fatty acids can be synthesized *de novo* from carbohydrates, which is regulated by acetyl-CoA carboxylase and fatty acid synthase [9]. Therefore, some metabolic changes could be triggered by the supplementation, although further enzyme expression and activity studies would be necessary to confirm the hypothesis.

Table 3. Effect of different larvae diet (W: wheat bran diet; C+W: cucumber waste supplemented diet; T+W: tomato waste supplemented diet) on flour vitamins content.

Insect Flours	Water-soluble vitamins (mg/100 g fresh weight)						Fat-soluble vitamins (mg/100 g fresh weight)				
	C	B1	B2	B3	B5	B6	A1	D2	D3	E	K
C+W	0.32 ± 0.06 a	1.10 ± 0.22 a	1.5 ± 0.3 a	12.0 ± 2.4 a	7.1 ± 1.4 a	0.34 ± 0.07 a	0.037 ± 0.007 a	<0.05 a	0.15 ± 0.03 b	0.38 ± 0.08 a	0.008 ± 0.002 b

T+W	0.48 ± 0.08 b	1.10 ± 0.22 a	1.6 ± 0.3 a	11.0 ± 1.8 ab	6.5 ± 1.0 a	0.34 ± 0.05 a	0.034 ± 0.005 a	<0.05 a	0.15 ± 0.03 b	0.70 ± 0.14 b	0.005 ± 0.001 b
W	0.29 ± 0.1 a	0.93 ± 0.19 a	1.4 ± 0.3 a	8.0 ± 2.2 b	6.3 ± 1.3 a	0.4 ± 0.03 a	0.038 ± 0.006 a	<0.05 a	0.10 ± 0.02 a	0.59 ± 0.11 ab	0.004 ± 0.001 a

Different letters indicate significant ($p < 0.05$) differences among flours.

Table 3 shows the vitamin contents in flours obtained after feeding *T. molitor* with different diets. *T. molitor* is a good source of vitamins since it contains high levels of vitamins B2, B3, B5 and C [10]. However, the vitamin content in diet and the applied drying conditions also influence their content in flours. All the flours had similar vitamin content, but some differences were found in vitamin C, B3, D3, E and K. Vitamin C was higher in T+W flours, which might be explained by the higher content found in tomato waste (16 mg/100 g FW) whereas cucumber and wheat bran had <1 mg/100 g FW. Similar results after feeding *T. molitor* with *Moringa olifeira* leaves were reported by Kotsou et al. [11]. Vitamin B3 was higher in C+W and T+W flours despite W had 5-fold times content than agricultural wastes. As vitamin B3 can be synthesized from tryptophan, the higher B3 content could be related to changes in the aminoacidic profile or the biosynthesis pathway in larvae. Higher vitamin D3 and K contents were found in C+W and T+W flours, although a correlation with the content in diets cannot be stated. Regarding vitamin E, the lowest content was found in C+W flour, probably due to the lower concentration in cucumber wastes compared to tomato wastes or W (0.1; 0.2 and 1.0 mg/100 g FW, respectively). Results may be also influenced by the fat contents of flours (C+W: 24.4%; T+W: 27.2%; W: 28.2%) and the possible degradation of vitamin E due to air, temperature, light exposure, and the role of this antioxidant vitamin during lipid peroxidation.

3.2. Quality Attributes

Table 4 shows the effect of different diets on the moisture and colour of flours obtained from *T. molitor*. Moisture was similar among flours since applied processing conditions for drying were maintained to obtain flours with <5% humidity. Regarding colour parameters, flours obtained from larvae fed with T+W and C+W showed slightly higher L* and b* values than those fed with W. However, a* and a*/b* ratio were lower in C+W and T+W flours. Despite the slight but significant differences detected in the colour of the flours, all of them were of acceptable quality to be used as food or feed ingredients.

Table 4. Effect of different larvae diet (W: wheat bran diet; C+W: cucumber waste supplemented diet; T+W: tomato waste supplemented diet) on flour moisture and colour parameters.

Insect Flour	Moisture (%)	L*	a*	b*	a*/b*
C+W	3.69 ± 0.17 a	37.84 ± 0.11 a	19.83 ± 1.11 c	20.13 ± 0.44 a	0.99 ± 0.08 c
T+W	3.88 ± 0.25 a	38.00 ± 0.22 ab	24.06 ± 1.33 b	18.79 ± 0.35 b	1.28 ± 0.08 b
W	3.81 ± 0.11 a	37.18 ± 0.35 b	26.23 ± 0.77 a	14.84 ± 0.5 c	1.77 ± 0.02 a

Different letters indicate significant ($p < 0.05$) differences among flours. L*: luminosity; a*: green-red chromaticity; b*: blue-yellow chromaticity; a*/b*: ratio among green-red and blue-yellow chromaticity.

C+W flour showed the highest lipid peroxidation, followed by T+W flour (Figure 1). Obtained results can be related to the fatty acids profile of flour, since the higher content in long-chain highly unsaturated fatty acids (Table 2) makes the flour more susceptible to oxidation. Besides, vitamin E acts as an antioxidant inhibiting the unsaturated fatty acid oxidation, so its lower content in C+W flour might be related to its susceptibility to lipid peroxidation. In addition, lipid oxidation can be triggered by different factors during flour processing and storage such as heat, light or oxygen.

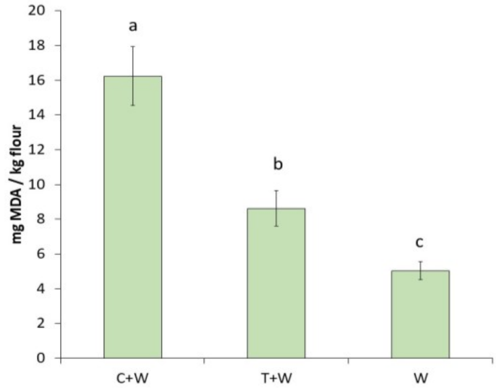

Figure 1. Effect of different larvae diet (W: wheat bran diet; C+W: cucumber waste supplemented diet; T+W: tomato waste supplemented diet) on flour lipid peroxidation (TBARs). Different letters indicate significant ($p < 0.05$) differences among flours.

4. Conclusions

Tomato and cucumber wastes have demonstrated to be excellent supplements for obtaining *T. molitor* flours with enhanced content in vitamins, protein, and unsaturated fatty acids while maintaining similar colour attributes than those obtained after conventional diets. Therefore, the revalorization of vegetable waste through *T. molitor* bioconversion is a feasible strategy for obtaining high-added value food products through a sustainable approach.

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