

BIO PROCESSING OF MICROALGAE USING BLACK SOLDIER FLY LARVAE FOR SUSTAINABLE PROTEIN PRODUCTION

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ABSTRACT

Microalgae cultivation offers promises for various applications, including biofuel production, nutrient sequestration, nutraceuticals, and biomass for animal feed. However, large-scale cultivation often generates significant amounts of residual biomass, creating a challenge for sustainable management. Bioprocessing using insect larvae presents a promising solution for converting algal biomass into valuable products. This study investigates the bioprocessing of algae using black soldier fly (BSF) larvae. We aimed to explore the efficiency of BSF in converting algal biomass and to evaluate the oxidative parameters of the proteins produced in the bioprocessing chain. The study compared BSF larvae fed on two different diets: fruit waste cake only and fruit waste supplemented with microalgae. We measured various oxidative parameters in the protein extracted from the resulting BSF larvae. The results showed that BSF larvae fed a mixed diet containing algae displayed a 14% increase in protein yield compared to those fed only fruit waste. Additionally, the results demonstrated that larvae fed only fruit waste had higher levels of hydrogen peroxide and lipid peroxidation, indicating oxidative stress and potential protein damage. Microalgae are naturally rich in antioxidants, which likely helped scavenge free radicals and protect protein in larvae fed the mixed diet. The study employed advanced statistical techniques like cluster analysis, general linear models (GLMs), artificial neural networks (ANNs), and radar analysis. These techniques helped to analyze the complex data and confirm the positive impact of microalgae on protein quality. The study concludes that incorporating microalgae into the fruit waste cake diet of BSF larvae can improve the quality of the protein produced. By reducing oxidative damage, the protein may have enhanced stability, shelf life, and potentially improved nutritional value. This research contributes to the development of sustainable protein production methods using bioprocessing of algal biomass and wastes to create more sustainable products.

KEY WORDS : Bioprocessing, Nutrient Recycling, Insect Biomass, Protein Production, Circular Bioeconomy, Meta-Analysis

INTRODUCTION

The circular bioeconomy emerges as a powerful response to the challenges of the existing linear economic model. It proposes a fundamental shift towards a more sustainable model, emphasizing a system where resources are continuously reused, recycled, and restored. Waste becomes a valuable input for new products, and nature thrives

alongside a thriving economy (Stegmann *et al.*, 2020). The core principles of the circular bioeconomy include the following renewable natural capital, minimized waste, and closed-loop systems. This can be done through prioritizing the use of renewable biological resources like insects, plants, animals, and microorganisms; striving to minimize waste generation throughout the production and consumption cycles; also, creating systems where

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resources are used efficiently and materials are cycled back into the production process after use (Tan and Lamers, 2021). Implementing a circular bioeconomy offers several advantages including environmental sustainability by reducing environmental impact through resource efficiency and waste reduction. Also, resource efficiency utilization through promoting the efficient use of resources throughout the entire life cycle (Carus and Dammer, 2018).

Challenges do exist in implementing the bioeconomy including investing in policy frameworks and infrastructure development, shifting consumer preferences towards sustainable products, and encouraging research and development (R and D) to improve the efficiency and scalability of bio-based processes (Negi and Kumar, 2021). The bio-based circular economy focuses on three key stages: inputs, processes, and outputs (Figure 1). It emphasizes utilizing renewable biological resources as inputs, transforming them through sustainable processes, and generating valuable outputs that can be reused or biodegrade safely back into the environment. The key aspect is that outputs, whether products or recycled materials, are designed to re-enter the system as inputs or decompose safely. This minimizes waste and promotes a closed-loop system. The circular bioeconomy offers a promising approach for achieving a more sustainable future. By harnessing the power of renewable biological resources and promoting circularity in production systems, we can create a more sustainable future. Optimizing the input-process relationship within the bio-based economy system is considered crucial for generating more affordable, sustainable, and economically viable products (Tan and Lamers, 2021; Vural *et al.*, 2022).

One of the most promising applications of the bio-based economy is the production of insect-based protein. This technology holds immense potential within the animal feed sector. Insect protein offers a sustainable and high-quality alternative to traditional protein sources, potentially contributing to improved quality of life (Ardo *et al.*, 2022; Sahrin *et al.*, 2022). Furthermore, by reducing reliance on resource-intensive protein sources like fishmeal, insect production can contribute to environmental sustainability by mitigating greenhouse gas emissions (Kee *et al.*, 2023). Therefore, selecting the most suitable feedstock for insect rearing becomes crucial for maximizing the positive social, economic, and environmental impacts of this technology. Previous research has demonstrated the potential of insects as bioreactors to recycle microalgae waste for protein production. Additionally, insects can be utilized to convert organic waste streams into valuable protein sources for animal feed, while also serving as a source of biofuel, biopolymers, and biofertilizers, all from the same bioreactor and input source (Rajendran *et al.*, 2018; Jensen *et al.*, 2021; Maroušková, 2021; Mahmoud *et al.*, 2022; Kee *et al.*, 2023).

The black soldier fly (*Hermetia illucens*) is considered a powerful tool for achieving a bio-based circular economy in a sustainable way. This widespread insect, found in tropical and temperate regions, offers a valuable solution for waste management. While previous research has explored its potential for protein bars, compost, and biodiesel production, focusing on the high content of macromolecules, including proteins, lipids, and amino acids in its larvae, offers further opportunities (Bortolini *et al.*, 2020; Mahmoud *et al.*, 2022). The growing field of bioprocessing seeks sustainable methods to convert organic materials into valuable

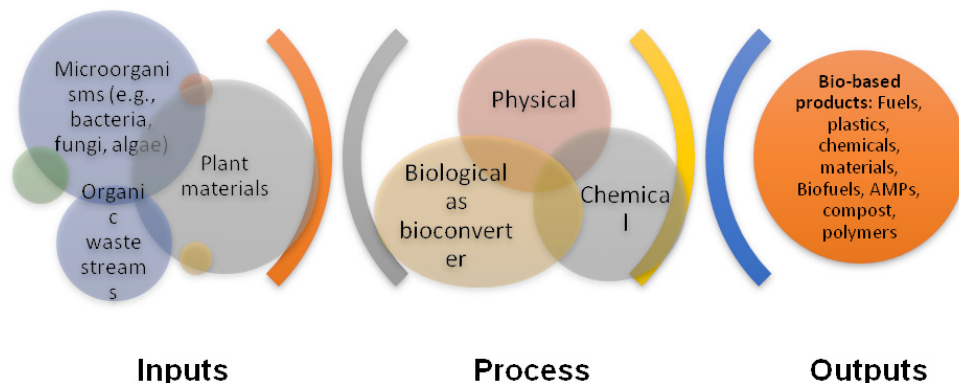


Fig. 1. The components of biobased economy system

products. Microalgae cultivation has gained traction for its potential in biofuel production, nutrient sequestration, and other applications. However, a significant challenge lies in managing the leftover biomass generated during harvesting and processing of algae. This biomass often ends up in landfills, presenting a waste management issue (Niccolai *et al.*, 2019; Geada *et al.*, 2021; Mahmoud *et al.*, 2022). BSF larvae demonstrate an exceptional ability to bio convert a variety of organic waste streams, including leftover biomass from algae cultivation. This remarkable capacity positions BSF as a key player in creating a circular system. By efficiently converting waste into protein-rich insect biomass, BSF offers a sustainable alternative protein source for animal feed or other applications (Lu *et al.*, 2022; Mahmoud *et al.*, 2022). One important aspect of protein quality is its oxidative stability. Proteins are susceptible to damage from free radicals, leading to a loss of nutritional value and functionality. Therefore, assessing the oxidative parameters of BSF protein is essential for several reasons, including improved nutritional value, enhanced shelf life, and functional properties. By evaluating the oxidative stability of BSF protein, we can optimize rearing practices and identify dietary strategies to enhance protein quality (Jensen *et al.*, 2021; Hu *et al.*, 2023). Briefly, this research contributes to the development of bioprocessing algal biomass using BSF to produce a high-quality protein source, promoting sustainable feed and food production systems. This current research assessed the role of BSF as a bioconverter of microalgae and organic waste on the oxidative parameters of the produced proteins. In this context, larvae of the black soldier fly were reared on organic waste

(fruits) alone, or the organic waste mixed with microalgae, *Chlorella vulgaris* (in a 1:0.5 ratio). Then, the evaluation of oxidative parameters of the produced proteins was investigated, focusing on both oxidant and antioxidant levels.

MATERIALS AND METHODS

Insect and Algae Source

Adult black soldier flies (BSF) (*Hermetia illucens*) were bred in a controlled environment (37 °C, 60% humidity, 12:12 light/dark cycle) at Cairo University's Entomology Department. This optimized setup ensured rapid development of the BSF population without compromising offspring health. Microalgae were obtained from a colony established and maintained for over five generations at the Agricultural Research Center, Egypt, for use in the experiment. The microalgae were cultured according to the methods described in (Wattrus, 2013; Rawindran *et al.*, 2022) by a team of microbiologists at the Agricultural Research Center, Egypt.

Experimental Setup

The proposed flowchart (Fig. 2) for total protein production from two treatment groups. Group A consisted of 500 first-instar BSF larvae fed on 0.5 kg of pure fruit waste (FW). Group B consisted of 500 first-instar BSF larvae fed on 0.5 kg of mixed fruit waste supplemented with microalgae, *Chlorella vulgaris* (1:0.5 ratio of FW to algae). After 14 days, the fifth-stage larvae were harvested from the waste, washed three times with distilled water, inactivated by freezing for 10 minutes, dried at 50 °C for 24

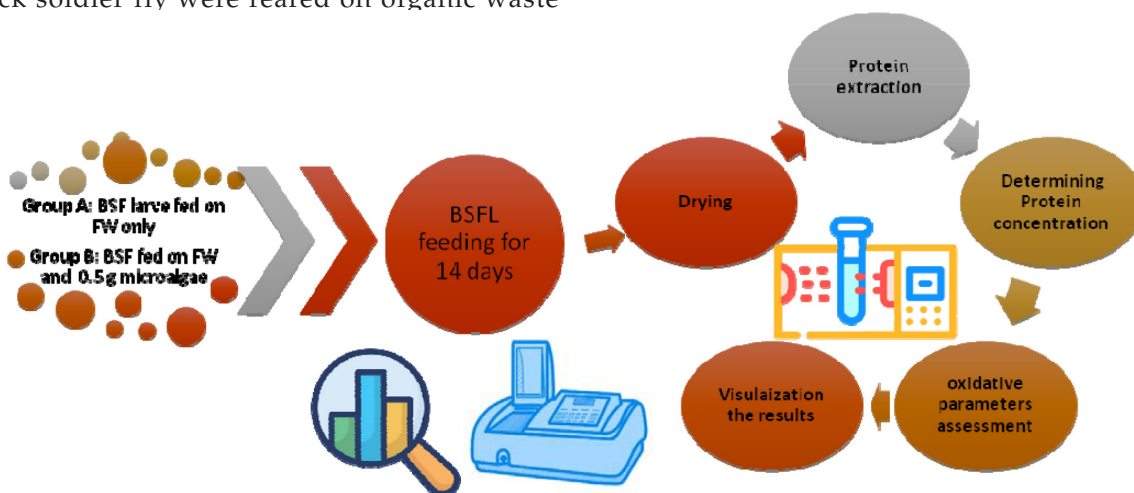


Fig. 2. Flowchart showing the experimental setup design

hours, and stored at 4 °C until further analysis.

Protein extraction and content

A 500 g sample of frozen insects was homogenized using a mortar and pestle at a rate of 30 strikes per second in deionized water (1200 ml) containing 2 grams of ascorbic acid and 2 ml of Triton-X 100. The resulting insect suspension was filtered through filter paper to remove tissue debris. The filtrates were then centrifuged at 15,000 g for 30 minutes at 4 °C. The protein content was determined from the freeze-dried supernatant according to Yi *et al.* (2013). The total protein content in the samples was measured using the Bradford colorimetric method (Bradford 1976). This method utilizes Coomassie Brilliant Blue G-250 (CBB) dye to bind to proteins, allowing for their quantification. The process involves the following steps: *Reagent preparation*: A dye reagent is created by mixing 10 mg of CBB dye with 5 ml of methanol, 10 ml of 85% phosphoric acid, and distilled water to reach a final volume of 100 ml. *Sample measurement*: 0.1 ml of each sample is added to a separate test tube. *Dye addition and incubation*: 0.9 ml of the prepared dye reagent is added to each test tube containing the sample. The contents are gently mixed and incubated for 2 minutes. *Optical density (OD) measurement*: The OD of each sample is measured at a wavelength of 595 nm using a spectrophotometer. Blank containing only distilled water is used for reference. *Protein standard*: Bovine Serum Albumin (BSA) fraction V (Sigma-Aldrich) dissolved in 0.15 M NaCl serves as the protein standard for calibration. By comparing the OD values of the samples to the standard calibration curve, the total protein concentration in the samples can be determined. The yield of biodiesel extraction is calculated using the following equation (1).

$$\text{Yield} = \text{Weight of } \frac{\text{Output}}{\text{Input}} \times 100 \quad \dots 1$$

Oxidative Stress Parameters Evaluation

Oxidants and oxidative damage contents

Hydrogen Peroxide (H₂O₂) Concentration can be done using a spectrophotometric method adapted from Junglee *et al.* (2014) was used to quantify H₂O₂ levels in the samples. Briefly, 150 µl of each sample was mixed with a solution containing 0.25 ml of 0.1% (w/v). Trichloroacetic acid (TCA), 0.5 ml of 1 M potassium iodide (KI), and 0.25 ml of 10 mM potassium phosphate buffer (pH 7.0) at 4 °C for 10

minutes. Following incubation at 20 °C for 20 minutes under light protection (aluminum foil), absorbance was measured at 240 nm for all samples and blanks (analyzed in triplicate). A calibration curve generated with H₂O₂ standard solutions prepared in 0.1% TCA was used to quantify H₂O₂ concentration, expressed in parts per million (ppm). The method of Hermes-Lima *et al.* (1995) was used to assess lipid peroxidation. The reaction mixture contained 350 µl of sample supernatant, 1750 µl of 1 mM FeSO₄, 700 µl of 0.25 M H₂SO₄, and 700 µl of 1 mM xylenol orange. After incubation in darkness at room temperature for 3 hours, the absorbance was measured at 580 nm. The absorbance was measured again after further 1-hour incubation with 10 µl of 0.5 mM cumene hydroperoxides. The difference in absorbance was determined to quantify lipid peroxidation.

Enzymatic and Non-Enzymatic Antioxidant Concentrations

Superoxide Dismutase (SOD) activity was measured according to the methodology of Misra and Fridovich (1972). The reaction mixture contained 0.4 ml of sodium carbonate buffer, 35 µl of EDTA, 87 µL of the sample supernatant, and 0.5 ml of freshly prepared epinephrine. The absorbance change of the reaction was monitored at 480 nm. The method of Prieto *et al.* (1999) was used to assess the antioxidant ability concentration of samples to neutralize free radicals. Homogenized tissue samples were mixed with solutions containing 0.25 ml of 0.6 M sulfuric acid, 0.5 ml of 28 mM sodium phosphate, and 0.25 ml of 4 mM ammonium molybdate. The mixture was incubated at 95 °C for 90 minutes, followed by absorbance measurement at 695 nm. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity method developed by Blois (1958); it was used to measure the ability of gut tissues to inhibit the formation of DPPH free radicals. Fifty microliters (µl) of experimental samples were incubated with 100 µl of 20 mM DPPH solution for 2 minutes. The absorbance of the mixture was then measured at 525 nm.

Statistical Analysis

All measurements related to antioxidants and oxidants in the protein products were performed in triplicate to ensure data accuracy. Results are presented as medians with standard deviations to indicate data variability. To assess the specific influence of adding algae to the fruit waste on the

measured oxidative parameters, a Mann-Whitney U test was employed. This non-parametric test is suitable for comparing data between two independent groups (algae vs. no algae). Statistical analyses were conducted using IBM SPSS software (version 17) and Microsoft Excel. A significance level (*p-value*) of less than 0.05 was considered statistically significant, indicating observed differences are unlikely due to chance. Advanced statistical techniques, including radar analysis, Artificial Neural Networks (ANNs), General Linear Models (GLMs), and cluster analysis Dendrogram, were employed to gain a deeper understanding of the role of algae in enhancing the oxidative status of BSF larval protein. Briefly, these analyses provide valuable information for optimizing the mixing ratio of algae in BSF larvae diets to produce protein with enhanced oxidative stability. This, in turn, could improve the BSF's ability as a bioconverter, potentially increasing the industrial protein's shelf life, functionality, and overall nutritional value.

RESULTS AND DISCUSSION

Protein yield and concentration

This study investigated how incorporating the microalgae *Chlorella vulgaris* into the black soldier fly larvae (BSFL) feed affects protein production yield. Equation 1 was used to calculate the yield based on the experimental data. The results showed that there was about 14% increase in protein production with the addition of algae to the larvae's food (Figure 3). The total protein concentration of BSF larvae as a result of feeding of fruit waste only and mixed fruit waste with micro algae, showed in Figure 4. The

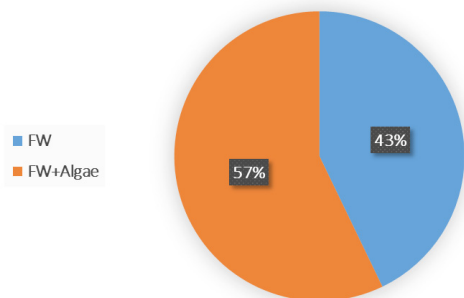


Fig. 3. Pie chart shows a comparison of protein production yield (%) from black soldier fly larvae (*Hermetia illucens*) in two feeding scenarios, larvae were fed a mixture of fruit waste only, larvae were fed the same fruit waste mixture mixed with 0.5 g of *Chlorella vulgaris* algae.

results presented that Larvae fed the supplemented diet had a protein concentration of 0.57, while those fed only fruit waste had a concentration of 0.43. The observed increase in macromolecules yield (Truzzi *et al.* 2020; Klüber *et al.*, 2023; Huseynli *et al.*, 2023) including protein with *C. vulgaris* supplementation may be attributed to *C. vulgaris* algae is a rich source of essential amino acids, vitamins, and minerals (Klüber *et al.*, 2023). Supplementation with algae might provide additional nutrients that can optimize protein synthesis pathways within the larvae. Also, the presence of *C. vulgaris* might influence the gut microbiome of the larvae, potentially promoting the growth of beneficial bacteria that contribute to improved protein digestibility and utilization. Besides that, algae may contain specific compounds that can influence the larvae's hormonal balance, potentially stimulating protein production pathways (Ahmad *et al.*, 2020). Microalgae can be used as a source of proteins³⁰, with estimates suggesting up to 70% of their dry biomass composed of protein. Compared to traditional protein sources like soybeans (38%), rice (10%), peas (2.8%), or even animal products like milk (4%) and eggs (13%), algae offer a significant advantage. Even the average protein concentration for generally recognized as safe (GRAS) algae species hangs around a substantial 40%. Also, the algae has a quality protein with a balanced profile of essential amino acids. This approach can contribute to the development of more efficient and sustainable insect protein production systems (Matos, 2019). The combination of fruit waste and algae may create a synergistic effect that goes beyond simply providing

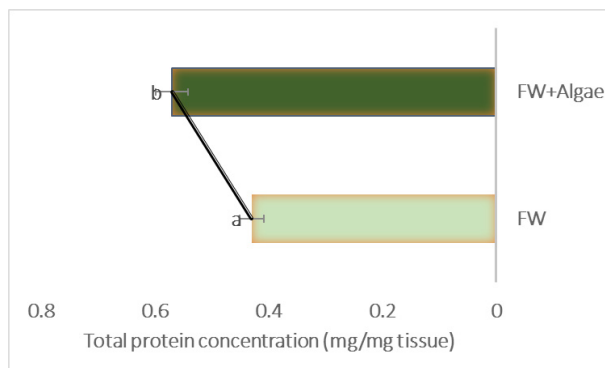


Fig. 4. Effect of additive addition of algae *Chlorella vulgaris* (0.5 g) to fruit waste on total protein concentration of black soldier fly larvae (*Hermetia illucens*). Median values marked with different letters are significantly different among algae additive addition (Mann-Whitney U revealed, $p < 0.05$).

a more complete nutritional profile. Studies have shown that *C. vulgaris* can increase macromolecules concentration as lipid content when grown on organic waste (Mahmoud *et al.*, 2022). Also, previous literature has shown that microalgae have the ability to detoxify the heavy metals and can be act as a bioremediator for wastewater treatment, besides algae can have an increased protein content when grown on organic waste (Kang *et al.*, 2021; Coronado-Reyes *et al.*, 2020). The mechanism of enhancing the protein production in presence of algae involves a multi-step process as organic waste may provide various nutrients like carbohydrates, amino acids, and organic matter. So, algae can trigger increasing gene expression for protein synthesis (Abreu *et al.* 2012) besides that, the presence of readily available carbon sources like carbohydrates in organic waste allows algae to redirect energy towards protein production. The results of Abreu *et al.* (2012) showed that the organic substrate have a key role in enhancing the biomass accumulation of *C. vulgaris* during the cultivation of microalgae. Also, the study of Shi *et al.* (1999) described that glucose, as the main sugar in fruit waste, can be reflected as the greatest organic C-substrate for the growth of *Chlorella* species. Furthermore, microalgae have the remarkable ability to utilize CO₂ from the air, along with light and water, to produce biomass and oxygen through photosynthesis. Municipal waste may also contain additional nutrients like nitrogen and phosphorus that can further support microalgae growth (Abreu *et al.* 2012; Chew *et al.*, 2018; Shi *et al.*, 1999). As mentioned earlier, the organic waste fermentation increases CO₂ concentration, which acts as a stressor for the microalgae. The study of Yang *et al.* (2023) described the ability of algae to convert CO₂ into proteins, the study results investigated that the high CO₂ may mimic nitrogen starvation for some algae species. This is because a key enzyme, Rubisco, used in the Calvin Cycle (CO₂ fixation process) also it has some affinity for oxygen (O₂) instead of CO₂. In high CO₂, rubisco can fix some oxygen instead of CO₂, leading to a decrease in usable organic molecules and effectively mimicking nitrogen deficiency. To compensate for the perceived nitrogen starvation, algae activate stress response pathways. These pathways involve the production of specific proteins and metabolites that help the algae survive under unfavorable conditions. One response might be to increase the production of specific nitrogen-rich proteins like enzymes involved in nitrogen fixation

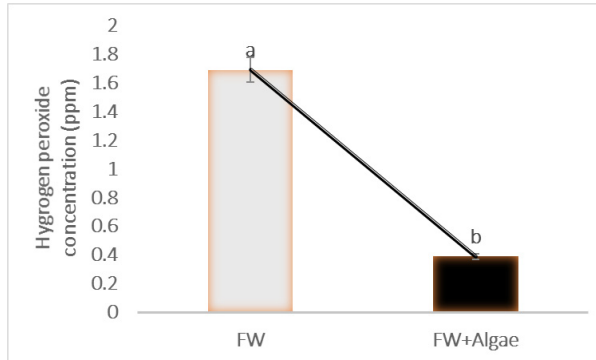
or alternative CO₂ fixation pathways. Additionally, the algae might increase the production of storage proteins to store excess carbon from CO₂ fixation (Schwartz *et al.*, 2003; Sayre and Donald, 2013; Yang *et al.*, 2023). Overall impact on protein production, adding microalgae to the BSFL diet offers a two benefits including that microalgae are a rich source of favorable proteins; the CO₂ extrapolated during fruit waste fermentation stimulates the microalgae to produce even more proteins within their cells. Consequently, larvae nourished with algae as an additive for its diet have advanced overall protein content, leading to a significant elevation in protein production yield.

Oxidative Stress Parameters Evaluation

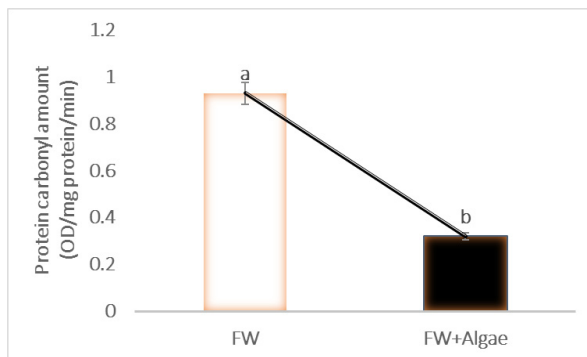
Oxidants and oxidative damage concentration

This study investigated the effect of supplementing fruit waste with microalgae on the oxidative stability of protein produced by black soldier fly larvae (*Hermetia illucens*). The results showed a significant improvement in protein quality when microalgae were added to the larvae's diet. Figure (5A and B) showed a significant increase in hydrogen peroxide and lipid peroxidation of BSFL fed on fruit waste only comparing to supplementary the diet with microalgae (*C. vulgaris*) with an elevation factor equal to 4.3-x fold for H₂O₂ concentration (Figure 5A), and 34% for lipid peroxidation concentration (Figure 5B) the results emphasized that the larvae fed only fruit waste cake had high level of oxidative stress, comparing to larvae fed a diet containing both fruit waste and microalgae. These findings suggest that incorporating microalgae into the black soldier fly larvae's diet can significantly enhance the oxidative stability of the resulting protein. The higher hydrogen peroxide concentration observed in protein from larvae fed solely on fruit waste indicates the presence of free radicals and potential protein damage due to oxidative stress. Previous studies investigated that microalgae are naturally rich in antioxidants, which can help scavenge free radicals and prevent protein damage in the larvae (Natrah *et al.*, 2007; Tiong *et al.*, 2020). Also, microalgae can provide essential nutrients like vitamins and minerals that may be lacking in fruit waste alone. These nutrients can contribute to the overall health and antioxidant defense system of the larvae, leading to the production of more stable proteins (Camacho *et al.*, 2019; Dhandwal *et al.*,

2024). Overall, this study highlights the potential of using microalgae to improve the quality of protein produced by BSFL reared on waste substrates like fruit waste cake. This approach could contribute to the development of more sustainable and high-quality insect protein sources for various applications especially animal food and feed.



A



B

Fig. 5. Effect of additive addition of algae *Chlorella vulgaris* (0.5 g) to fruit waste on (A) hydrogen peroxide (H_2O_2) concentration, and protein carbonyls amount (B) of black soldier fly larvae (*Hermetia illucens*). Median values marked with different letters are significantly different among algae additive addition (Mann-Whitney U revealed, $p < 0.05$).

Antioxidants amounts

The activity of superoxide dismutase (SOD), an enzyme that combats oxidative stress, and the concentration of antioxidant ability were significantly increased in larvae fed a diet containing both fruit waste and microalgae than fruit waste only with the fold of 75, and 17% (Figure 6). Also, the results of DPPH inhibition percentage increased with the fold of 1.65-x fold (Figure 7). The substantial increase in SOD activity observed in larvae fed the microalgae-supplemented diet suggests an enhancement of their antioxidant

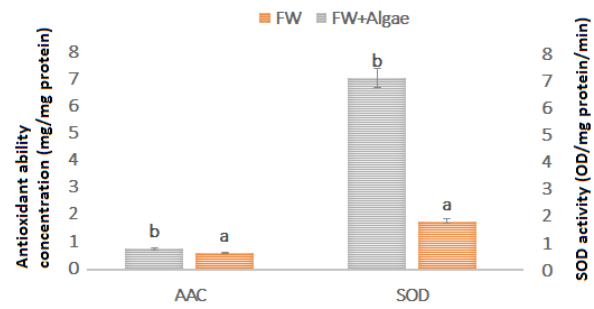


Fig. 6. Effect of additive addition of algae *Chlorella vulgaris* (0.5 g) to fruit waste on antioxidants concentration of black soldier fly (*Hermetia illucens*)- protein, expressed as enzymatic superoxide dismutase (SOD), and non-enzymatic antioxidant ability concentration. Median values marked with different letters are significantly different among algae additive addition (Mann-Whitney U revealed, $p < 0.05$).

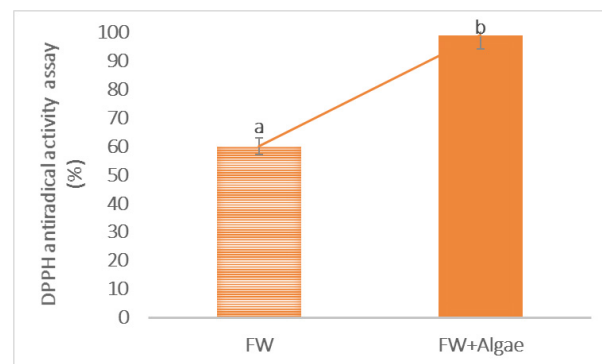


Fig. 7. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) antiradical activity assay of *Hermetia illucens* larval protein as a results of two feeding scenarios fruit waste only; and additive addition of algae *Chlorella vulgaris* (0.5g) to fruit waste. Median values marked with different letters are significantly different among algae additive addition (Mann-Whitney U revealed, $p < 0.05$).

defense system. Microalgae are known to be rich in various antioxidants, including enzymes like SOD. When consumed by the larvae, these antioxidants can get incorporated into their bodies, leading to improved protection against oxidative damage. Besides that, microalgae are known to be rich in various antioxidants, including carotenoids, phenolic, and tocopherols. When consumed by the larvae, these antioxidants can be incorporated into their body tissues, including the protein fraction. This finding can help scavenge free radicals in the body, which can reduce oxidative stress and the risk of chronic diseases. Additionally, antioxidants may

contribute to improved shelf life of protein-based foods by delaying lipid oxidation and rancidity. Overall, this study demonstrates that supplementing fruit waste cake with microalgae can be a promising strategy to enhance the antioxidant properties of protein produced by BSFL. This approach has the potential to improve the nutritional value and marketability of BSFL protein as a sustainable and functional food ingredient (Cha *et al.*, 2010; Assunção *et al.*, 2017; Camacho *et al.*, 2019; Zhang *et al.*, 2022; Dhandwal *et al.*, 2024)

Advanced statistical analysis

Cluster analysis with Dendrogram visualization was analyzed in Figure 8 as sections of oxidants and oxidative damage parameters in 8. A1 and 8. A2 for BSFL fed on fruit waste only and mixed FW-microalgae, respectively. While the sections of antioxidants presented in 8. B1 and B2 for BSFL fed on FW only and mixed FW-microalgae, respectively.

This technique might have been used to group protein samples based on their oxidative profiles. The resulting Dendrogram, a tree-like structure, could reveal distinct clusters of protein samples, potentially differentiating those derived from larvae fed with and without algae. The results demonstrated the similar pattern between LP and H₂O₂ in case A2 unlike the difference pattern between two oxidative parameters in A1. However, the general tendency was similar in the sections B1 and B2 where the SOD is similar to AAC and DPPH is different both in both experimental cases. The results of Marzorati *et al.* (2023) demonstrated that the effects of various protein sources on intestinal health using a cluster analysis approach. Three distinct clusters were identified based on their impact on inflammatory markers and intestinal permeability, including Cluster 1 (Soybean Meal): This cluster exhibited pro-inflammatory cytokine production (CXCL-10 and MCP-1) and weak

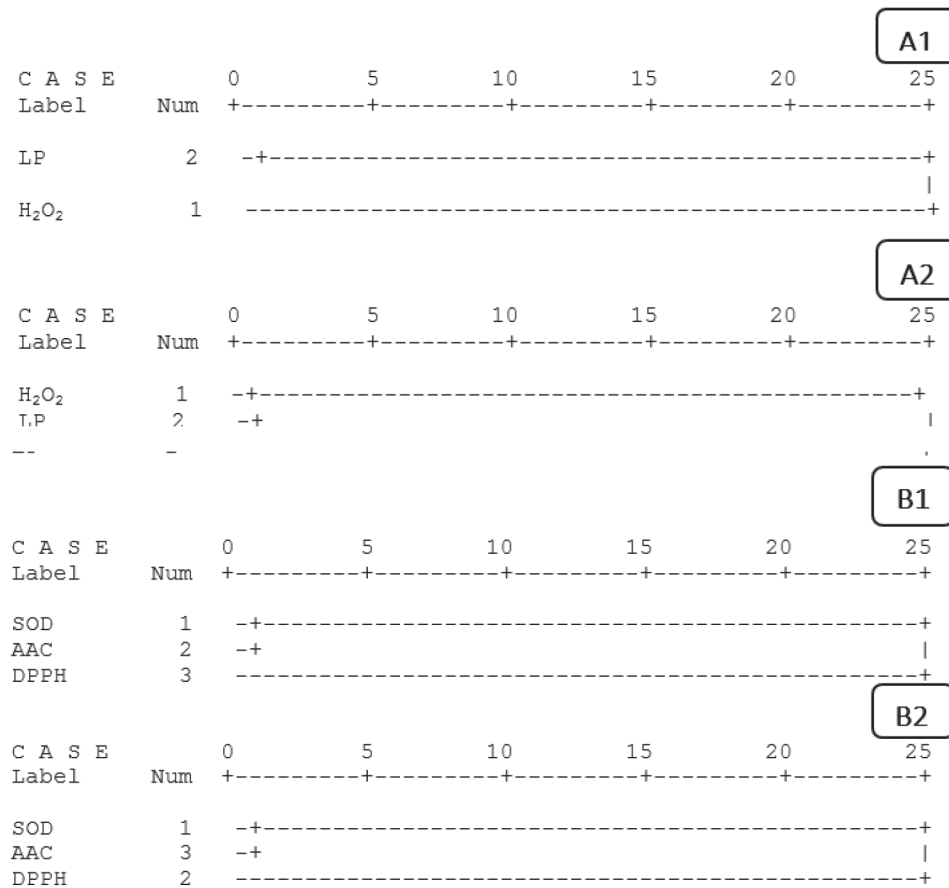


Fig. 8. Dendrogram of the cluster analysis (using Ward’s Method) applied for oxidative stress parameters (enzymatic and non-enzymatic antioxidants (A); oxidants as hydrogen peroxide (H₂O₂), oxidative damage as lipid peroxidation (B) of *Hermetia illucens* larval as a results of two feeding senarioes fruit waste only (1); and additive addition of algae *Chlorella vulgaris* (0.5 g) to fruit waste (2).

intestinal barrier protection (low TEER values). However, it also showed the highest levels of the anti-inflammatory cytokine IL-10. While, Cluster 2 (Bacterial Meals): This cluster was associated with the strongest pro-inflammatory response (TNF- α , IL-1 β , IL-8, and NF- γ B) and is suspected to be linked to the bacterial cell wall components. The Last cluster (Yeast, Microalgae, Fish Meal, Insect Meal): This cluster displayed generally neutral or anti-inflammatory properties and strong intestinal barrier protection (high TEER values). Yeast, in particular, showed the most pronounced anti-inflammatory effects (increased IL-10 and reduced pro-inflammatory cytokines). The research suggested that Soybean meal may have both beneficial and detrimental effects on gut health. Further research is needed to understand the specific components responsible for these contrasting effects. Bacterial meals, in their current form, might worsen gut health due to their pro-inflammatory properties. Investigating ways to mitigate these effects through processing or selecting specific bacterial strains is important. And finally, Yeast, microalgae, fish meal, and insect meal appear to be promising protein sources for promoting gut health due to their anti-inflammatory properties and ability to strengthen the intestinal barrier. General Linear Model (GLM) was showed in Table 1. This

statistical approach likely served to identify significant factors affecting the protein's oxidative status. The GLM could account for the effects of algae addition and other potential variables, helping

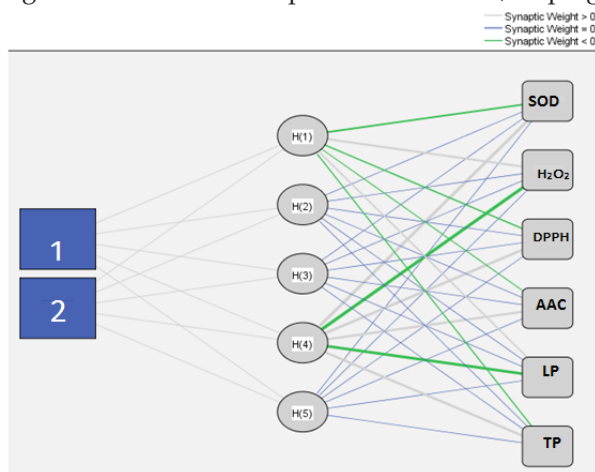


Fig. 9. An Artificial Neural Networks with three layer, two inputs, four bias terms, and six outputs applied for oxidative stress parameters of *Hermetia illucens* larval as a results of two feeding senarioes fruit waste only; and additive addition of algae *Chlorella vulgaris* (0.5 g) to fruit waste. TP: total protein; H₂O₂: hydrogen peroxide; LP: lipid peroxidation; SOD: superoxide dismutase; AAC: antioxidant ability concentration; DPPA: 2,2-Diphenyl-1-picrylhydrazyl antiradical assay.

Table 1. General Linear Model to analyze the corrected model, intercept, treatment of addition of algae *Chlorella vulgaris* (0.5g) to fruit waste for feeding *Hermetia illucens* larval on the oxidative stress parameters and protein concentration of the BSFL

Parameters	Source	Type III Sum of Squares	df	Mean Square	F	p value
TP	Corrected Model	0.034	5	0.03	675	<0.001
	Intercept	1.47	5	1.47	29403	<0.001
	F	0.03	5	0.03	675	<0.001
H ₂ O ₂	Corrected Model	2.53	5	2.53	76050	<0.001
	Intercept	6.44	5	6.44	193442	<0.001
	F	2.53	5	2.53	76050	<0.001
LP	Corrected Model	0.55	5	0.55	8281	<0.001
	Intercept	2.3	5	2.33	34969	<0.001
	F	0.55	5	0.55	8281	<0.001
SOD	Corrected Model	43.74	5	43.74	8661	<0.001
	Intercept	121	5	121	24059	<0.001
	F	43	5	43	8661	<0.001
AAC	Corrected Model	0.01	5	0.01	450	<0.001
	Intercept	2.58	5	2.58	77618	<0.001
	F	0.01	5	0.01	450	<0.001
DPPA	Corrected Model	2242	5	2242	3364	<0.001
	Intercept	37762	5	37762	56644	<0.001
	F	2242	5	2242	3364	<0.001

TP: total protein; H₂O₂: hydrogen peroxide; LP: lipid peroxidation; SOD: superoxide dismutase; AAC: antioxidant ability concentration; DPPA: 2,2-Diphenyl-1-picrylhydrazyl antiradical assay.

to isolate the specific contribution of algae to protein quality. Artificial Neural Networks (ANNs) was demonstrated in Figure 9. This powerful computational tool may have been used to model the complex relationships between various factors, such as algae inclusion level, protein quality markers, and environmental factors. By analyzing the ANN model, researchers could gain insights into how algae supplementation influences the protein's oxidative stability. This study employed advanced statistical techniques to comprehensively analyze the influence of dietary algae on the oxidative status of protein derived from black soldier fly (BSF) larvae. Radar analysis was demonstrated in (Figure 10). This method likely provided a visual representation of multiple variables related to the oxidative status of the protein simultaneously. By comparing the radar plots for larvae fed with and without algae, the results demonstrated that the addition of microalgae into BSFL diet has a significance increase in the level of antioxidants levels.

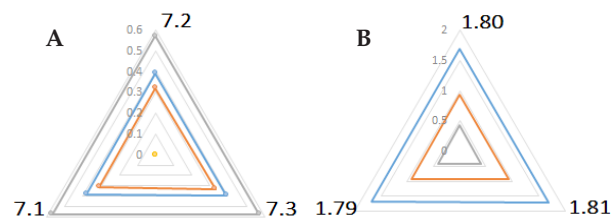


Fig. 10. Radar analysis applied for oxidative stress parameters of *Hermetia illucens* larval as a results of two feeding senarioes fruit waste only (A); and additive addition of algae *Chlorella vulgaris* (0.5g) to fruit waste (B).

Conclusion and Further Research

This study investigated the impact of supplementing fruit waste cake with microalgae (*C. vulgaris*) on the oxidative stability of protein produced by black soldier fly larvae (BSFL). Our findings demonstrate that incorporating microalgae into the BSFL diet significantly improves protein quality. BSFL larvae fed a mixed diet containing microalgae exhibited a substantial increase in protein stability, as measured by DPPH radical scavenging activity, compared to those fed only fruit waste. Also, the improved protein stability is likely due to the presence of antioxidants from the microalgae, which can be directly incorporated into the larvae's tissues or stimulate their own antioxidant production pathways. This enhanced

protein stability offers potential benefits like extended shelf life, improved functionality in food products, and potentially even additional health advantages due to the presence of dietary antioxidants within the protein itself. These findings highlight the potential of using microalgae to enrich insect-derived protein sources with valuable properties. Further research can explore the most effective types and levels of microalgae supplementation to maximize protein quality and identify the specific antioxidant compounds contributing to the observed effects. To gain a deeper understanding of the mechanisms behind the observed increase in protein yield, future studies could identify the specific components of *C. vulgaris* that most significantly influence protein production in the larvae. Also, investigating the changes in the gut microbiome composition of larvae fed with and without algae supplementation. And finally, analyzing the hormonal profiles of larvae in both groups to identify potential regulatory effects of the algae.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Disclosure of potential conflict of interest

The authors declare that they have no conflict of interest. Informed consent Informed consent was obtained from all individual participants included in the study.

Research data for this article

The raw data of the article are available from the authors upon request.

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