DOTTORATO IN SCIENZE VETERINARIE

XXXVII CICLO

Simone Vozzo

Black soldier fly: Growth Performance and Nutritional Profile on Different Types of Organic Substrates. Possible Imfplications and Uses for Sustainable Production

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PhD Thesis

"Black soldier fly: Growth Performance and Nutritional Profile on Different Types of Organic Substrates. Possible Implications and Uses for Sustainable Production"

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Abstract

The expansion of animal-derived protein production to meet global demand presents significant environmental challenges, including heightened greenhouse gas emissions, increased water consumption, and extensive land requirements. In both developing and developed nations, the issue of sourcing alternative protein sources is becoming increasingly urgent. Urban population growth in developed regions necessitates the development of large-scale production systems with minimal land use, which in turn leads to increased consumption and a corresponding rise in organic waste production. One of the main and strongest answers nowaday seems to be the circular economy model, which can provide to the consumption of the great amount of waste and at the same time evaluate it producing value and work. Insects seem to be a great candidate in this sense: due to their evolutionary adaptation to diverse environments, many insect species have the potential to be used in sustainable systems for biomass conversion. Insect farming offers substantial environmental advantages compared to conventional livestock production in terms of power consuption, land and water. Among the most studied bioconverting insects the black soldier fly (*Hermetia illucens*, Linnaeus, 1758) has garnered significant attention for its ability to efficiently convert a wide range of organic waste into high-quality biomass. The *H. illucens* larvae are rich in proteins, lipids, and bioactive compounds, such as chitin, lauric acid, and antimicrobial peptides, which have demonstrated beneficial effects on intestinal health and overall animal welfare.

This PhD project aims to study *Hermetia illucens*, analyzing its growth performance and nutritional profile at the larval stage on different types of organic substrates. The project will evaluate the potential applications and implications for the sustainable production of this species on a large scale. Each study contributing to this work focuses on a different chemical analysis of BSFL (Black Soldier Fly Larvae) grown on various substrates, in order to assess the insect's potential. Therefore, one of these works aimed to evaluate how different food wastes used as growth substrates affected the lipid composition of *Hermetia illucens* larvae; the relationship among substrates, larvae, and frass fatty acid (FA) composition was studied. Six thousand five-day-old *Hermetia illucens* larvae were allotted to one of four different substrates named CTRL (a control substrate made of broiler feed), V50 + B50 (vegetable and butchery wastes, 1:1 ratio), V75 + B25 (vegetable and butchery wastes, 3:1 ratio), and V100 (entirely composed of vegetables). Saturated FAs (SFAs) prevailed in all the groups. The CTRL larvae had the highest SFA (64.3%), while the V100 and V50 + B50 larvae had the lowest percentages. The V100 frass recorded a significantly higher SFA value (44.8%) than the others. Lauric acid (C12:0) was high in CTRL (0.2%) and V100 (0.3%) substrates and amount at 7.6 and 9.6% in their correspondent frass, respectively.

However, C12:0 content was higher in the CTRL larvae than in the V100 ones (36.7 and 24.5%, respectively), while it had an intermediate value (28.9%) in the V75 + B25 larvae. Finally, the n-3 polyunsaturated FAs (PUFAs) level was high in the V100 substrate and larvae but not in the V100 frass. The relationship between frass FAs and their correspondent amounts in substrates and larvae was significant for C12:0, C18:2n-6, MUFAs, and n-6 PUFAs. There was a positive relationship for C12:0 and MUFAs with both substrates and larvae, while for C18:2n-6 and n-6 PUFAs, the relationship was positive for substrates but negative for larvae. In the end the V100 substrate appeared to be the most suitable treatment for rearing *Hermetia illucens* because of the positive effects on the fatty acid content of larvae (low SFA and high n-3 PUFA content) and frass (optimal lauric acid levels for fertilizers). After the fatty acids, the second work shown how BSFL amino acid composition is strictly related to the amino acid composition of the substrates themselves. For this research the same substrates as the fatty acid one were used, and their influence on larvae amino acid profile was evaluated: vegetable and butchery wastes show interesting potential for BSF larvae, in terms of growth and amino acid composition. As result of larvae growth, the insect frass (excreta, exuviae and residual substrate) resulting at the end of the production cycle, offers new and interesting aspects, from the perspective towards circular economy. In BSF, the amount of protein ranged from 20.0% in V75B25 to 43.7% in V100 ($P < 0.01$). The larvae on V100 had the highest ($P < 0.01$) amount of essential and non-essential amino acids (EAA) except for hydroxyproline which has been detected only in V75B25 group. V75B25 group had the lowest amount ($P < 0.05$) for almost all the AA. Out of the composition, BSF larvae appeared to be a useful tool for the bioconversion of a wide range of food wastes although significant differences in protein fraction and amino acid profile have been observed depending on the rearing substrate. In a third work, with same substrates that were previously used in fatty acid and amino acid research, the mineral profile of the larvae and frass, and the potential bioaccumulation of toxic elements (As, Cd, Pb, Hg) were valuated. The V100 larvae had the highest concentrations of all minerals, except for Se and P, higher in the control group (poultry diet as substrate) ($p<.01$). The V50B50 and V75B25 larvae showed lower ($p<.01$) mineral concentrations than the other groups. The V50B50 group had the highest ($p<.01$) bioaccumulation factor (BAF) for Cd (8.52%), the V100 for Pb ($p<.01$). The equations to estimate the mineral content of larvae from the substrate were significant only for Ca and Mg ($p<.05$). The V100 frass had the highest ($p<.01$) levels of As, Cd, Pb, Fe, K and Mg, while the control group showed the highest ($p<.01$) levels of Se, Zn, Ca, P and K. Toxic elements can accumulate in black soldier fly larvae and frass, with the exception of Hg. However, organic wastes from V and B had a very low content of toxic elements and that strongly reduced the risk of excessive bioaccumulation in larvae. Indeed, the content of toxic elements in larvae is lower than the maximum values established for feed materials by EU regulations. In conclusion, organic substrates from vegetables or butchery wastes can be used for BSF larvae growth without the risk of accumulation of toxic elements.

The latest contribution to this PhD project aimed to observe how BSF larvae behave, under a nutritional and bioconvertion profile, with pet food, specifically with cat wet food. This work was born looking at some data about the great petfood industry: the European pet population is substantial, with an estimated 340 million animals, comprising 26% dogs and 25% cats, so is the pet food industry as a business, with annual sales of approximately 10.9 million tons, and a growth rate of 5.1% over the last three years. The market is expected to continue growing in the coming years. As the global pet industry expands, it is likely that waste generation from this sector will also increase, including expired products, so this trend is expected to have significant environmental implications. For this purpose, a total of 4 substrates were used in the trial. A commercial broiler diet was used for the control group. The other three substrates were represented by three wet complete food for adult cats named beef, chicken and salmon mousses. In terms of growth performance the results showed that the larvae fed on salmon-based cat food had a shorter life cycle (20 days) and higher final weight (+14.98% and +8.24% compared to beef and chicken, respectively) than the other two groups. Moreover, the mortality rate of the salmon group was significantly lower (-19.7%) compared to the control group. The diverse growing substrates affected the chemical-nutritional characteristics of the resulted larvae. The decrease of larval protein content due to use of beef substrate is in line with the previous reasearch in larvae grown on butchery waste, consisting of meat from the trimming of bovine carcasses and cuts of meat, in comparison to larvae grown on a vegetable diet. However, the amount of protein content in larvae ranged from 43.12 to 45.64 %. The lipid content of the larvae on the diverse substrates was not different and only larvae on beef had a lower lipid content and, despite the similar carbohydrate content than the other substrates, larvae on beef cat food showed a lower amount of lipids stored in the body. Among all the results, one of the most interesting to see was the presence of polyunsaturated lipids: the control and salmon group showed a higher content of total PUFA than the other two groups, in particular the PUFA n-3 content in the diet and, as a consequence, improve the n6/n3 ratio, confirming that the fatty acid profile of BSF larvae can be modulate according to the fatty acid profile of the substrates. The results also indicate that not all cat foods, despite similar chemical-nutritional characteristics, can produce the same outcomes. A closer examination of the ingredients reveals that the main difference among the three cat foods is the inclusion of specific animal proteins from chicken, beef, or salmon. Therefore, it is likely that the differences in larvae growth can be attributed to the varying ability of the larvae to utilize these protein sources.

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Introduction

The prospects for global population growth in the near future, along with recent global crises (such as the COVID-19 pandemic, the Russo-Ukrainian war, and the Middle Eastern conflict), have drawn attention to potential challenges in the supply of raw materials and energy, thereby increasing the overall cost of food and feed, and underscoring the need for local supply chains. Nevertheless, the increased production of animal-derived proteins to meet the needs of the global population will have a negative environmental impact. It is sufficient to consider the emissions of gases, water consumption, and space requirements for increasing the production of animal-derived proteins. Furthermore, the effects on soil acidification, erosion, compaction, and nitrification should not be underestimated (GROSSI et al. 2019).

In fact, while the issue in developing countries is the sourcing of new protein sources, the situation is not too different in the First World, where population concentrations will increase in urban areas, necessitating the development of systems for massive production with low land use, increasing consumption and thus a greater production of organic waste. In the European Union alone, approximately 246 million tons of municipal waste are produced annually (with constant growth over the past three years); of this, about 88 million tons are food waste, nearly one-third of the total, with an estimated value of 143 billion euros. Successfully disposing of and simultaneously valorizing urban organic food waste, therefore, presents one of the greatest challenges of our time (SALANDIN 2009).

Circular Economy

One of the most promising tools for waste disposal and valorization is undoubtedly the promotion of a transition to a circular economy. The European Parliament defines it as “a production and consumption model involving sharing, lending, reusing, repairing, reconditioning, and recycling existing materials and products for as long as possible,” capable of using production waste as raw materials. In recent years, environmental awareness has driven consumers to increasingly favor this model.

In March 2020, under the European Green Deal and in line with the proposal for a new industrial strategy, the European Commission presented the Action Plan for a new circular economy, which includes proposals for the design of more sustainable products, waste reduction, and empowering citizens with tools like the 'right to repair'. Resource-intensive sectors like electronics, ICT, plastics, textiles, and construction receive special attention.

In February 2021, the European Parliament voted for the new Action Plan for the circular economy, calling for additional measures to achieve a carbon-neutral, environmentally sustainable, toxic-free,

and fully circular economy by 2050, including stricter recycling standards and binding targets for 2030 concerning material usage and ecological footprint.
However, the benefits of the circular economy go beyond environmental advantages: since innovation and the adoption of new technologies require new skills, this stimulates job creation. Technological innovation, renewable energy, waste valorization, and product reuse create a virtuous circle that promotes economic development.
The general population can directly benefit from such an economic model: money can be saved through the availability of more durable products with longer lifespans that are easier to repair or reuse. The circular economy could be a new virtuous model for managing citizen services, such as smart lighting solutions, energy efficiency, resource sharing, digital communication, and electric mobility.
A circular city is one that produces no waste or reuses what it does generate, that utilizes renewable energy flexibly, and where shared resources like cars, buildings, and machinery are used to their fullest potential. However, before implementing a circular approach to urban services, it must be noted that the agri-food industry has been a pioneer in this sector: the use of biodigesters to recover methane from organic waste is well-established within the agri-food supply chain, with only a minor portion of waste being discarded. Nonetheless, to limit the recovery of such biomass for low-value applications (such as soil amendments or biomass for digesters), it is essential to identify efficient, competitive, low-impact solutions for diversifying its use into secondary-grade products (e.g., animal feed or oil production); among these, insect farming on a large scale stands out as one of the most promising.

Insects

Insects are a class of animals belonging to the phylum Arthropoda, phylogenetically closely related to crustaceans. This class represents the most numerous group of living organisms on the planet, with about one million described species, accounting for five-sixths of the entire animal kingdom. Not all insect species are known, and many (estimated between 4 and 5 million) become extinct before they are even discovered, mainly due to massive deforestation by humans. Insects not only make up a biomass that far exceeds that of vertebrate animals, particularly in tropical regions, but they also occupy extremely diverse ecological niches and biomes at various levels, which is why they are involved in all terrestrial food chains.
They constitute a significant part of the diet of many animals subsequently farmed by humans, and, as reported by in-depth FAO studies, insects have recently entered the human diet in various emerging and Third World countries (FAO Insect for Food and Feed 2003). The success of insects

is due to their extraordinary adaptability: they occupy all terrestrial environments, various freshwater environments, and even aerial environments. This is facilitated by the different developmental cycles that each specimen undergoes to reach the adult stage. Consider the holometabolous cycle (or complete cycle) of butterflies, which develop from caterpillars and thus occupy different ecological niches depending on the stage of their life. In contrast, insects with an "incomplete" life cycle (hemimetabolous) have larval forms that do not differ much from adults and, therefore, occupy ecological niches from the very first day of life (e.g., order Mantodea).
This premise is crucial as it defines the only probable obstacle to breeding various insect species for commercial purposes: large-scale farming must be specialized according to the environment that each species occupies in its developmental stage (where this is a complete cycle) since juvenile specimens will need to be housed (and often fed) differently from adults. However, the need to create different sectors for breeding certain species also presents opportunities: for insects with an incomplete life cycle, the same stabling system is required, but the same type of production is also obtained. Conversely, different developmental stages offer different types of production (larval or adult).

European regulation on edible insects

The history of regulations on insect consumption in animal husbandry reflects a path of progressive acceptance and regulation of a practice that is increasingly seen as a sustainable solution for feed production. The consumption of insects is a millennia-old practice in many parts of the world, especially in Africa, Asia, and Latin America, where insects are a traditional food source for humans and animals. However, in Europe and the Western world, the use of insects in food and feed was virtually unknown or stigmatized until recent times.
It can be said that the scientific community took its first steps in trying to understand the potential uses of insects as bioconverters and new protein sources in the early 2000s. Insects have been recognized for their resource efficiency and ability to reduce the environmental impact of livestock farming.
During this period, no specific regulations existed regarding the use of insects for animal feed in Europe, and the matter was left to the discretion of individual states. However, European laws on animal feed banned the use of processed animal proteins (PAP) in ruminant feed following the bovine spongiform encephalopathy (BSE or "mad cow disease") crisis of the 1990s.
An important turning point came with Regulation (EU) 2015/2283, which governs so-called Novel Foods, including insects among foods that require authorization before being placed on the

European market. Although the regulation was primarily aimed at human consumption, it laid the foundation for regulating insects in the livestock sector.

In 2017, the European Union authorized the use of proteins derived from insects for aquaculture through Regulation (EU) 2017/893, a crucial step in the legalization of insects as a protein source for farm animals. The authorized insects included seven species, among them the black soldier fly (*Hermetia illucens*) and the mealworm (*Tenebrio molitor*).

Subsequently, in 2021, the EU further extended the possibility of using insect-derived proteins in feed for pigs and poultry, established by Regulation (EU) 2021/1372, opening up new opportunities for the insect farming industry.

In addition to the authorization of insects in feed, the European Union has established strict rules to ensure food safety and traceability. Insects must be farmed under controlled conditions and fed authorized plant-based by-products. Moreover, insect farming and processing must comply with sanitary standards similar to those required for other animal-derived products. Outside the European Union, several countries are adopting similar regulations or developing their own guidelines. In Switzerland, for example, since 2017, certain insect species have been allowed for human and animal consumption. Similarly, in Asia, countries like Thailand and China are exploring the use of insects in feed in response to growing protein demand and resource scarcity.

In the future, it is expected that regulations on insect-based feeds will expand further, potentially including other animal species and ensuring greater integration of these protein sources into the global livestock supply chain.

Insects and sustainability

Historically, insects have been used in a wide range of sectors, from forensic entomology to the use of saprophagous fly larvae for wound treatment, as well as the vast array of products derived from beekeeping in the food, cosmetic, and artisanal industries. There are numerous examples of how insects are and can be employed, mainly due to their adaptation to thousands of different habitats over millions of years of evolution. However, the species that seem to be growing the most in terms of consumption and transformation of biomass into renewable materials are those used for the consumption and transformation of biomass.

This type of farming requires significantly less water than any other known type of farming. It is known that 1 kg of chicken meat requires 2,300 liters of water, 1 kg of pork requires 3,500 liters, and 1 kg of beef requires between 22,000 and 43,000 liters, while many insects can survive for several days without added water because they can obtain it directly from their food or environment. In terms of emissions, farmed insects produce significantly fewer greenhouse gases (carbon dioxide and methane) compared to any traditional livestock farming system. At the same time, insects can be bred in confined spaces, reducing land consumption. The same Life Cycle Assessment (LCA), which evaluates the environmental impact associated with all stages of a product's life, has estimated that edible insects have a lower impact in every respect compared to protein production from traditional livestock farming.

Consuption comparison

As ectothermic animals, insects' body temperature is heavily influenced by the surrounding environment. Most insect species bred for human consumption, such as crickets and black soldier fly larvae, thrive at temperatures between 25°C and 30°C. Maintaining these temperatures may require heating, especially in colder climates, which can be achieved using electric heaters, radiant panels, or underfloor heating systems.

However, as highlighted in the FAO report *"Edible Insects: Future Prospects for Food and Feed Security"* (2013), insect farming is still a highly energy-efficient solution. The energy consumption for producing 1 kg of insect biomass is significantly lower compared to other intensive farming systems, varying between 15 and 4 kWh, compared to 6-12 kWh/kg for cattle farming, 4-10 kWh/kg for pigs, and even 3-9 kWh/kg for poultry. This is important to keep in mind, as noted in a study published in the *Journal of Insects as Food and Feed* in 2015 (*"Energy and Resource Use Efficiency in the Insect Industry"*) which highlights that overall, insect farming uses resources much more efficiently than traditional livestock farming, reducing the overall environmental impact and leaving a much smaller ecological footprint.

Assuming an average consumption of 1 kWh of electricity to heat 10 square meters of space for one hour, and considering that insect farming may require continuous heating for 12 hours a day, the daily consumption for 10 square meters would be approximately 12 kWh—a relatively manageable consumption when considering the high ratio of biomass produced to space occupied. Furthermore, with efficient technologies and good insulation, this consumption can be further reduced. All this is part of a farming system with extremely low costs in all other aspects, as previously mentioned, compared to traditional livestock farming.

Species	Heating (kWh/kg)	Lighting (kWh/kg)	Total (kWh/kg)
Cattle	5-10	1-2	6-12
Swine	3-8	1-2	4-10
Poultry	2-6	1-3	3-9
Insects	1-3	0.5-1	1.5-4

In their activity of consuming biomass, insects help diversify agricultural activities sustainably. For years now, many farmers and producers overseas have allowed various companies (in sectors like poultry and pig farming, for example) to breed larvae of different insect species, helping dispose of the company's waste and slurry, which the larvae feed on, while the producer processes them into feed once they reach optimal size.

In addition to mere waste disposal, the push towards these solutions stems from the high efficiency of nutritional conversion (i.e., how much feed is required to produce a 1 kg weight gain in an animal). While this conversion varies widely for meat depending on the type of animal and farming practices, it tends to be as follows:

- Cattle: 6-8 kg of feed for 1 kg of meat produced
- Pigs: 2.5-3.5 kg of feed for 1 kg of meat produced
- Poultry: 1.6-1.8 kg of feed for 1 kg of meat produced
- Crickets: 1.7-2 kg of feed for 1 kg of biomass produced
- Black soldier fly: 1.5-2 kg of feed for 1 kg of biomass produced

Insects can convert about 2 kg of feed into 1 kg of biomass, even with low-nutritional-value feed. A well-known example is the cricket *Acheta domesticus*, which, in converting feed substrate, shows performance twice that of chicken, five times that of pigs, and 12 times that of cattle. Additionally, insects provide a greater percentage of edible fractions compared to traditionally farmed animals, due to the lack of bones, hair, hooves, and other inedible parts. In terms of health benefits for both humans and animals, it is widely recognized that insects provide high-quality proteins and nutrients comparable to those found in meat and fish. Many species have high levels of fatty acids and micronutrients such as copper, iron, magnesium, manganese, phosphorus, selenium, and zinc. Furthermore, based on current knowledge, they have a low risk of transmitting zoonoses. However, when studying insect species and developing techniques for their production, certain key points must be considered. Firstly, there is a need to enhance innovation in mechanization, automation, processing, and logistics to reduce production costs to levels comparable to other food and feed sources. Additionally, it is essential to develop guidelines for insect farming and the nutritional value of growth substrates, conduct more in-depth research on the life cycles of a wide range of insect species to facilitate comparisons with traditional food and feed sources, and maintain a broad genetic diversity to avoid the collapse of farming systems.

Another crucial aspect that cannot be overlooked is food and feed safety. Investigating potential allergic reactions to insects and the digestibility of chitin is important, emphasizing the potential contribution to human and animal health. It is essential, from a large-scale production perspective, to conduct research on the risk of possible zoonoses, pathogens, toxins, and heavy metal contamination due to insect consumption. As with any food product, it will be necessary to develop tools to extend the shelf life of these products. To manage the production and consumption of entomological food and feed in the context of human health and animal welfare (see, for example, the Codex Alimentarius), it will be necessary to develop voluntary codes and regulations at both national and international levels, as well as improve risk assessment methods associated with mass agriculture and wild harvesting to prevent the introduction of exotic and invasive species into natural insect populations.

One of the most promising aspects is the great availability and accessibility of various insect species in nature. This, combined with the simplicity and cost-effectiveness of breeding systems and the high reproductive rate of insects (thus allowing structured breeding starting from just a few specimens), represents a significant financial advantage for starting a business. Moreover, it offers an opportunity—both for developing countries and for lower-income social classes—to generate jobs and products with minimal space, funds, and resource requirements, regardless of the installation area (rural or urban). These activities can directly improve diets and provide income by selling excess production in local markets, thereby offering business opportunities in developed, transitioning, and developing economies. Insects can be used directly as food for humans or as animal feed relatively easily: some species can be consumed whole, while others can be ground or mashed before consumption, or their proteins can be extracted and used separately.

Hermetia illucens

Among the bioconverting insects that have garnered general attention as a response to the enormous demand for sustainable protein sources (while also addressing the significant issue of food waste and the resulting production of organic waste), we find the black soldier fly (Hermetia illucens, Linnaeus, 1758). This species is capable of converting a wide range of organic waste into high-quality biomass in a short time. The larvae are rich not only in proteins and lipids but also in bioactive compounds such as chitin, lauric acid, and antimicrobial peptides that can positively influence intestinal morphometry and animal health.

Hermetia illucens is a dipteran insect of the Stratiomyidae family, originally from the Americas, where it is known as the black soldier fly, but it now has a cosmopolitan distribution. The larvae of this dipteran are frequently found in composting and waste disposal facilities, where they play a useful role in reducing the mass and pollutant load of the waste itself.

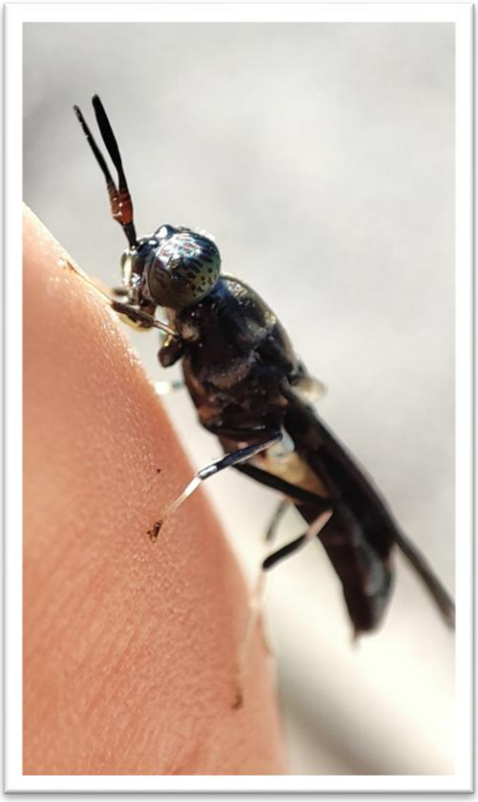
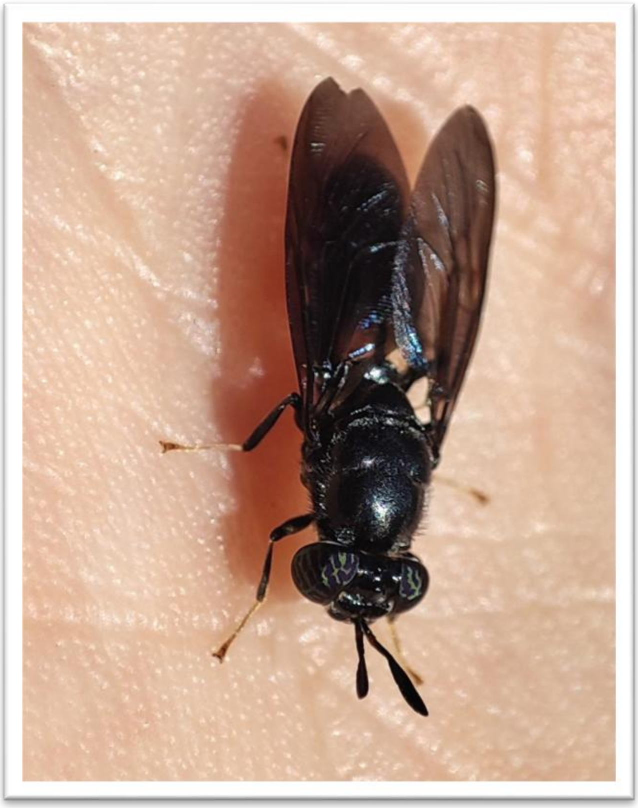

Fig. 1. and Fig. 2. *Black soldier fly adult specimens*

Biological Cycle

The biological cycle of Hermetia illucens is holometabolous, meaning it develops through distinct larval stages following hatching, a pupal stage, and finally metamorphosis into a perfect insect. Each of these stages is characterized by specific growth and transformation processes.

It all begins with the adult female laying eggs. After mating, the female carefully selects a suitable place for egg-laying, usually near decomposing organic materials that will provide nourishment for the newly hatched larvae. The eggs, oval-shaped and whitish in color, are extremely small, measuring about one millimeter in length. The female lays between 500 and 900 eggs in groups, preferring crevices or surfaces that offer protection and proximity to food.

Fig. 3. *Black soldier flies mating*

Fig. 4. *Black soldier flies eggs layed in cardboard*

After an incubation period of 2 to 4 days, the eggs hatch. The first larval stage (L1) is the longest and most significant in the life cycle of the black soldier fly, generally lasting from 14 to 28 days, depending on environmental conditions such as temperature and food availability. The initially whitish larvae progressively darken and can reach a length of about 25 mm. During this stage, the larvae intensely feed on decomposing organic matter, rapidly converting it into body biomass and a residue that can be used as compost, called frass. The larvae are extremely efficient at transforming food, accumulating nutrients that they will utilize in the next developmental stage.

When the larvae are ready for metamorphosis, they enter the prepupal stage, during which they stop feeding and seek a dry and safe place to pupate. In this stage, the larva undergoes a series of significant physiological changes that prepare it for the subsequent transformation into an adult. The pupal stage lasts about 10-14 days. During this period, within the pupa, the insect undergoes a complete reorganization of its body, transforming from a larva to an adult fly. The pupa is dark in color and remains motionless, providing rigid protection for the fragile metamorphosis process occurring inside.

At the end of the pupal stage, the adult emerges, representing the final stage of the biological cycle of Hermetia and also the shortest, lasting on average between 5 and 8 days. During this time, their primary activity is reproduction. Adults do not actively feed, instead living off reserves accumulated during the larval stage. Once mating occurs and eggs are laid, the cycle begins anew.

Early Studies on Hermetia Illucens

Initially noted for their remarkable ability to recycle manure, the larvae of this dipteran were proposed in 2005 by Newton et al. for a project called the "Black Soldier Fly Manure Management System," not only to reduce livestock waste but also to generate a food source for fish and other animals. In the outlined program, pig manure was used as food for black soldier fly larvae, which reduced the amount of manure by 50%. Besides their extensive use in waste disposal, the black soldier fly has proven to be an excellent source of oil and proteins for animal feed and extremely useful for improving organic waste into rich fertilizers. The larvae, by feeding on the provided organic matrix, reduce the levels of phosphorus and nitrogen, suppress harmful organisms (bacteria and other insects), and even reduce unpleasant odors; the residue resulting from the insects' development is a high-quality amendment compost (known as frass). Depending on the type of growth substrate, the mature larvae and prepupae (the stage preceding the adult) constitute a biomass rich in proteins and fats, which can be harvested for various purposes, including animal feed and biofuels. The ability of insects to convert plant biomass into high-nutrient compounds is increasing interest in their use as a low-impact environmental nutrient source, although currently in Italy, there are only a few small-scale operations capable of producing black soldier flies. These companies breed these insects on a small scale, not at an industrial level, so products derived from this farming are currently not available on the market. Finally, Hermetia has also proven extremely useful for biological control programs against the common and harmful housefly, for which Hermetia is the primary competitor in many terrestrial ecosystems.

Breeding

The breeding of *Hermetia illucens* is extremely simple and low-cost: adults are ready to mate 48 hours after emerging, and the eggs, once laid, take about four days to hatch. The biological cycle consists of six larval stages, a pupal stage, and an adult stage. Temperature plays a crucial role in the breeding of this insect; indeed, within a range of 27 to 30°C, the soldier fly exhibits an extremely short larval cycle (ranging from a minimum of fourteen days to a maximum of thirty) and a complete cycle of just forty days. This distinction between the larval and complete cycles is fundamental to delineate two realms of production that can be generated within a single facility: the production of larvae intended for feed and organic waste disposal and the breeding of adults, useful for producing new larvae.

The management of adults is certainly the least labor-intensive aspect of breeding, as they do not require feeding but only environmental humidity for hydration, having an extremely short lifespan during this phase of the life cycle (males die within about 48 hours after mating and females shortly after laying eggs), and do not have high space requirements. To date, the only real limit to adult breeding seems to be the photoperiod, which plays a crucial role in mating and egg-laying; indeed, the positioning of the adult containment cages is closely tied to their exposure to sunlight, which, in winter, leads to a reduction in mating and consequently a slowdown in egg production. Recent studies on *Hermetia illucens* show that the use of artificial lighting and heating systems can effectively increase egg production under unfavorable conditions. However, despite the increase in egg-laying, the mating index remains about 40% lower than under optimal natural conditions, such as those present in summer with natural light, heat, and humidity. This is because, although artificial lighting may induce egg-laying, the conditions created are not completely equivalent to natural ones, having a significant impact on mating quality and the vitality of the bred populations.

While the environmental values of larvae do not seem to differ significantly from adults, their housing needs are completely different: larvae require a system that is not so much wide vertically but rather extensive, thus a tank system would be the most suitable for breeding the entire larval cycle of the insect; these tanks must be equipped with an organic substrate to host the larvae, insulate the temperature, and above all provide them with nutrition. The substrate can be supplied in two main ways: daily feeding, where food is provided in small quantities but at regular intervals, and batch feeding, designed so that the larvae can consume a single large mass of food until they reach the pupal stage.

We can thus delineate the phases of the entire production cycle based on the insect's growth stage:

Egg Hatching

The life cycle of *Hermetia illucens* begins with the hatching of the eggs. These, laid by the female near moist, organic-rich materials, hatch in a period ranging from 3 to 4 days. Optimal environmental conditions for hatching include a temperature between 26 and 30°C and a relative humidity around 70-80%. Newly hatched larvae, known as "neonates," immediately start feeding on the organic substrate on which they were laid, initiating their rapid development.

Fig. 5. *Hermetia illucens eggs*

Larval Growth

Hermetia illucens larvae pass through five larval stages (excluding the prepupal stage), during which their size increases significantly. This growth phase lasts about 14-18 days and represents the period when larvae accumulate most of their mass. During this time, larvae are fed a rich organic substrate, which can include food waste, plant residues, or by-products from the agro-food industry. The substrate must be constantly monitored to maintain optimal humidity and temperature conditions, essential for ensuring rapid and uniform growth of the larvae.

Separation of larvae from Substrate

Once maturity is reached, the larvae must be separated from the substrate for utilization. This process occurs through mechanical methods such as sieving, where the substrate is sifted to separate the larvae from the organic residues. Alternatively, self-separation techniques can be used, exploiting the natural behavior of mature larvae, which tend to migrate to dry areas away from the moist substrate in preparation for pupation.

Fig. 6. *Black soldier fly larvae*

Pupation and Collection of Exuviae

Mature larvae that are not collected for immediate use enter the pupal stage. During this phase, larvae stop feeding and undergo metamorphosis leading to adulthood. The exuviae, or the remains of the larvae's outer skin that detach during pupation, can be collected and used as an agricultural amendment or for bioplastic production, due to their high chitin content.

Final Use of Larvae and By-products

Mature larvae of *Hermetia illucens* can be utilized in various ways. Their high protein and lipid content makes them ideal as animal feed, particularly for aquaculture and poultry farming. Additionally, they can be processed into protein flour for human consumption after appropriate processing. By-products of their processing, such as frass (organic residue), can be used as organic fertilizer, thereby contributing to nutrient cycle closure. The breeding of *Hermetia illucens* thus represents an example of a circular economy, where organic waste is transformed into valuable resources, reducing environmental impact and promoting sustainability in the agro-food sector.

Applications

The flour and oil produced from soldier fly larvae (BSFL) are already considered a high-quality animal alternative to fishmeal and fish oil, used to feed carnivorous fish and in other animal diets, due to their high protein and lipid content even when fed on streams of vegetable waste (Kroeckel, S. et al., 2012). The importance of fishmeal and fish oil in aquaculture is well known, but competition with the demand for fish for human consumption and overfishing, among other factors, have reduced the supplies of fishmeal and oil and increased costs, pushing the fishing sector to seek alternatives such as vegetable oils (Li, SL; Ji, H.; Zhang, BX; Tian). The applications and trials of implementing *Hermetia* have also been extended to poultry feeding, where the soldier fly has shown promising results, especially in terms of sustainability and improvement of animal health. The larvae are a high-quality protein source, rich in essential fatty acids, vitamins, and minerals, which can positively contribute to poultry nutrition. Furthermore, specific components like chitin and medium-chain fatty acids found in insect flour exhibit antimicrobial and prebiotic properties, supporting the immune system of birds.

One study highlighted that the partial or total replacement of soybean oil with oil derived from *Hermetia illucens* larvae had no negative effects on poultry performance, while another found that the inclusion of this flour can improve the lipid quality of quail meat without compromising the sensory or physical quality of the final product (Elangovan et al., 2021). In summary, the use of *Hermetia illucens* in poultry feeding is not only a step toward more sustainable production but also offers tangible benefits in terms of animal health and performance.

Other Products

One of the best-known and widely used by-products of *Hermetia* (and other species) is certainly frass: this is generally described as the product of insect digestion, but in a commercial context, it often refers to a mixture primarily of insect feces, substrate residues, and shed exoskeletons. Frass can account for up to 75% of the total weight of the substrate during the insect production cycle (Diener, S. et al., 2016) and is often marketed as a fertilizer product. In recent years, a growing number of studies have begun to focus on significant applications of frass (Quilliam, R.S. et al. 2020; Alattar, M. et al., 2013; Sarpong, D. et al., 2019), and the first large-scale field studies have provided promising perspectives for its application in agriculture, particularly regarding nutrient availability for plants (Beesigamukama, D. et al., 2020; Beesigamukama, D. et al., 2020; Alattar, M.

et al., 2016). The substrate used to grow insects affects the properties of the excreta, as non-degradable residues remain unused while the digested fraction is modified by the intestinal microbiota as it passes through the gastrointestinal tract (Klammsteiner, T. et al., 2020; Osimani, A. et al., 2018). Frass from *Hermetia illucens* not only provides nutrients directly available to plants but also has profound effects on soil microbiology. It contains organic compounds that can stimulate the growth of beneficial microorganisms, including bacteria and fungi, which play a crucial role in the soil nutrient cycle. Increased microbial activity can enhance the mineralization of organic matter and improve nutrient availability for plants. This not only boosts agricultural productivity but also enhances soil structure, contributing to resilience against erosion and other degradation factors. An interesting aspect related to the use of frass is the stimulating action that the chitin contained in it can have on microorganisms that degrade this polysaccharide. Chitin is a substance that, once introduced into the soil, can promote the proliferation of specific microorganisms that produce chitinase enzymes, capable of breaking down the structure of chitin and releasing important nutrients. This process has long-term effects on soil, improving its biological activity and making it more fertile. Finally, the use of frass contributes to climate change mitigation: one study showed that using organic amendments like frass can increase the soil's capacity to sequester carbon, thus reducing carbon dioxide emissions into the atmosphere. Furthermore, increased soil biodiversity can improve resilience to abiotic stresses, such as drought, thereby reducing the need for intensive irrigation (Dabbou, Sihem, et al. 2019).

Another great byproduct of Hermetia illucens is the oil: the oil derived from Hermetia illucens larvae is one of the most promising products due to its lipid composition. The production process involves collecting mature larvae, which are then subjected to either mechanical pressing or solvent extraction. In these methods, the larvae are dried and pressed to separate the lipids, or treated with chemical solvents to improve yield. The crude oil is then purified to remove any organic impurities. This oil is rich in lauric acid, a saturated fatty acid that gives it stability and makes it highly versatile, especially in animal feed, where it is used to enhance nutritional value and provide a dense energy source (Spranghers et al., 2017). Additionally, its lipid profile makes it an ideal candidate for biodiesel production through transesterification processes, proving to be a renewable resource for the energy sector (Surendra et al., 2016). In the cosmetics industry, Hermetia illucens oil is increasingly used in emollient and moisturizing products, such as creams and soaps, taking advantage of its antibacterial and nourishing properties due to fatty acids like lauric acid (Li et al., 2012).

Chitosan, derived from the chitin present in the exoskeletons of Hermetia illucens larvae is another product with broad applications. The production process begins with the extraction of chitin, which

is isolated by treating the larval material with alkaline substances to remove proteins and mineral acids. Subsequently, the chitin undergoes a deacetylation process to be converted into chitosan, a more soluble and versatile form. This biopolymer is notable for its biodegradable and antimicrobial properties, enabling its use in numerous fields (Huang et al., 2020). In the bioplastics industry, chitosan can be utilized to create biodegradable materials suitable for sustainable packaging, offering an eco-friendly alternative to traditional plastics (Islam et al., 2017). Moreover, in the biomedical field, it is widely used in hemostatic dressings and controlled drug delivery due to its ability to promote coagulation and interact with human tissues without adverse effects. In cosmetics, chitosan is also employed for its hydrating and antibacterial properties, particularly valued in skincare and haircare products, where it forms protective films that retain moisture.
Hermetia illucens represents a promising solution for environmental sustainability and organic waste management. Its biological cycle and bioconversion capabilities offer various opportunities for a circular economy, transforming organic waste into high-value resources such as proteins and lipids for animal feed and biofuels.
The larvae of *Hermetia illucens* can rapidly convert large quantities of organic waste into biomass, simultaneously reducing the environmental impact associated with greenhouse gas emissions, land use, and water resource consumption. This makes the farming of these insects one of the most sustainable options compared to traditional livestock practices, such as cattle, pigs, and poultry farming.

Fig. 7. *Hermetia illucens frass*

Fig. 8. *Hermetia illucens extracted oil*

European regulations are progressively recognizing the value of insects as a source of protein, particularly for animal feed, with an increasing focus on aquaculture and poultry farming. However, challenges remain regarding large-scale production, especially in automation, energy efficiency, and the quality of breeding conditions, which necessitate further research and innovation.
Beyond its applications in livestock farming, *Hermetia illucens* offers potential uses in agriculture (with frass as a natural fertilizer), biofuel production, and even in the cosmetic and biomedical sectors due to its derivatives like chitin and oil extracted from the larvae. These by-products exhibit antimicrobial and biodegradable properties, further contributing to sustainability and the diversification of products obtainable from this species.
In conclusion, *Hermetia illucens* stands out as a key candidate for the development of a more circular and sustainable economy. Its large-scale use could play a crucial role in addressing global challenges related to food production, waste management, and energy supply, significantly contributing to the reduction of the overall environmental impact.

Objectives

The main objectives of this PhD project are:

• To evaluate Hermetia illucens lavae by analyzing in detail their nutritional characteristics and assessing their potential as a sustainable feed source.

• To evaluate the ability of Hermetia illucens larvae to growth on substrates with different chemical-nutritional characteristics.

• To theorize the potential applications of Hermetia illucens, such as animal feed and waste management, in the perspective of the circular economy.

• To verify if the growth of H. illucens larvae on waste can affect some aspects of their safety.

This project therefore aims to study the response of Hermetia illucens larvae growing on different substrates (waste) with various nutritional profiles, evaluating the possible effects on breeding performance, chemical-nutritional characteristics and safety, with a view to a possible use of certain types of waste on large scale production systems. In this way, the research also intends to contribute to providing more information to the European Community for a possible review of the current legislation which prohibits the use of certain types of substrates in the feeding of these insects. Studies conducted so far on Hermetia illucens larvae and their by-products have highlighted a certain variability in the composition of macro and micronutrients in relation to the stage of development and the substrate on which they are bred; this makes it difficult to generalize about its nutritional value and possible use in feed production. However, what are currently considered limitations in defining the standard nutritional characteristics of Hermetia illucens can actually be seen, in the light of more in-depth studies, as a great opportunity: the identification of possible waste substrates on which to breed the larvae up to a certain stage would make it possible to use Hermetia illucens as a multipotential insect, capable of satisfying different chemical requirements through the "simple" choice of diet and the stage at which the larvae are harvested; in essence, different products from the same species. In order to achieve this objective, it is fundamental and urgent to promote laws that regulate the use of insects as raw materials for feed production, and this can be achieved through research efforts to understand the possibilities (as well as the limitations) of this sector, which can be said to be barely explored today. Given their high nutritional value and, according to the knowledge acquired so far, the clearly superior efficiency compared to conventionally bred animal species in terms of feed conversion into body weight, it is evident that interest in large-scale insect breeding as an alternative feed source mainly for monogastric livestock (but not only) use is growing. Although the numerous positive aspects of using insects as feed are already known, the literature on the subject requires a lot of work, in particular in order to asses possible problems for feed and/or food safety.

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RESEARCH ARTICLE

Fatty acid profile of black soldier fly larvae and frass as affected by different growing substrates

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Abstract

This study aimed to evaluate how different food wastes used as growth substrates affected the lipid composition of *Hermetia illucens* larvae; the relationship among substrates, larvae, and frass fatty acid (FA) composition was tudied. Six thousand five-day-old *Hermetia illucens* larvae were allotted to one of four different substrates named CTRL (a control substrate made of broiler feed), V50 + B50 (vegetable and butchery wastes, 1:1 ratio), V75 + B25 (vegetable and butchery wastes, 3:1 ratio), and V100 (entirely composed of vegetables). Lipids were extracted from substrates, larvae, and frass separately and the FAs were quantified. Saturated FAs (SFAs) prevailed in all the groups. The CTRL larvae had the highest SFA (64.3%), while the V100 and V50 + B50 larvae had the lowest percentages. The V100 frass recorded a significantly higher SFA value (44.8%) than the others. Lauric acid (C12:0) was high in CTRL (0.2%) and V100 (0.3%) substrates and amount at 7.6 and 9.6% in their correspondent frass, respectively. However, C12:0 content was higher in the CTRL larvae than in the V100 ones (36.7 and 24.5%, respectively), while it had an intermediate value (28.9%) in the V75 + B25 larvae. Finally, the n-3 polyunsaturated FAs (PUFAs) level was high in the V100 substrate and larvae but not in the V100 frass. The relationship between frass FAs and their correspondent amounts in substrates and larvae was significant for C12:0, C18:2n-6, MUFAs, and n-6 PUFAs. There was a positive relationship for C12:0 and MUFAs with both substrates and larvae, while for C18:2n-6 and n-6 PUFAs, the relationship was positive for substrates but negative for larvae. In conclusion, the V100 substrate appeared to be the most suitable treatment for rearing *Hermetia illucens* because of the positive effects on the fatty acid content of larvae (low SFA and high n-3 PUFA content) and frass (optimal lauric acid levels for fertilizers).

1. Introduction

Over the last decade, interest in insect production has increased to address the shortage of protein resources for humans and animals due to the expected growth of the world population in the near future (FAO, 2021). In addition, the recent global crises (the pandemic COVID-19, the Russian-Ukrainian war) have drawn attention to the possible difficulties in the supply of raw materials and energy, which has also increased the overall cost of food and feed (Attia *et al*., 2022), thus supporting the need of local supply chains. Much attention has been paid to some bio-converting insects, such as the black soldier flies (BSF, *Hermetia illucens*, L.), which are able to convert, in a short time, a wide range of organic wastes into valuable biomass (larvae). Larvae are rich not only in proteins and lipids (Scieuzo *et al*., 2023) but also in bioactive compounds such as chitin, lauric acid, and antibacterial peptides that can positively influence the intestinal morphometry and animal health (Candian *et al*., 2023; Saviane *et al*., 2021). After harvesting larvae to obtain the different products (meal, oils, etc.), the residual frass (containing insect exuviae and excrements, as well as residual substrate) might be used for various purposes such as fertilizer or to produce biogas or biofuel (Carrol *et al*., 2023; Mohan *et al*., 2023; Wedwitschka *et al*., 2023). In the context of the circular economy principle, the greenest choice could be to grow *H. illucens* (HI) larvae on organic wastes from different industries, considering the great bioconversion power of this insect species. However, the chemical and nutritional characteristics of insect larvae and frass can largely vary according to the different growing substrates. This can be particularly true for lipids, whose content in *H. illucens* larvae can vary from 5 to 40% of dry matter (DM) due to the substrate characteristics (Li *et al*., 2022). In addition, even if the fatty acid profile of *H. illucens* lipids can be partially modulated depending on the substrates (Cattaneo *et al*., 2023), this species is notoriously able to endogenously synthesized some saturated fatty acids (SFA), as lauric (C12:0) and myristic (C14:0) acids. During the last decades, several beneficial effects of lauric acid emerged both for animal and human health, such as its antibacterial properties (Borrelli *et al*., 2021; Dayrit, 2015) and a possible role against cancers spread through lymphatics (Sleeman, 2000). Actually, coconut oil is the main natural source of C12:0 (Dhanya *et al*., 2023); hence, it could be interesting to locate another source high in lauric acid and suited for varied industries. Regarding myristic acid, it seemed to decrease hyperglycemia in patients affected by type 2 diabetes (Jung *et al*., 2016). In addition, other fatty acids have potential or already established uses, such as palmitic acid (C16:0), which enhances the effect of anti-viral therapy (Akoh, 2017); stearic acid (C18:0) that can protect against oxidative stress (Chow, 2007) and oleic acid (C18:1n-9) that seemed to act as a tumoricidal agent (Jung *et al*., 2016). However, the richness in lipids of *H. illucens* larvae, prominently composed by SFAs, not only makes it complicated to balance the diet when the integral larvae are utilized in animal feeding, but it has also responsible for the augmented content of SFAs in meat of the animals fed with *H. illucens* (Cattaneo *et al*., 2023). Due to this, two strategies have been adopted to limit these disadvantages of the use of HI. From one hand, the fat content of larvae generally is extracted as oil. The oil extracted from *H. illucens* may be an interesting resource for the pharmaceutical industry (Khan *et al*., 2021), biodiesel and cosmetic industry (Franco *et al*., 2021). On the other side, attempts to enrich BSF with polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 series by enriching the growth substrate have been multiplied (Ewald *et al*., 2020; Truzzi *et al*., 2020), sometimes without an efficient resource utilization. Considering the possible industrial use of lipids obtained from *H. illucens*, the relationship among the fatty acid profile of larvae, substrate, and residual frass was investigated by the present research, which specifically aimed to evaluate the fatty acid profile of black soldier fly larvae and frass according to growing substrates that are entirely different in terms of origin, lipid percentage, and fatty acid profile. The possibility to estimate the lipid composition of larvae based on the substrate one was also investigated in order to enhance the knowledge and guide the potential applications in various industries, aligning with the study's objectives and broader implications.

2. Material and methods

2.1 Experimental design

The *Hermetia illucens* larvae utilized here derived from the growing trial described in Addeo *et al*. (2021) where readers can find all the details about the environmental conditions, the growing performances, and larvae survival rate. To sum up the core of the study, five-day-old BSF larvae were purchased from a commercial insectrearing company (Smart Bugs, Treviso, Italy) and randomly allotted to one of four different substrates, named CTRL (a control substrate made of broiler feed), V50 + B50 (a substrate made by vegetable and butchery wastes in a 1:1 ratio), V75 + B25 (a substrate made by vegetable and butchery wastes in a 3:1 ratio), and V100 (a substrate entirely composed by vegetables). The vegetable wastes, irrespective the amount contained in the diet, consisted of a mix containing 75% vegetables (40% broccoli, 35% celery, 25% cabbages) and 25% fruits (50% oranges and 50% apples). The butchery wastes included the trim- Journal of Insects as Food and Feed 0 (2024) 1–15 Growing substrates affect *Hermetia illucens* larvae and frass fatty acids 3 mings of bovine carcasses and cuts of meat. Each diet was put into nine plastic containers (60 × 40 × 15 cm), resulting in nine replicates for each diet; each replicate had 1 kg of substrate and 6,000 five-day-old larvae. At 22 days old, larvae were separated from the frass and fasted for 24 hours. Two sieve metal meshes were used to separate larvae and frass at the end of the trial. Initial manual sieving (to separate the coarser particles) was done using a mesh spacing for 1 × 4 mm sieve (were 1 is the bar thickness and 4 the square spacing between bars). Thus, the second mesh (1 × 2 mm) was used to separate the smaller particles of frass from larvae. Samples of diets, larvae, and frass were collected from each replicate, freeze-dried, and stored up to the chemical analyses.

2.2 Chemical analyses

The dry matter content of the substrates, larvae, and frass was quantified using AOAC (2007) standard procedure. The total lipids of each matrix were subdued to a cold extraction with chloroform:methanol (2:1, v:v) as solvent, as suggested by the Folch *et al*. (1957) method. The extracted lipids were firstly gravimetrically quantified, then they were saponified and methylated in order to perform the analysis of the fatty acid content using gaschromatography. The details of the run were reported in Secci *et al*. (2021). To quantify the fatty acid methyl esters (FAME), we utilized an internal (C23:0; Supelco, Bellefonte, PA, USA), and an external standard (Supelco 37 component FAME mix; Supelco, Bellefonte, PA, USA) necessary to compare the FAME retention time and obtain calibration curves. The oxidative status of the substrates, larvae, and frass was evaluated considering the conjugated dienes (CD) content, expressed as mmol hydroperoxides/100 g samples (Srinivasan *et al*., 1996), and the thiobarbituric acid reactive substances (TBARS), analyzed on fresh larvae, expressed as mg of malondialdehyde equivalent (mg MDA-eq.)/100 g sample (Vyncke, 1975).

2.3 Statistical analysis

The efficacy of sample size and the normal distribution of the data and their error were tested using the Shapiro-Wilk test (SAS, 2002). The random distribution of the data confirmed the relevance of the 4 analysis of variance (ANOVA) assumptions: (1) each observation was mutually independent, (2) the data adhered to an additive-effect statistical model comprising fixed effects and random errors, (3) the random errors are normally distributed, and (4) they have homogenous variations. The homoscedasticity (variance homogeneity) was evaluated using Levene's test (SAS, 2002). One-way analysis of variance using the procedure GLM of SAS (2002) was applied, according to the following model (Equation 1):

$Yij = m + Si + eij$ (1)

where:

Y = is the single observation

m = the general mean

S = the effect of substrates

e = the error.

Each replicate was considered as an experimental unit. The selected post-hoc test was the Tukey's (SAS, 2002). The relationship among the lipid content and fatty acid profile of substrate, larvae and frass was studied using the PROC REG of SAS (2002), comparing, for each item, the average values of the substrate with the correspondent average values obtained from the nine replicates of the larvae and/or frass (n = 4). *P*-values lower than 0.05 were considered significant.

3. Results

The total lipid content of the four substrates was 3.98% DM, 5.12% DM, 36.97% DM, and 49.76% DM in V100, CTRL, V75 + B25, and V50 + B50, respectively; hence, the group including the highest level of butchery wastes was the richest in fat. Their fatty acid profile is reported in Table 1.

The total lipid content of the BSF increased while increasing the content of butchery wastes in the substrates (*P* < 0.01). The V50 + B50 showed the highest lipid content (35.56% DM), followed by the V75 + B25 and CTRL, which did not differ each other, having a lipid content equal to 27.08 and 22.12% DM, respectively. The V100 larvae were the leanest (6.01% DM). Table 2 reports the fatty acid profile of BSF larvae raised on the four substrates. All the groups showed a high level of lauric acid (C12:0), even if the V50 + B50 group had the lowest one (*P* < 0.01). In all the tested groups, the most abundant fatty acids were the SFAs, followed by MUFAs, n-6 PUFAs, and n-3 PUFAs. However, the V100 and V50 + B50 groups had the lowest content of SFAs than the other groups (*P* < 0.01), and V50 + B50 showed the highest proportion of MUFAs (*P* < 0.01) compared to the other groups. The V100 group had the highest content of n-3 PUFAs (*P* < 0.01). Relevant differences among groups (*P* < 0.01) were found for the total lipids of the residual frass obtained from farming BSF larvae on the four substrates. The V50 + B50 showed the highest lipid value (39.32% DM) and the CTRL and V100 frass the lowest (1.77% and 2.47% DM, respectively). The lipid content of V75 + B25 amounts to 19.98% DM, thus significantly lower than the V50 + B50 frass, but significantly higher than the V100 and CTRL. The fatty acid profile of the frass is depicted in Table 3. The V100 frass had the highest content of C12:0 (*P* < 0.01), while the CTRL frass showed higher contents of lauric acid than the V50 + B50 and V75 + B25 ones. CTRL and V100 frass had a proportion of SFAs higher than MUFAs, while the opposite was for the frass obtained from substrates including butchery wastes. In Table 4, the content of conjugated dienes and TBARS of substrates, larvae and frass is summarized. The larvae grown on the CTRL and V50 + B50 substrates showed the highest values of CD, followed by V75 + B25 and V100 groups (*P* < 0.01). The TBARS values measured in the V75 + B25 group were higher than those of the CTRL one (*P* < 0.01). The CD value was the highest in the V50 + B50 frass, followed by the V75 + B25, V100, and CTRL groups (*P* < 0.01). The TBARS value in the V75 + B25 frass was higher than that measured in the V50 + B50 one. In Table 5, the equations to estimate the fatty acid profile of BSF larvae from that of the growing substrate are reported. Significant relationships were found for C16:0, C18:1n-9, C18:2n-6, n-6 PUFAs, n-3 PUFAs (*P* < 0.05) and C18:3n-3 (*P* < 0.01). Table 6 reports the equations able to estimate the fatty acid percentages on the total FAME of frass starting from both growing substrates and larvae. The relationship was significant for C12:0, C18:2n-6, MUFAs and n-6 PUFAs (*P* < 0.05). For C12:0 and MUFAs, there was a positive relationship with both substrates and larvae, while for C18:2n-6 and n-6 PUFAs the relationship was positive for substrates but negative for larvae. Figures 1, 2, and 3 compare the fatty acid profile of the substrates with those obtained for larvae and frass. For C12:0 and C14:0, larvae showed very high contents compared to substrate and frass, whilst for C18:0 larvae had lower values than substrate and frass.

An intermediate condition was found for C16:0. In general, for MUFAs and PUFAs, larvae had low contents of fatty acids compared with both substrate and frass.

Table 1 Dry matter (g/100 g substrate) and fatty acid profile (g/100 g of total FAME) of the four tested substrates

	CTRL	V50+B50	V75+B25	V100
Drymatter	29.95	36.35	29.87	14.63
Fattyacids				
C10:0	0.00	0.04	0.03	0.05
C12:0	0.23	0.08	0.06	0.29
C13:0	0.01	0.01	0.01	0.01
isoC14:0	0.01	0.02	0.03	0.01
C14:0	0.21	2.02	2.07	0.97
C14:1n-5	0.04	0.41	0.52	0.10
isoC15:0	0.03	0.08	0.11	0.14
anteisoC15:0	0.01	0.10	0.13	0.16
C15:0	0.11	0.20	0.27	0.19
isoC16:0	0.00	0.10	0.14	0.08
C16:0	19.45	23.75	23.49	19.00
C16:1n-9	0.11	0.18	0.18	0.09
C16:1n-7	0.34	3.01	3.10	1.81
C16:4n-1	0.09	0.07	0.06	0.14
anteisoC17:0	0.03	0.33	0.48	0.32
C17:0	0.15	0.63	0.75	0.39
C17:1	0.01	0.00	0.01	0.85
C18:0	2.68	16.20	17.38	8.83
C18:1n-9	29.00	43.63	42.22	21.38
C18:1n-7	1.29	3.91	4.06	2.79
C18:2n-6	40.11	3.50	3.06	23.11
C18:3n-6	0.03	0.01	0.01	0.04
C18:3n-3	1.50	0.14	0.31	13.28
C20:0	0.46	0.16	0.13	0.45
C20:1n-11	0.09	0.12	0.13	0.12

C20:1n-9	0.82	0.62	0.30	0.38
C20:2n-6	0.18	0.12	0.03	0.34
C20:3n-3	0.02	0.01	0.00	0.08
C20:3n-6	0.00	0.05	0.12	0.04
C20:4n-3	0.02	0.00	0.01	0.08
C20:4n-6	0.01	0.04	0.07	0.04
C20:5n-3	0.03	0.08	0.01	0.10
C21:5n-3	0.00	0.02	0.00	0.00
C22:0	0.32	0.02	0.03	0.43
C22:1n-11	0.86	0.03	0.01	0.06
C22:1n-9	1.10	0.04	0.01	0.14
C22:5n-6	0.01	0.00	0.003	0.010
C24:0	0.41	0.01	0.01	0.64
ΣSFA	24.12	43.73	45.11	29.95
ΣMUFA	33.66	51.95	50.53	27.71
Σn-6PUFA	40.40	3.73	3.33	23.63
Σn-3PUFA	1.57	0.25	0.34	13.54
ΣUnknown	0.16	0.27	0.64	5.04

CTRL = broiler feed diet,

V50 + B50 = diet consisting of 50% of vegetable diet + 50% of butchery wastes,

V75 + B25 = diet consisting of 75% of vegetable diet + 25% of butchery wastes,

V100 = vegetable mix diet; SFA = saturated fatty acids,

MUFA = monounsaturated fatty acids,

PUFA = polyunsaturated fatty acids.

Table 2 Dry matter (g/100 g larvae) and fatty acid profile (g/100 g of total FAME) of *Hermetia illucens* larvae according to substrate

	CTRL	V50+B50	V75+B25	V100	RMSE	*P*-value
Drymatter	33.78c	42.31a	40.90b	22.54d	0.14	<0.0001
Fattyacids						
C10:0	0.89a	0.26d	0.58b	0.35c	0.03	<0.0001
C12:0	36.68a	11.83d	28.82b	24.54c	0.66	<0.0001
C13:0	0.014c	0.017bc	0.022ab	0.024a	0.01	0.0002
isoC14:0	0.04b	0.02c	0.02c	0.07a	0.05	<0.0001
C14:0	8.40a	3.73d	5.30c	6.26b	0.17	<0.0001
C14:1n-5	0.17d	0.58a	0.51b	0.35c	0.23	<0.0001
isoC15:0	0.08b	0.07b	0.04c	0.12a	0.01	<0.0001
anteisoC15:0	0.22a	0.09c	0.07d	0.18b	0.01	<0.0001
C15:0	0.11c	0.25a	0.20b	0.25a	0.01	<0.0001
isoC16:0	0.18a	0.08c	0.05d	0.10b	0.01	<0.0001
C16:0	13.76c	22.61a	17.60b	13.27c	0.76	<0.0001
C16:1n-9	0.27d	0.41c	0.90a	0.47b	0.02	<0.0001
C16:1n-7	2.55d	4.36c	4.69b	5.12a	0.14	<0.0001
C16:4n-1	0.07a	0.05b	0.06ab	0.07a	0.01	0.0021
anteisoC17:0	0.38a	0.30b	0.16c	0.14c	0.04	<0.0001
C17:0	0.20d	0.60a	0.29c	0.34b	0.03	<0.0001
C17:1	0.13b	0.01c	0.02c	0.17a	0.01	<0.0001
C18:0	3.07b	10.18a	3.52b	3.29b	0.51	<0.0001
C18:1n-9	13.77d	32.71a	28.46b	24.50c	0.73	<0.0001
C18:1n-7	0.58d	2.41a	0.96c	1.24b	0.05	<0.0001
C18:2n-6cis	15.56a	5.38c	5.59c	13.50b	0.30	<0.0001
C18:3n-6	0.01b	0.02b	0.08a	0.01b	0.01	<0.0001
C18:3n-3	0.96b	0.43d	0.58c	2.54b	0.06	<0.0001
C20:0	0.18a	0.12c	0.07d	0.16b	0.02	<0.0001
C20:1n-11	0.07a	0.09a	0.02b	0.10a	0.01	0.0003
C20:1n-9	0.10c	0.23a	0.05d	0.14b	0.01	<0.0001
C20:2n-6	0.02b	0.07a	0.01c	0.03b	0.01	<0.0001
C20:3n-3	0.006ab	0.007ab	0.003b	0.011a	0.01	0.0051

C20:3n-6	0.01d	0.07a	0.05b	0.03c	0.01	<0.0001
C20:4n-3	0.011ab	0.010ab	0.005b	0.014a	0.01	0.0044
C20:4n-6	0.01d	0.13b	0.29a	0.04c	0.01	<0.0001
C20:5n-3	0.01c	0.03b	0.07a	0.03b	0.01	<0.0001
C21:5n-3	0.09a	0.01c	0.01c	0.05b	0.01	<0.0001
C22:0	0.08b	0.03c	0.03c	0.12a	0.01	<0.0001
C22:1n-9	0.014b	0.013bc	0.006c	0.03a	0.01	<0.0001
C22:5n-6	0.01	0.21	0.02	0.02	0.19	0.0855
C24:0	0.016b	0.006c	0.006c	0.027a	0.06	<0.0001
Σ*SFA*	*64.30a*	*50.19c*	*56.77b*	*49.24c*	*1.21*	*<0.0001*
Σ*MUFA*	*17.66d*	*40.82a*	*35.62b*	*32.13c*	*0.89*	*<0.0001*
Σ*n-6PUFA*	*15.63a*	*5.88c*	*6.04c*	*13.64b*	*0.35*	*<0.0001*
Σ*n-3PUFA*	*1.07b*	*0.49d*	*0.67c*	*2.66a*	*0.06*	*<0.0001*
Σ*Unknown*	*1.27c*	*2.57a*	*0.83d*	*2.26b*	*0.08*	*<0.0001*

CTRL = broiler feed diet,

V50 + B50 = diet consisting of 50% of vegetable diet + 50% of butchery wastes,

V75 + B25 = diet consisting of 75% of vegetable diet + 25% of butchery wastes,

V100 = vegetable mix diet;

SFA = saturated fatty

Table 3 Dry matter (g/100 g of total frass) and fatty acid profile (g/100 g FAME) of frass obtained by *Hermetia illucens* larvae reared on the four experimental substrates

	CTRL	V50+B50	V75+B25	V100	RMSE	*P*-value
Drymatter	66.82b	57.61c	78.72a	58.37c	0.64	<0.0001
Fattyacids						
C10:0	0.23a	0.02b	0.03b	0.21a	0.01	<0.0001
C12:0	7.60b	0.36c	0.78c	9.55a	1.51	<0.0001
C13:0	0.004b	0.007b	0.005b	0.013a	0.01	0.0002
isoC14:0	0.07a	0.01b	0.03b	0.06a	0.01	<0.0001
C14:0	2.14b	1.60c	1.31d	2.74a	0.13	<0.0001
C14:1n-5	0.05d	0.40a	0.31b	0.10c	0.03	<0.0001
isoC15:0	0.38b	0.10c	0.12c	0.69a	0.07	<0.0001
anteisoC15:0	0.78a	0.14c	0.17c	0.59b	0.06	<0.0001
C15:0	0.11b	0.20a	0.22a	0.23a	0.03	<0.0001
isoC16:0	0.87a	0.11b	0.21b	0.84a	0.11	<0.0001
C16:0	14.83b	17.03ab	18.76a	17.33a	1.75	0.0005
C16:1n-9	0.19b	0.21b	0.27a	0.20b	0.03	<0.0001
C16:1n-7	0.94d	3.41a	2.61b	1.84c	0.18	<0.0001
C16:4n-1	0.09a	0.07b	0.06b	0.10a	0.01	<0.0001
anteisoC17:0	1.93a	0.47c	0.84b	0.95b	0.12	<0.0001
C17:0	0.26c	0.64b	0.86a	0.55b	0.09	<0.0001
C17:1	0.11b	0.01d	0.04c	0.27a	0.02	<0.0001
C18:0	5.23d	12.49b	16.53a	9.98c	1.68	<0.0001
C18:1n-9	21.61c	47.35a	46.18a	27.22b	2.50	<0.0001
C18:1n-7	2.10c	4.55a	3.50b	3.82b	0.27	<0.0001
C18:2n-6	34.66a	6.12c	4.07d	14.30b	1.33	<0.0001
C18:3n-6	0.16a	0.01c	0.14a	0.07b	0.03	<0.0001
C18:3n-3	2.19a	0.38c	0.48c	1.53b	0.09	<0.0001
C20:0	0.57a	0.11d	0.18c	0.42b	0.03	<0.0001
C20:1n-11	0.09c	0.18a	0.20a	0.16b	0.02	<0.0001
C20:1n-9	0.46ab	0.50a	0.33c	0.39bc	0.06	<0.0001
C20:2n-6	0.10b	0.14a	0.04c	0.12ab	0.02	<0.0001
C20:3n-3	0.02b	0.02b	0.01b	0.12a	0.01	<0.0001

C20:3n-6	0.03c	0.13a	0.14a	0.06b	0.01	<0.0001
C20:4n-3	0.02b	0.01bc	0.01c	0.04a	0.01	<0.0001
C20:4n-6	0.04c	0.12b	0.18a	0.05c	0.02	<0.0001
C20:5n-3	0.13a	0.02b	0.04b	0.17a	0.07	0.0001
C21:5n-3	0.020a	0.007b	0.004b	0.018a	0.01	<0.0001
C22:0	0.46a	0.02c	0.06c	0.36b	0.04	<0.0001
C22:1n-11	0.12a	0.01c	0.01c	0.03b	0.01	<0.0001
C22:1n-9	0.22a	0.03c	0.03c	0.12b	0.06	<0.0001
C22:4n-6	0.04c	0.05bc	0.06ab	0.08a	0.02	0.0002
C22:5n-6	0.006a	0.001b	0.004ab	0.004ab	0.00	0.042
C24:0	0.40a	0.01d	0.06c	0.34b	0.04	<0.0001
Σ*SFA*	*35.86bc*	*33.32c*	*40.17ab*	*44.85a*	*3.71*	*<0.0001*
Σ*MUFA*	*25.89c*	*56.66a*	*53.47a*	*34.14b*	*2.9*	*<0.0001*
Σ*n-6PUFA*	*35.04a*	*6.56c*	*4.64d*	*14.68b*	*1.33*	*<0.0001*
Σ*n-3PUFA*	*2.38a*	*0.44c*	*0.54c*	*1.88b*	*0.10*	*<0.0001*
Σ*Unknown*	*0.73d*	*2.96b*	*1.12c*	*4.35a*	*0.28*	*<0.0001*

CTRL = broiler feed diet,

V50 + B50 = diet consisting of 50% of vegetable diet + 50% of butchery wastes,

V75 + B25 = diet consisting of 75% of vegetable diet + 25% of butchery wastes,

V100 = vegetable mix diet;

SFA = saturated fatty

4. Discussion

How to combine healthy diet, access to food (food safety), environmental impact mitigation, and the increase in world population is a still debated issue. Insects have been proposed as a strategic pillar to reach the solution; however, the creation of a solid circular supply chain should be supported by scientific evidence. It is well known that insects – in particular the bioconverter species, such as the black soldier fly – can grow on a wide range of substrates, even when unbalanced for protein, carbohydrate and lipid contents (Li *et al*., 2022). Lu *et al*. (2021) revealed an increased larval weight gain in *H. illucens* when the dietary fat level in the growing substrate of larvae increased from 3 to 12%, suggesting that lipids might play an important role in larval growth and development. Focusing on the fatty acid composition, some authors observed an increased accumulation of lauric acid in BSF larvae with increasing weight (Ewald *et al*., 2020; Liu *et al*., 2017); however, our results only partially agree with the previous findings. In fact, looking at the growth performance of larvae involved in our study and reported by Addeo *et al*. (2021), at the end of the growing period the live weights recorded for CTRL, V50 + B50, V75 + B25, and V100 groups were 0.216, 0.205, 0.216, 0.184 g, respectively, and the relative abundance of lauric acid was 36.68, 11.83, 28.82, 24.54% of total FAME, respectively. Hence, the two groups having the highest weight, CTRL and V75 + B25, had high C12:0 content but at two different levels, whilst the smallest larvae (V100) were not the poorest in C12:0. Overall, the increase of lipids and the decrease in carbohydrates, as reported in Addeo *et al*. (2021), in substrates containing animal sources (butchery wastes) strongly decreased the amount of lauric acid in larvae (−67.7% in V50 + B50 compared to CTRL). This pattern, previously also observed by Oonincx *et al*. (2015), could be explained by the role of C12:0 in the energetic metabolism of this species and the possible lauric acid accumulation as stored energy during larval development, as reported by Zhu *et al*. (2019), that is the way insects preferentially stored the energy for their adult life, during which, as known, they do not feed. In addition, Spranghers *et al*. (2017) suggested that lauric acid content is increased by carbohydrates increasing in feed. Several authors (Giannetto *et al*., 2020; Truzzi *et al*., 2020; Zarantoniello *et al*., 2020) showed that BSF larvae tend to accumulate some of MUFAs and PUFAs, in particular oleic, linoleic, and α-linolenic acids, to convert them mainly in C12:0. This is in line with our results. which showed a significant relationship between fatty acid concentrations of oleic, linoleic and α-linolenic acids in larvae and substrates. The substrates in which butchery wastes were included showed an almost double content of C18:1n-9 than the other two substrates involved in the trial, whereas C18:2n-6 and C18:3n-3 were low, and this reflected the fatty acid profile of the larvae. The BSF metabolic pathways to endogeny synthesize fatty acids are scarcely known (Hoc *et al*., 2020). However, considering that V50 + B50 larvae had a lower C12:0 content than the other groups, it can be hypothesized that C18:1n-9 is less utilized for conversion in C12:0 instead of the other two fatty acids. A possible confirmation of our hypothesis can be obtained by looking at the research of Li *et al*. (2022). In fact, our results only apparently disagree with the findings of Li *et al*. (2022), who found that C12:0 and C14:0 can be prominently accumulated in BSF larvae as a consequence of high dietary fat content. In this regard, two interesting considerations can be drawn. First, the range of total lipid contents in the diets studied by Li *et al*. (2022) varied from 5.8 to 10.3%, with very few alterations compared to the levels of lipids in the diets used in the present trial. On the other hand, Li *et al*. (2022) detected the lowest percentage of lauric acid in larvae reared on the substrate enriched with peanut oil, which had the highest concentration of C18:1n-9. From this dissertation, it is possible to hypothesize that more than one factor is involved in determining the fatty acid profile of BSF larvae, particularly regarding the synthesis of lauric acid. Altogether, our results seemed to indicate that the lauric acid in BSF larvae is unrelated to its content in the substrates, which were very poor in C12:0, and that both the amount of energy and the source of energy might modulate the synthesis of lauric acid. As shown by Hoc *et al*. (2020), SFAs from C12:0 to C18:0 are known to be *de novo* biosynthesized by several insect species from carbohydrates contained in their diet, and this justifies why no significant relationship was found for C12:0, C14:0 and C18:0 contents between larvae and substrates. Regarding palmitic acid (C16:0), an accumulation from the substrate, corresponding to approximately 50% of the palmitic acid in larvae, has been described by Hoc *et al*. (2020), and this might explain the significant relationship observed between substrates and larvae for this fatty acid. Combining the results for larvae and substrates, it is possible to reach significant equations for estimating the lauric acid content in frass. Frass obtained from CTRL and V100 substrates is particularly rich in lauric acid. This is interesting for potential applications of functional plant amendments, as suggested by Liang *et al*. (2021) who affirmed that lauric acid is safe for soybean plants and, at lesser than 0.3 g a.i./plant, can promote soybean growth and control plant pathologies. Looking at Figures 1, 2 and 3, representing the fatty acid contents of larvae, substrates, and frass in the same graph, different behavior of the three fatty acids groups (SFAs, MUFAs, PUFAs) can be observed. For SFAs, larvae had a greater proportion than substrates and frass; for MUFAs, frass showed in general (except for CTRL) the highest proportion, while for PUFAs, the substrates had higher contents than frass and larvae.

Table 4 Conjugated dienes (mmol Hp/100 g of sample) and TBARS (mg MDA-eq./100 g of sample) in substrates, larvae, and frass

	CTRL	V50+B50	V75+B25	V100	RMSE	*P*-value
Substrates						
Conjugated dienes	0.85	4.98	2.37	0.47	–	–
TBARS	0.40	0.57	0.27	0.51	–	–
Larvae						
Conjugateddienes	1.64a	1.69a	0.86b	0.56c	0.29	<0.0001
TBARS	0.18b	0.21ab	0.27a	0.22ab	0.04	0.0032
Frass						
Conjugated dienes	0.36c	1.89a	1.30b	0.43c	0.27	<0.0001
TBARS	0.15ab	0.11b	0.22a	0.16ab	0.02	<0.0001

CTRL = broiler feed diet,

V50 + B50 = diet consisting of 50% of vegetable diet + 50% of butchery wastes,

V75 + B25 = diet consisting of 75% of vegetable diet + 25% of butchery wastes,

V100 = vegetable mix diet; a,b,c = $P < 0.05$; RMSE = root mean square error.

Table 5 Equations to estimate fatty acid percentages on total FAMEs in BSF larvae from growing substrates

Y	X	Intercept	r2	SE	*P*-value
C12:0	37.71	19.25	0.1775	11.54	0.5787
C14:0	−2.02	8.59	0.8609	0.89	0.0721
C16:0	0.27	13.16	0.9014	1.91	0.0492
C18:0	0.26	2.17	0.3006	3.53	0.4517
C18:1n-9	0.72	0.46	0.9065	3.22	0.0486
C18:2n-6	0.29	4.97	0.9419	1.56	0.0294
C18:3n-3	0.15	0.55	0.9813	0.16	0.0093
Σ*SFA*−0.30	68.89	0.1993	7.64	0.5535	
Σ*MUFA*	0.53	9.78	0.4216	9.25	0.3507
Σ*n-6PUFA*	0.27	5.38	0.9461	1.44	0.0272
Σ*n-3PUFA*	0.15	0.63	0.9740	0.19	0.0130

Y = fatty acid concentration on total FAME in BSF larvae; X = fatty acid percentage on total FAME in the substrate; r2 = coefficient of determination; SE = standard error; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

Table 6 Relationship among frass fatty acid percentages on total FAME and their correspondent amount in feeding substrate and larvae

Y	X	Z	Intercept	r2	SE	*P*-value
C12:0	38.39	0.05	−2.97	0.9987	0.28	0.0346
C14:0	−1.19	−0.34	5.56	0.6685	0.63	0.5757
C16:0	−0.21	0.47	15.98	0.8488	1.09	0.3888
C18:0	0.75	−0.51	5.51	0.8757	1.27	0.1559
C18:1n-9	2.51	−1.98	−0.65	0.8576	4.95	0.2179
C18:2n-6	1.52	−2.62	14.48	0.9994	0.59	0.0243
C18:3n-3	−0.80	5.67	−2.19	0.6741	0.50	0.3296
Σ*SFA*	−0.20	−0.37	66.29	0.2455	7.62	0.8686
Σ*MUFA*	0.68	0.82	−11.20	0.9967	1.48	0.0475
n-6PUFA	1.57	−2.93	17.55	0.9994	0.59	0.0243
n-3PUFA	−0.67	4.91	−2.06	0.8742	0.60	0.3547

Y = fatty acid concentration on total FAME in BSF larvae; X = fatty acid percentage on total FAME in the substrate; r2 = coefficient of determination; SE = standard error; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

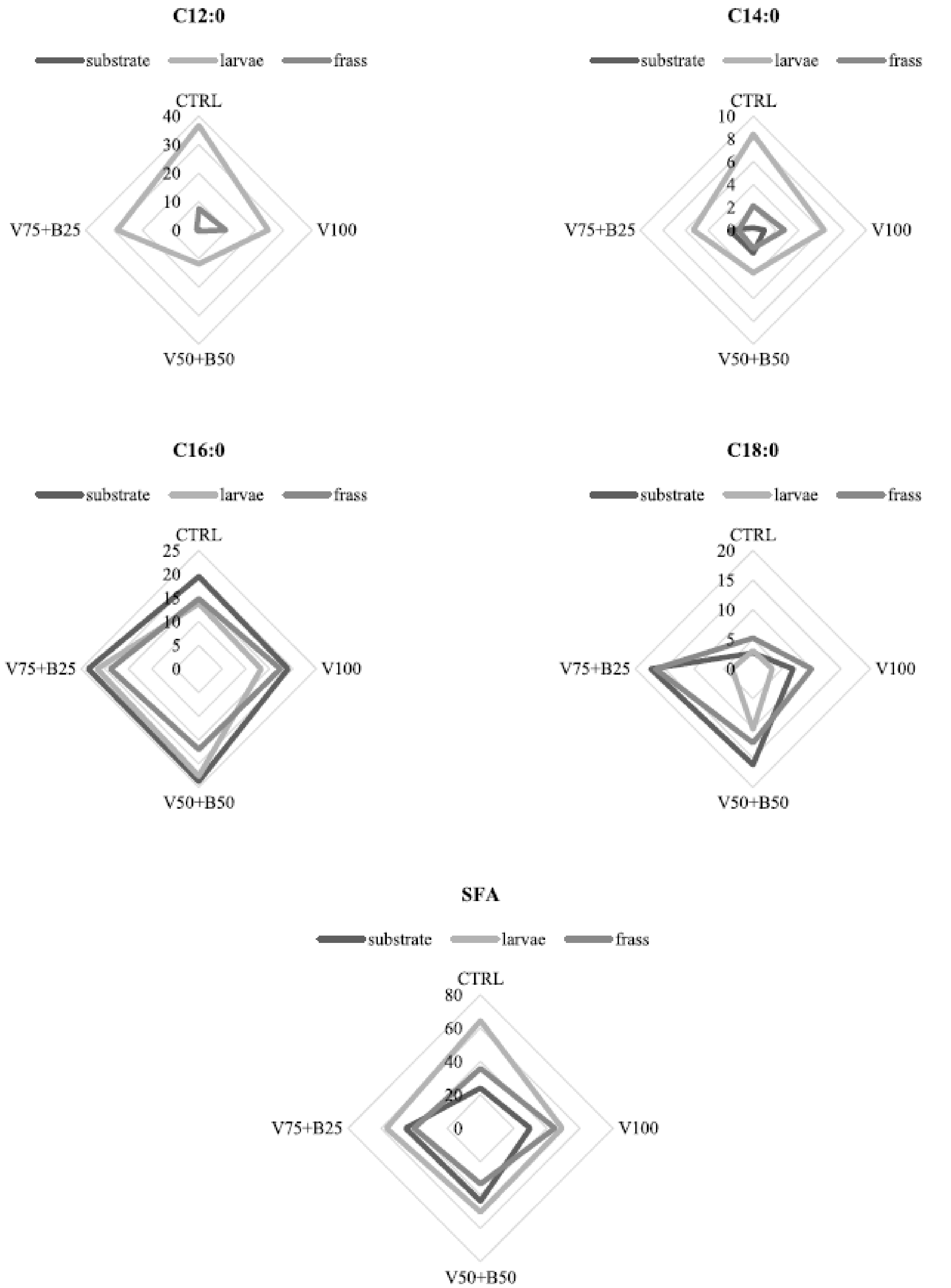

Figure 1 Comparison of saturated fatty acid percentages among substrate, larvae, and frass.

According to Ewald *et al*. (2020), the percentage of some fatty acids (such as C20:3, C22:0, and C22:1) was very low in the tested substrates (<0.5%); however, disagreeing with the author, low percentages of these fatty acids also in larvae and frass were detected. Very few studies are available in the literature on the fat oxidation of *H. illucens* larvae, and almost all were focused on processed products (Hurtado-Ribeira *et al*., 2023; Larouche *et al*., 2019; Mouithys-Mickalad *et al*., 2021; Vandeweyer *et al*., 2022). Despite the scarce amount of unsaturated fatty acids, both MUFAs and PUFAs can be vulnerable to lipid oxidation which can lead the reduction of nutritional values, the development of unpleasant odor and taste, and even the devel opment of toxic molecules (Domínguez *et al*., 2019). Hence, lipid oxidation can thwart the efforts of using alternative and sustainable ingredients in feeds but also the PUFAs enriching trials. Nevertheless, as far as we know, none had proposed a threshold to discriminate between rancid or no rancid BSF products. Considering the rancidity ranking showed by Connell (1975), lipids can be classified as not rancid (TBARS value 1.5 mg MDA/kg), slightly rancid (TBARS value from 1.6 to 3.6 mg MDA/kg), and rancid (TBARS value > 3.7 mg MDA/kg). During this study, the lipids of larvae and frass were slightly rancid. Nevertheless, TBARS are wellestablished oxidative stress markers since they have been associated with stress-related diseases and disorders (Dahama *et al*., 2019) in animals, and the control of oxidative stress in invertebrates is still debated. In some insect species, for example, it has been reviewed that oxidative stress is induced by pesticides/insecticides. Also, flavonoids and tannins can increase free radical content, thus potentially unbalancing the antioxidant defense of the organism. In this regard, further studies on the relationship between BSF metabolic pathways and chemical compounds in the substrates should be encouraged.

5 Conclusion

According to the analyses of lipid and fatty acids, black soldier fly lipids show a particular fatty acid profile in which lauric acid is uppermost. The V100 substrate might be the most suitable treatment for rearing *Hermetia illucens* larvae for two main reasons. Firstly, V100 larvae had the lowest SFA percentages and the highest n-3 PUFAs content, both positive results for the wholesomeness of *Hermetia illucens* when used as an ingredient for feeds. In the second place, frass obtained from V100 substrates is particularly rich in lauric acid, an interesting aspect for potential applications of frass as plant amendments and fertilizer. Thus, considering these factors, the V100 substrate seemed to promote desirable fatty acid profiles in both larvae and frass, making it a potentially suitable choice for rearing *Hermetia illucens* larvae for subsequent use as an ingredient in feeds and for producing an optimal frass, useful as fertilizer. In conclusion, the BSF larvae and frass' fatty acid profile is confirmed to be modulable, but the pursuit of the route of supplementation of substrates with long-chain polyunsaturated fatty acids both of the n-3 and n-6 series does not seem the best way considering the relationship between the fatty acid profile of substrates and larvae. Additional strategies, not addressed in this study, should therefore be considered to optimize the use of the biomasses obtained in terms of larvae and BSF oils, whose potential use as an alternative to drugs, particularly antibiotics, should be further considered with the aim of improving the sustainability of farmed animals.

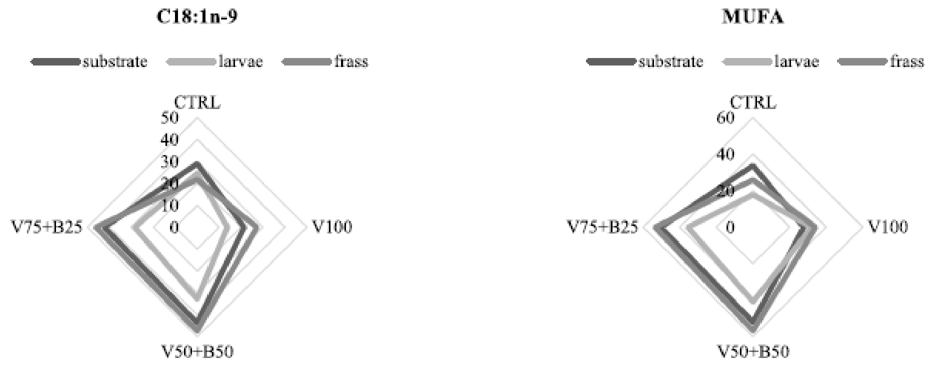

FIGURE 2 Comparison of monounsaturated fatty acid percentages among substrate, larvae, and frass.

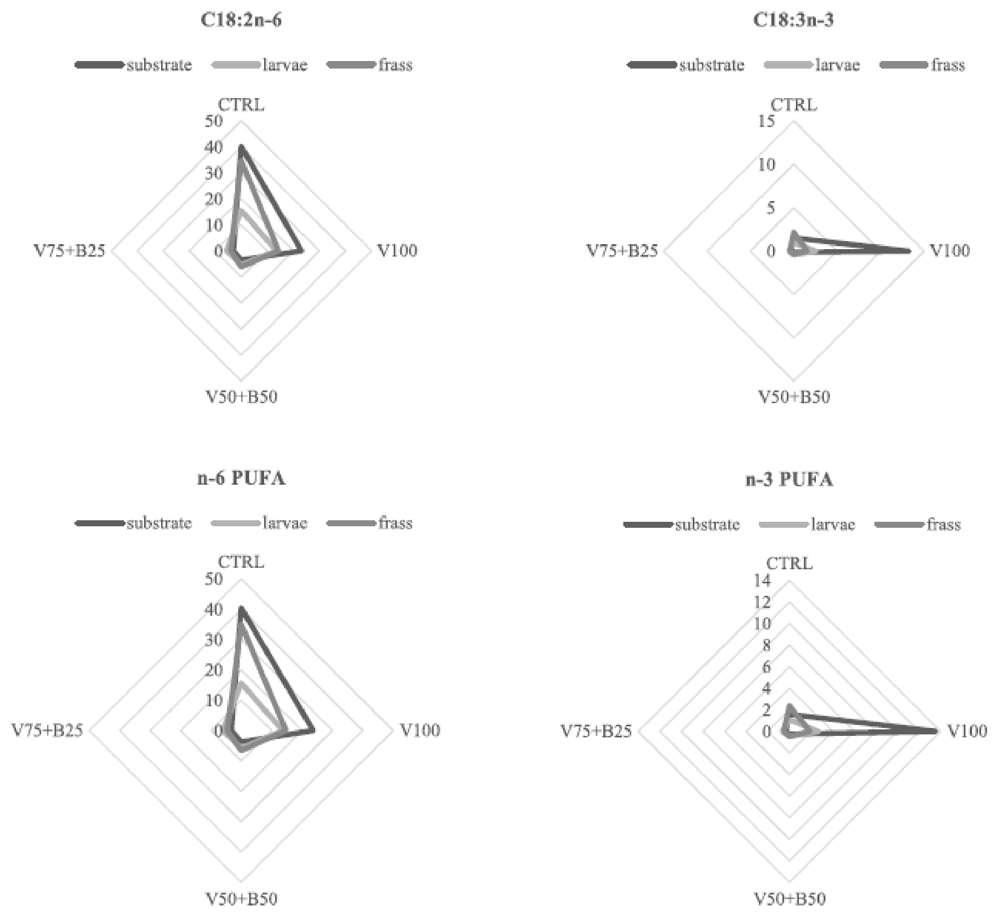

Figure 2 Comparison of polyunsaturated fatty acid percentages among substrate, larvae, and frass.

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Article

Amino Acid Profile of *Hermetia illucens* Larvae Reared on Different Combinations of Butchery and Vegetable Wastes and Resulting Frass

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Simple Summary

Black soldier fly (BSF) larvae can grow on a wide range of substrates, transforming them into valuable biomass, and their amino acid composition is strictly related to the amino acid composition of the substrates themselves. In this research, four different substrates were used, and their influence on larvae amino acid profile was evaluated: broiler feed as control diet, a 100% vegetable diet, a diet composed of 50% of vegetables and 50% of butchery waste, and a diet consisting of 75% of vegetables and 25% of butchery waste. Vegetable and butchery wastes are easy to find and collect and they show interesting potential for BSF larvae, in terms of growth and amino acid composition. As result of larvae growth, the insect frass (excreta, exuviae and residual substrate) resulting at the end of the production cycle, offers new and interesting aspects, from the perspective towards circular economy.

Abstract: Black soldier fly larvae (BSF, *Hermetia illucens*) are largely used to convert organic waste into edible biomass for use as animal feed and the substrate of growth may affect its proximate composition. In this research, larvae were raised on four different substrates: broiler feed as control diet; a 100% vegetable mix diet (V100); a diet composed of 75% of vegetables and 25% of butchery wastes (V75B25); and a diet consisting of 50% of vegetables and 50% of butchery wastes (V50B50). Samples of substrates, larvae and frass were freeze-dried, and crude protein content and protein amino acids (AA) profile were determined. In BSF, the amount of protein ranged from 20.0% in V75B25 to 43.7% in V100 ($P < 0.01$). The larvae on V100 had the highest ($P < 0.01$) amount of essential and non-essential amino acids (EAA) except for hydroxyproline which has been detected only in V75B25 group. V75B25 group had the lowest amount ($P < 0.05$) for almost all the AA. BSF larvae appear to be a useful tool for the bioconversion of a wide range of food wastes although significant differences in protein fraction and amino acid profile have been observed depending on the rearing substrate.

1. Introduction

Given the present speed of human population development, the mass farming of insects for feed and food is in full industrial development, as a valuable alternative to conventional livestock to produce animal protein (Poveda et al., 2021). The Commission Regulation (EU) 2017/893 recognize that black soldier fly (*Hermetia illucens*) is a species of insect safe for use as feed, between others. Current small-scale breeding of insects for pet food can be adequately managed within the framework of existing national control plans; furthermore, Union provisions relating to animal and plant health, public health or environmental risks are appropriate to ensure that insect farming within the Union on a larger scale is safe. Insect species farmed in the Union must not be pathogenic or have other adverse effects on human, animal, or plant health; they must not be recognized as vectors of human, animal or plant pathogens and must not be either protected species or species defined as invasive aliens. In this activity, insect frass (insect excreta, exuviae and residual substrate) represents one of the main residues of the productive process, being the final volume up to 40 times higher than the production of animal biomass (Poveda et al., 2021). Actually, its use as organic fertilizer to replace the use of agrochemicals is considered a positive alternative in the development of sustainable agriculture and circular economy (Poveda et al., 2021). Insect frass has been approved as a fertilizer in Europe. In November 2021, Commission Regulation (EU) 2021/1925 amended the annexes of Regulation (EU) N. 142/2011, establishing the requirements for the marketing of insect frass as a fertilizer/soil improver. This means that insect frass can be sold and used as an organic fertilizer or soil conditioner in all countries of the European Union. Non-EU countries may have different regulations, so it is important to check them locally. In line with the principles of circular economy, the breeding of black soldier fly larvae on organic waste streams from various industries presents a promising green solution, leveraging this insect species' remarkable bioconversion capacity (Addeo et al., 2024 a). Nonetheless, the chemical and nutritional profiles of insect larvae and frass can exhibit substantial variability contingent upon the specific growth substrates employed (Addeo et al., 2024 a). Maximizing the utilization of this residual organic secondary stream could not only present an environmental opportunity but also prove to be economically advantageous for insect producers. By exploiting this by-product efficiently, additional income could indeed be generated (Beesigamukama et al., 2022). The population growth and rapid urbanization have also increased the global demand for animal feed and protein sources. To meet this growing demand, it becomes essential to increase the production of animal protein (Sogari et al., 2023). As the prospect of incorporating insects into the human food system gains traction, it is imperative to thoroughly assess the potential hazards associated with their consumption, particularly in the context of diverse cultivation substrates (Addeo et al., 2024 b). In line with the growing momentum towards circularity, research has directed the efforts towards utilizing organic waste as a nutrient source for *H. illucens* larvae growth (Addeo et al., 2024 b). Therefore, traditional animal feed production should be increased using alternative nutrient sources, such as insects. This step becomes crucial to reduce dependence on conventional resources and to ensure a sustainable supply of animal feed. In this situation, Amino Acids (AA) play a significant functional role, acting as the foundational components for proteins and serving as materials for the generation of low-molecular-weight substances. Balance of amino acids (AA) in diets is essential for healthy nutrition (Wu et al., 2021). Insufficient or excessive intake of specific AAs can cause health problems in animals (Wu et al., 2009). Therefore, it is essential to consider the functions of amino acids in dietary formulations in order to optimize nutrient utilization efficiency, promote growth, development, reproduction and contribute to well-being overall of animals (Wu et al., 2014). Depending on growth patterns or nitrogen equilibrium, Amino Acids (AA) were traditionally categorized as either nutritionally indispensable or dispensable for animals. Animal diets require the presence of nutritionally essential amino acids (EAA) (Wu et al., 2014), and insects offer a significant supply of energy, protein, EAA, fatty acids, and micronutrients (Verkerk et al., 2007 - Van Huis et al., 2013). Moreover, the EAA profile of insects aligns with the necessary amino acid profiles for optimal growth in broiler chickens and piglets (Makkar et al., 2014).

Insect frass is fundamental in natural cycle of nutrients, being important in the exchanges between plant material and soil (Poveda et al., 2021). Although insect production is highly efficient in converting biowaste into biomass, it also yields a waste stream of moulting skins (exuviae) and, more importantly, insect feces and residual substrate (Dicke, 2018). Frass has a great potential to be used as a partial or a complete substitute of mineral NPK (nitrogen, phosphorus, and potassium) fertilizer (Houben et al., 2020). This perspective is linked to the interesting observation regarding insect excrement, which represents a source of nutrients easily assimilated by plant roots. This assimilation is made possible by the specific physiological mechanisms of insects, which shape their body waste to facilitate absorption by plants (Poveda et al., 2021). The qualitative attributes of insect residue, particularly its amino acid composition, exert a substantial influence on its efficacy as a fertilizer through various mechanisms (Poveda et al., 2021). Insect frass serves as a nitrogen-rich substrate, crucial for optimal plant development. The quantity and spectrum of amino acids within insect frass play a pivotal role in modulating the accessibility and assimilation of nitrogen by plants (Beesigamukama et al., 2020). Additionally, the insect-derived fraction encompasses essential nutrients, including phosphorus, potassium, calcium, and magnesium, all of which contribute to plant nourishment (Beesigamukama et al., 2020). The amino acid profile of insect residues further shapes the accessibility and assimilation of these vital nutrients by plants

(Beesigamukama et al., 2022). Notably, insect frass harbors a diverse array of beneficial microorganisms that confer improvements to soil health (Houben et al., 2020). The amino acid composition of insect residues also exerts an influence on the structure and functionality of the soil microbiota. Several factors, such as insect species, dietary regimen, growth conditions, and harvesting and processing methodologies, can impact the amino acid composition of insect frass (Poveda et al., 2021 - Beesigamukama et al., 2020). Consequently, insect frass emerges as a naturally derived, environmentally sustainable fertilizer with the potential to enhance both plant growth and soil vitality. The mineral composition of *Hermetia illucens* excrement varies depending on the growing medium used to feed the larvae. This variation can significantly affect the quality and quantity of nutrients present in their excrement (Romano et al., 2023 b). Studies have shown that feeding larvae with different organic residues, such as food waste or other agricultural by-products, can result in a different mineral composition in their excrement (Addeo et al., 2024 b - Romano et al., 2023 b). In general, *Hermetia illucens* droppings are known to contain high amounts of nitrogen, phosphorus, potassium, and other micronutrients essential for plant growth (Poveda et al., 2021). These elements make excrement a high-quality natural fertilizer, potentially usable in sustainable agriculture. Applications in the circular bioeconomy include using excrement as fertilizer to improve soil fertility and increase crop yields (Amorim et al., 2024). Furthermore, their use can contribute to the sustainable management of organic waste, transforming waste materials into useful resources for agriculture (Czekała et al., 2020). Despite some farmers have already reported several beneficial effects of frass to crops (Beesigamukama et al., 2020), there is currently very limited information on the ability insect farms derived frass to ameliorate soil fertility and, consequently, plant growth (Poveda et al., 2019). As highlighted by Berggren et al. (Berggren et al., 2019), the fertilizer potential of frass needs to be deeply investigated before that a major upscaling of the industry is undertaken. This action would be important given the urgent need to find cost-saving and environmental-friendly options to conventional chemical nourishments, based on fossil fuels and finite reserves (Houben et al., 2020). BSF larvae frass is a by-product that can enhance agricultural sustainability, as could be compared to synthetic or organic fertilizers (Kebli et al., 2017- Choi et al., 2009). This aspect is particularly crucial in the perspective of addressing the current challenges related to agricultural production and the need to reduce the environmental impact associated with the use of conventional fertilizer. In contrast to studies on growth performance and nutritional quality of BSF larvae, there is a limited attention on the resulting frass (Fischer et al., 2021). The presence of important nutrients in insect frass makes of strong interest its inclusion in fish feed, highlighted by the use of *Hermetia illucens* frass in the feed for channel catfish (*Ictalurus punctatus*), where growth performances were significantly improved (Yildirim-

Aksoy et al., 2020 a) or juvenile hybrid tilapia (*Oreochromis niloticus x O. mozambique*) diets, in which resistance to bacterial disease and growth were improved (Yildirim-Aksoy et al., 2020 b). Based on this scenario, the aim of the present research was to evaluate the amino acid profile of BSF larvae fed on different substrates, of vegetable and/or animal origin, and of the resulting frass.

2. Materials and Methods

2.1 Larvae and Substrates

Five-days-old black soldier fly larvae (BSF, *Hermetia illucens*, Linnaeus, 1758; Diptera: Stratiomyidae) were bought from an industrial insect farm (Smart Bugs, Treviso, Italy) and used in a growing trial. For the trial, which lasted 17 days, four different substrates were employed: broiler feed (I period feed, Mignini & Petrini SpA, PG, Italy) as control diet; a 100% vegetable mix diet (V100); a diet composed of 75% of vegetables and 25% of butchery wastes (V75B25); and a diet consisting of 50% of vegetables and 50% of butchery wastes (V50B50). The vegetable diet consisted of a mixture of wastes collected from supermarkets in the province of Naples (Italy), containing 75% of vegetables (40% broccoli, 35% celery, 25% cabbages) and 25% fruits (50% apples, 50% oranges). The butchery wastes were acquired from butchers in the Naples area and mainly consisted of meat and fat deriving from the finishing of beef cuts. The protein content of residues from slaughter, calculated on the basis of dry matter, was found to be equal to 7.12 ± 0.65 as reported by Addeo et al., 2024. This value indicates the percentage of proteins present in the materials considered, with a standard variation that reflects the possible difference between different samples, thus underlining the consistency of the results obtained during the analysis. For such research,a batch feeding strategy was used, which means that the substrates were placed in plastic containers (60x40x15cm) one day prior to placing the larvae, at the beginning of the experimental trial. The experimental protocol forecasted groups (Control, V100, V50B50, and V75B25) consisted of 9 replicates, for a total of 36 trays. Ten kg of substrate were placed in each replicate. After weighing the larvae, 6,000 five-day-old individuals were transferred on each substrate. The trays were equipped with a perforated cap featuring a black nylon grid and positioned in an environment under controlled conditions (T 27 ± 0.5 °C; RH: 70 ± 5%). Other details on insect farming have been described by the Authors (Addeo et al., 2021 a). Maintaining of larvae was continued until more than 25% of them in a dish had developed into prepupal stage.

2.2 Moisture, Crude Protein and Amino Acids determination

At the beginning of the trial, moisture content was measured in each substrate. Ten grams aliquot was constituted by mixing 3 randomly collected samples of each substrate and then analyzed using an electric oven for 24 h at 65 °C. At the end of the trial, moisture content of the frass was measured with the same method.

At the end of the trial, samples of substrates, larvae and frass were collected from each tray. All the products were freeze-dried using a Micromudulyo freeze drier (Thermo Electron Corporation, Thermo Fisher Scientific Inc., Whaltham, MA, USA). Dry matter (DM) and crude protein (CP) contents were analyzed according to a standardized protocol (AOAC, 2005). Only for larvae, the nitrogen to crude protein conversion ratio was 4.67 according to the literature (Janssen et al., 2017). Twenty grams of each sample were used for the determination of the amino acid profile, using a HPLC procedure after acid hydrolysis (6 N HCl for 24 hours at 110°C) followed by ion exchange chromatography using an amino acid analyser (L-8800 Auto-analyser, Hitachi, Tokyo, Japan).

For methionine and cystine analysis performic acid oxidation with acid hydrolysis-sodium metabisulfite method was utilized as previously reported (AOAC, 2005 - (Addeo et al., 2021 b). The frozen substrates sampled at the start of the trial, were submitted to the same procedure of analysis applied for larvae and frass.

2.3 Statistical Analysis

The normal distribution of the data and their error, as well as the efficacy of sample size were tested using the Shapiro-Wilk test (Kebli et al., 2017). The random distribution of the data confirmed the relevance of the 4 analysis of variance (ANOVA) assumptions: (1) each observation was mutually independent, (2) the data adhered to an additive-effect statistical model comprising fixed effects and random errors, (3) the random errors are normally distributed, and (4) they have homogenous variations. The homoscedasticity (variance homogeneity) was evaluated using Levene's test (Kebli et al., 2017).

Data, concerning crude protein content and protein amino acids profile of the larvae at the end of the trial, were analyzed by a one-way ANOVA, using the GLM procedure of SAS (Kebli et al., 2017) according to the following model: $Y_{ij} = m + S_i + e_{ij}$, where Y = is the single observation, m = the general mean, S = the effect of substrates, e = the error. Differences were considered significant at $p < 0.0001$ and means were separated by Tukey's test.

3. Results

After 17 days (22 days-old larvae), all the groups reached the end of the experiment, consisting of the development of 25% of prepupal stage.

Moisture resulted 85.37 ± 1.12% and 38.92 ± 2.85%, in vegetable and butchery wastes, respectively. Thus, for Control, V100, V75B25, and V50B50 diets, the moisture was 70.05, 85.37, 70.13, and 63.65%, respectively. At the end of the trial, for frass deriving from these substrates, moisture resulted 33.18, 41.63, 21.28, 42.39 %, respectively.

Table 1 shows the crude protein content and the amino-acid profile of the four substrates used in this trial.

Table 1. Crude protein content and protein amino acids profile of the substrates used in this trial (g/100g DM)

	Control	**V100**	**V75B25**	**V50B50**
Crude protein	12.6	17.9	13.4	12.5
Essential amino acids				
Phenylalanine	0.568	0.553	0.492	0.509
Isoleucine	0.404	0.54	0.448	0.464
Histidine	0.327	0.221	0.312	0.303
Leucine	1.00	0.986	0.867	0.882
Lysine	0.436	0.655	0.769	0.714
Threonine	0.429	0.636	0.474	0.471
Valine	0.586	0.744	0.574	0.564
Methionine	0.220	0.170	0.204	0.238
Tryptophan	0.164	0.202	0.0854	0.11
Non-essential amino acids				
Aspartic acid	0.898	1.55	1.21	1.12
Glutamine	2.46	2.24	1.94	2.12
Alanine	0.733	0.949	0.957	0.749
Arginine	0.815	0.575	0.857	0.715
Glycine	0.629	0.706	1.46	0.827
Hydrossiproline	-	0.194	0.605	0.251
Proline	0.872	0.522	0.962	0.749
Serine	0.652	0.659	0.584	0.570
Tyrosine	0.379	0.361	0.317	0.347
Cystine	0.277	0.139	0.132	0.170

Control: broiler feed; V100: diet with 100% vegetables; V75B25: diet with 75% vegetables + 25% butchery wastes; V50B50: diet with 50% vegetables + 50% of butchery wastes.

Table 2 reports the crude protein and the amino acid profile of the BSF larvae fed with the different substrates. In general, the V100 larvae had the highest ($P < 0.01$) amount of essential and non-essential amino acids except for hydroxyproline which was detected only in V75B25 group. V75B25 group had the lowest amount ($P < 0.05$) for almost all the amino acids. The amount of protein, ranged from 20.0% in V75M25 to 43.7% in V100 ($P < 0.01$).

Table 2. Crude protein content and protein amino acids profile of larvae at the end of the trial (g/100g DM)

	Control	**V100**	**V75B25**	**V50B50**	**RMSE**	***P* value**
Crude protein	42.17^{B}	43.7^{A}	20.01^{D}	29.69^{C}	0.24	<0.0001
Essential amino acids						
Phenylalanine	1.38^{B}	2.05^{A}	0.58^{D}	1.15^{C}	0.09	<0.0001
Isoleucine	1.42^{B}	1.88^{A}	0.68^{D}	1.23^{C}	0.07	<0.0001
Histidine	0.87^{C}	1.18^{A}	0.40^{D}	0.87^{B}	0.03	<0.0001
Leucine	2.49^{B}	2.89^{A}	1.07^{D}	2.02^{C}	0.02	<0.0001
Lysine	2.16^{B}	2.36^{A}	0.83^{D}	1.67^{C}	0.03	<0.0001
Threonine	1.40^{B}	1.65^{A}	0.58^{D}	1.30^{C}	0.05	<0.0001
Valine	2.14^{B}	2.40^{A}	0.98^{D}	1.75^{C}	0.05	<0.0001
Methionine	0.60^{B}	0.72^{A}	0.22^{D}	0.47^{C}	0.01	<0.0001
Tryptophan	0.51^{B}	0.68^{A}	0.23^{D}	0.37^{C}	0.03	<0.0001
Non-essential amino acids						
Aspartic acid	3.24^{B}	3.79^{A}	1.33^{D}	2.67^{C}	0.05	<0.0001
Glutamine	4.09^{B}	4.91^{A}	1.78^{D}	3.83^{C}	0.04	<0.0001
Alanine	2.53^{B}	3.13^{A}	1.55^{D}	2.32^{C}	0.15	<0.0001
Arginine	1.39^{C}	2.13^{A}	0.66^{D}	1.54^{B}	0.05	<0.0001
Glycine	1.77^{B}	2.35^{A}	0.99^{D}	1.64^{C}	0.04	<0.0001
Hydroxyproline	-	-	0.05^{A}	-	0.0007	<0.0001
Proline	1.73^{C}	2.37^{A}	0.93^{D}	1.87^{B}	0.05	<0.0001
Serine	1.48^{B}	1.97^{A}	0.71^{C}	1.47^{B}	0.03	<0.0001
Tyrosine	2.10^{B}	2.97^{A}	0.77^{D}	1.74^{C}	0.03	<0.0001
Cystine	0.36^{A}	0.37^{A}	0.18^{C}	0.29^{B}	0.02	<0.0001

Control: broiler feed; V100: diet with 100% vegetables; V75B25: diet with 75% vegetables + 25% butchery wastes; V50B50: diet with 50% vegetables + 50% of butchery wastes; within rows: A, B, C, D: $p < 0.01$; RMSE: Root Mean Square Error.

Table 3 shows the crude protein content and the amino-acid profile of the frass resulted from the 4 substrates at the end of the trial.

Table 3. Crude protein content and protein amino acids profile of frass (g/100g DM)

	Control	**V100**	**V75B25**	**V50B50**	**RMSE**	**P value**
Crude protein	25.4^{B}	10.1^{D}	12.8^{C}	33.3^{A}	0.20	<0.0001
Essential amino acids						
Phenylalanine	0.942^{A}	0.386^{C}	0.272^{D}	0.864^{B}	0.02	<0.0001
Isoleucine	0.932^{A}	0.368^{C}	0.257^{D}	0.780^{B}	0.02	<0.0001
Histidine	0.476^{A}	0.177^{C}	0.921^{D}	0.392^{B}	0.007	<0.0001
Leucine	2.10^{A}	0.655^{C}	0.487^{D}	1.43^{B}	0.02	<0.0001
Lysine	1.22^{A}	0.463^{B}	0.281^{C}	1.17^{A}	0.09	<0.0001
Threonine	0.913^{A}	0.415^{C}	0.266^{D}	0.850^{B}	0.01	<0.0001
Valine	1.21^{A}	0.534^{C}	0.382^{D}	1.14^{B}	0.03	<0.0001
Methionine	0.348^{A}	0.175^{B}	0.091^{C}	0.318^{A}	0.03	<0.0001
Tryptophan	0.285^{A}	0.166^{B}	-	0.170^{B}	0.01	<0.0001
Non-essential amino acids						
Aspartic acid	2.28^{A}	0.856^{C}	0.545^{D}	1.85^{B}	0.02	<0.0001
Glutamine	3.87^{A}	1.28^{C}	0.930^{D}	3.23^{B}	0.05	<0.0001
Alanine	1.54^{B}	0.733^{D}	0.956^{C}	2.23^{A}	0.04	<0.0001
Arginine	1.11^{B}	0.357^{C}	0.255^{D}	1.45^{A}	0.05	<0.0001
Glycine	1.12^{B}	0.672^{D}	0.976^{C}	3.34^{A}	0.06	<0.0001
Hydroxyproline	-	0.058^{C}	0.427^{B}	1.60^{A}	0.018	<0.0001
Proline	1.20^{B}	0.445^{D}	0.691^{C}	2.26^{A}	0.09	<0.0001
Serine	1.08^{A}	0.452^{C}	0.288^{D}	1.04^{B}	0.02	<0.0001
Tyrosine	0.648^{A}	0.329^{C}	0.178^{D}	0.622^{B}	0.01	<0.0001
Cystine	0.354^{A}	0.167^{C}	0.093^{D}	0.276^{B}	0.01	<0.0001

Control: broiler feed; V100: diet with 100% vegetables; V75B25: diet with 75% vegetables + 25% butchery wastes; V50B50: diet with 50% vegetables + 50% of butchery wastes; within rows: A, B, C, D: $p < 0.01$; RMSE: Root Mean Square Error.

In figures 1, 2, 3 and 4, the EAA contents of larvae is compared to the correspondent level of the same amino acids in substrates. All the substrates had a lower amount of Threonine compared to the profile of the larvae. The V100 substrate had 5 AA lower than the correspondent profile of the larvae, the V50B50, 4, the V75B25, 3 and the Control only 2.

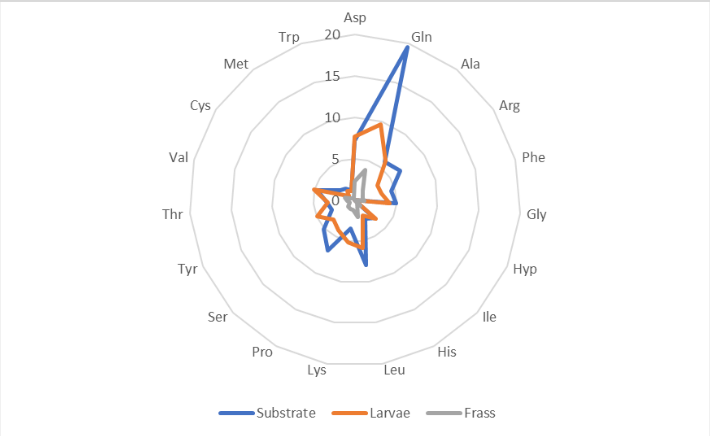

Figure 1. Control group: comparison of essential amino acids contents between substrate, larvae and frass.

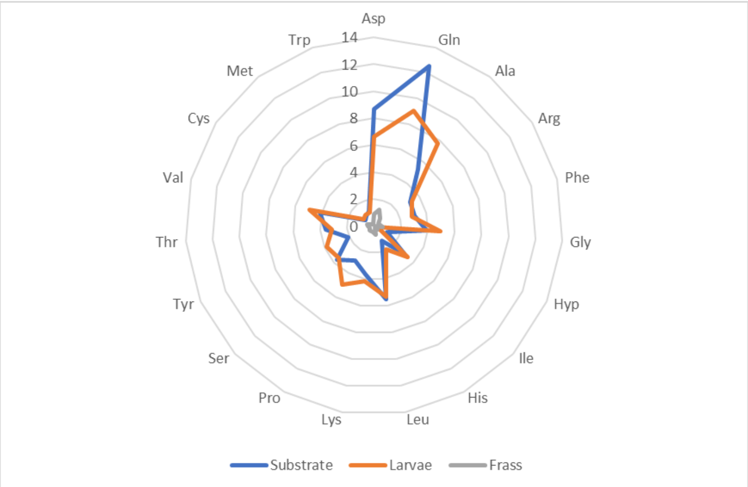

Figure 2. V100 group: comparison of essential amino acid content between larvae, substrate, and frass.

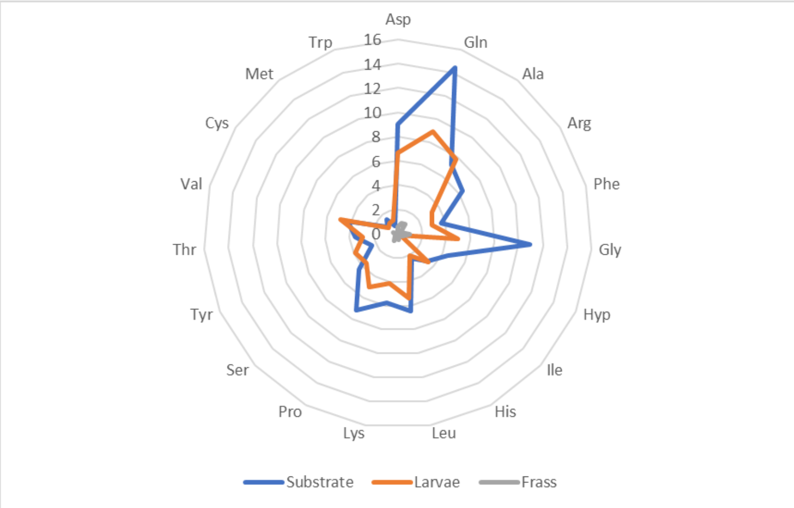

Figure 3. V75B25 group: comparison of essential amino acid content between larvae, substrate, and frass.

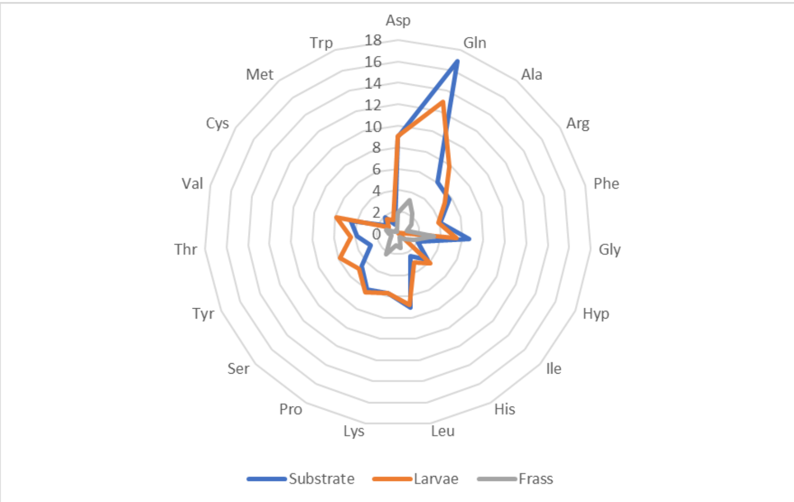

Figure 4. V50B50 group: comparison of essential amino acids contents between substrate, larvae and frass.

4. Discussion

Amino acids, the building blocks of proteins, are important molecules to produce fly larvae tissue, hormones, and transport proteins (Cohen, 2005). To ensure proper functioning, it is necessary to maintain physiological concentrations of amino acids and their metabolites. Based on dietary requirements for nitrogen balance or growth, amino acids are traditionally classified as essential or nonessential for humans and animals. Essential amino acids (EAA) are amino acids whose carbon skeleton cannot be synthesized or is not produced adequately by the body compared to needs, making their intake through the diet crucial to meet optimal needs (Wu et al., 2009). In general, insects contain high amounts of lysine, threonine, and methionine, which are major limiting essential amino acids in low-protein cereal- and legume-based diets for pigs and poultry (Verkerk et al., 2007-Van Huis et al., 2013). Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are considered EAA for insects (Cohen, 2005). Protein has been identified as the pre-eminent macronutrient in organic waste, exerting a significant impact on the process performance of black soldier fly larvae (Nguyen et al., 2013-Tinder et al., 2017). Many authors have studied the influence of rearing substrates on BSF protein content, while for the amino acid composition, studies in the literature are often inconsistent, and this is true also for other insect species (Gere et al., 2019-Fuso et al., 2021). Regarding the protein levels, our values obtained with V100 diet agree with other studies in the literature involving larvae raised on vegetable diets, reporting a range of 32-58% (Gold et al., 2018). In literature, few papers reported the use of butchery waste as a substrate for BSF. Regulation (EC) No 1069/2009 is a fundamental pillar for the management of animal by-products (ABPs) not intended for human consumption. Article IV of this regulation lists the ABPs that are prohibited for animal feed, thus establishing strict criteria to ensure food safety and public health. In the context of our research, we adopted an approach compliant with these regulatory provisions. The butchery wastes used as substrate were subjected to targeted treatments aimed at eliminating any presence of prohibited ABPs, thereby ensuring the integrity of the starting material. These treatments were implemented using advanced screening and sanitization techniques, aimed at removing any contaminants from the meat residues. It was crucial to pursue a preventive and proactive approach to ensure compliance with the regulation's provisions, to avoid any risk to food safety and consumer health. The careful selection of treatment methods allowed us to obtain a safe and contaminant-free substrate, ready to be used in the rearing of insects like *Hermetia illucens*. The use of meat from ruminants as feed is banned in many countries, but in another study the meat component was included in a substrate, based on fast food waste, to evaluate the feasibility of different waste streams to grow BSF, giving the best larval performance and composition (Naser El Deen et al., 2023). Our results agree with a report that demonstrated that larvae grown in a substrate with a high lipid content had a low protein content (in % of dry mass) (Barragán-Fonseca, 2018). In larvae, adipose tissue serves as a depot for nutrients such as proteins, carbohydrates, and fats, essential for growth and metamorphosis (Arrese et al., 2010- Hahn et al., 2008). However, a high amount of fat does not necessarily indicate a sufficient nutrient reserve, as insect lipid tissue contains trophocytes with lipid and protein droplets of various sizes and staining reactions, highlighting the complexity of its composition (Coppe Pimentel et al., 2017). Considering the different moisture levels in the tested substrates and that an equal amount of substrate has been supplied to the larvae in each trial, the grams of protein available for each group were 37.7, 26.2, 40.0, and 45.4 g, respectively for control, V100, V75B25, and V50B50 groups. In our trial, a high amount of available protein in the substrate did not correspond to a high amount of protein in the larvae, indicating that a well-balanced availability of other nutrients is important to improve the protein efficiency of the larvae. In agreement with our study if fed on a vegetable mix, BSF showed high amounts of aspartic acid, glutamate, and arginine (Cappellozza et al., 2019), but also of leucine (Spranghers et al., 2017). However, significant differences in the amino acid profile were observed according to the rearing substrate (Fuso et al., 2021).

Our results show that the levels of lysine and methionine were significantly higher in the larvae subjected to a V100 diet compared to other experimental groups. It is important to underline that the V100 diet has been observed as particularly efficient in the conversion of starting substrates into lysine and methionine, when compared with other dietary regimes. This suggests that the specific composition of the V100 diet, combined with the metabolic processes of the larvae, favored greater synthesis and accumulation of these essential amino acids. This confirms, in line with Cappellozza et al. (Cappellozza et al., 2019), that BSF larvae demonstrate a remarkable ability to adapt to various food sources, including plant waste. Methionine and lysine are very important for animal and human nutrition (Ozimek et al., 1985) and are considered limiting amino acids for pigs (Baker, 1996). Lysine is a key amino acid involved in protein synthesis, which is critical for muscle growth and body development; it is especially important in chickens and pigs, as their requirements for this amino acid are often higher than other animal species. Lysine deficiency can limit growth and meat production, negatively affecting the animal's performance (Baker, 1996). Methionine is another essential amino acid that plays a key role in protein synthesis and has crucial implications for animal metabolism and health; it is a source of sulfur, which is essential for the formation of keratin, a protein found in feathers and fur. Furthermore, methionine is involved in the regulation of lipid metabolism and may help improve feed efficiency (Baker, 1996).

It is interesting to note that in our trial V75B25 expressed the lowest protein content in larvae. This could be attributed to the specific composition of this diet, probably containing a suboptimal proportion of ingredients or substrates for protein synthesis, or negatively affecting the metabolic processes of the larvae. In C and V50B50, protein content was higher in frass than in the respective substrates. In this case, a key role could be attributed to decomposition processes and changes in the chemical composition in the digestive tract of the larvae. Although this study highlighted the role of BSF larvae in the management of food waste and in the development of protein alternatives, their application in the nutritional field is still in its infancy.

De Marco et al. (De Marco et al., 2015) showed that insect meals derived from *Tenebrio molitor* and *Hermetia illucens* are valuable sources of apparent metabolizable energy and digestible amino acids for broiler chickens. This further highlights the nutritional potential of insects in livestock production, emphasizing their ability to significantly contribute to the dietary needs of farm animals. Also, for fish, they are two of the most limiting amino acids whose deficiencies can lead to reduced growth and high mortality (Mai et al., 2022). Some authors (Fuso et al., 2021) demonstrated that the highest content of lysine in the insects can only be obtained when the rearing substrate has at least 90% dry matter of carbohydrates and a slightly higher amount of lipids compared to protein and fibers. Using a rearing medium consisting of 75% dry matter in carbohydrates leads to larvae with reduced levels of lysine. These considerations are in line with Addeo et al. (Addeo et al., 2021 a) who reported, even if with slightly lower data, higher percentages of carbohydrates (CHO) in the control and vegetable substrates (68.50 and 66.50% dry matter, respectively), compared to the groups V75B25 and V50B50, which had lower CHO percentages (43.24 and 33.52% dry matter, respectively). The amino acid composition of BSF larvae is similar to that reported in the literature for most amino acids, including those with relevance for animal feed, like lysine, isoleucine, threonine, valine, and methionine (Newton et al., 2005- Sealey et al., 2011). BSF larvae can be easily reared and propagated on different nutrient substrates (Lu et al., 2022). Substrate composition affects amino acid content in harvested larvae (Palma et al., 2020), and this was mainly shown for methionine content. BSF larvae kept on animal manure were lacking different essential amino acids, such as threonine, methionine, and cysteine (Caligiani et al., 2018), whereas larvae acquired sufficient threonine amounts when farmed on a standard diet (a plant-based material consisting of processed wheat (particle size: 50–2000 μm), supplemented with increasing quantities of seaweed (Liland et al., 2017). For this reason, it is important to investigate the optimization of substrates for BSF larvae. Our results confirm what was reported in a recent review on potential uses of BSF larvae and its nutritional composition in animal diets, methionine and tryptophan were considered the least abundant essential amino acids independently of the kind of substrate (Lu et al., 2022). In the case of almond by-products employed in the initial feedstock, it was demonstrated that, when non-fiber carbohydrates (as starch and sugar) decreased, methionine content increased (Palma et al., 2020).

BSF larvae are a complete and balanced protein source, capable of satisfying human and animal needs. Different studies have shown that BSF larvae guarantee optimal amounts of all the essential amino acids (EAA) to satisfy nutritional requirements of human adult, as in the case of histidine and tryptophan as established in the reference of FAO/WHO [40,44,51]. Furthermore, the amino acid composition of BSF larvae features the majority of amino acids, including those relevant to animal nutrition, such as lysine, isoleucine, threonine, valine, and methionine (Spranghers et al., 2017). Tryptophan can regulate feed intake, reproduction, immunity, neurological function, and anti-stress responses; additionally, tryptophan may modulate gene expression and nutrient metabolism to

impact whole-body homeostasis in organisms (Yao et al., 2011). The deficiency in methionine + cystine and threonine has also been reported in the amino acid profiles of BSF larvae (Makkar et al., 2014). Among the non-EAAs, glutamine was the most represented in V100, followed by aspartic acid 4.91 and 3.79 (g/100g dry matter) respectively, in accordance with the findings reported for other insect species (Addeo et al., 2021 b - Okaraonye et al., 2009). The high levels of aspartic and glutamic acids were responsible for the flavor in a variety of foods, including meat, cheese, seafood, and vegetables (Phat et al., 2016). During the waste decomposition process, BSFLs carry out an effective transformation of organic materials, generating components of considerable value such as amino acids, peptides, proteins, oils, chitin, and vitamins. This ability not only converts waste into useful resources but also plays a fundamental role as a control mechanism against pathogenic bacteria (Erickson et al., 2004 - Liu et al., 2008). Therefore, the use of organic waste from this perspective not only constitutes a sustainable nutritional option but also constitutes a significant contribution to the mitigation of environmental pollution (Erickson et al., 2004-Purba et al., 2020-Purba et al., 2021). Indeed, if larvae production is intended for feed or food, they cannot be fed on any waste, not even if it constitutes an important source of some nutrient molecule for the larvae, nor if particularly attractive for them, nor if they gain considerable weight or improve their nutritional profile. If, conversely, the larvae are used to produce energy, they can feed on any waste, and they can be very useful to mitigate pollution (Pas et al., 2022-Wedwitschka et al., 2023).
As part of our insect culture research, analyzing the quantities and availability of selected food by-products plays a crucial role in understanding their feasibility and applicability on a large scale. The selection of food by-products as nutrient sources for insects is a key element that directly affects the sustainability and effectiveness of this process (Addeo et al., 2024 a). Regarding quantities, it is essential to provide detailed data on supply metrics, considering both production batch sizes and replenishment frequency. This will allow us to evaluate the economic and logistical feasibility of insect cultivation on a large scale, providing clear indications on the adaptability of the process to a broader production context. Regarding availability, the description of the sources and methods of obtaining food by-products is essential. This includes assessing the stability of supplies over time and diversifying sources to ensure a reliable supply chain (Ortiz et al., 2016- Varelas, 2019). This approach aims to mitigate any risks associated with fluctuations in the by-product market, ensuring continuous and sustainable production over time. Detailed quantitative analysis of the quantities of food by-products used in the culture of *Hermetia illucens* provided promising results (Varelas, 2019). The specific amounts of waste used were shown to be effective in supporting healthy insect growth during the study period. Furthermore, the diversification of supply sources and attention to seasonality have guaranteed continuous availability of food by-products, crucial elements for a

future expansion of the cultivation process (Czekała et al., 2020). The effectiveness of this diet suggests that the process could be easily adapted and implemented on a larger scale without compromising the insects' well-being. Local accessibility of the selected plant food by-products was confirmed as a key element for the sustainability of the process (Czekała et al., 2020). These findings not only consolidate the validity of our conclusions, but also offer concrete perspectives for the future, indicating that our approach could be replicated on a large scale to contribute to a sustainable food waste management and insect biomass production initiative.

In the current study, the amino acid content of different types of frasses was lower than those determined in the frass included as feedstuff in the diet for juvenile channel catfish (*I. punctatus*) (Romano et al., 2023 a). These differences could probably be ascribed to the fact that the BSFL frass employed in that study was produced using wastes deriving not only from vegetables, as in our trial, but also from expired commercial fish diet. This diet, of fish origin, could have provided the content of lysine and the other EAAs higher than that detected in the frass assayed in our trial. Differently, frass obtained in our trial showed a content of EAAs higher than that employed in Florida Pompano (*Trachinotus carolinus L*.) diets, in which the frass was derived from a mixture made up of exclusively vegetable feedstuff as equal parts of corn, wheat, and soybean meal [65]. The good balance of amino acid profile of the substrates/frass used in our trial also emerged comparing the content of the frass, produced from the larvae of black soldier flies fed dried distillers' grains and employed for Pacific white shrimp (*Litopenaeus vannamei*) feeding (Yildirim-Aksoy et al., 2022). In summary, the results of our study contribute to the detailed understanding of the complex relationship between substrate, amino acid composition of BSF larvae, and their potential use as a nutritional resource. This information could be key to developing sustainable farming practices and maximizing the use of BSF larvae in various contexts.

5. Conclusions

The black soldier fly could be in feed production, owing to its advantageous physiological and behavioral attributes, along with the optimal nutritional characteristics exhibited by its larvae. Notably, a vegetable-based diet has been observed to enhance the protein content in larvae, whereas substituting vegetable proteins with animal counterparts results in a decline in protein levels, indicating a discernible minimum protein threshold within the diet. Suboptimal nutritional content in utilized leftovers correlates with reduced protein production. Threonine and lysine emerge as the primary limiting amino acids in larvae reared on vegetable waste, alignprepuping with established amino acid profiles in the literature. Consequently, the cultivation of larvae on diverse substrates necessitates meticulous control over waste ingredient proportions to attain a standardized product applicable not only in animal feed but also in human nutrition. Moreover, given the anticipated expansion of insect farming, insect frass assumes significance as a sustainable resource capable of enhancing plant nutrition and promoting health in sustainable crop systems. It represents a promising alternative to chemical fertilizers, contributing to the "closing of the circle" in the context of the circular economy, particularly in scenarios of mass insect production where waste accumulation is a concern. Analogous to the manure derived from zootechnical animals, the utilization of insect frass in sustainable agriculture offers nutrients, beneficial microorganisms, and valuable biomolecules for soil enhancement. Furthermore, the environmental impact of using insect droppings as fertilizer extends beyond their nutrient content. Helps reduce dependence on synthetic fertilizers, which are often associated with environmental degradation. Integrating insect excrement into agricultural practices is in line with the principles of sustainable agriculture by recycling waste materials into valuable inputs, thus improving soil health and fertility. Nonetheless, to fully exploit the potential of insect excrement in agriculture, comprehensive investigations are imperative to delineate the advantages and constraints associated with the application of frass in agricultural practices. Research should focus on the long-term effects of excreta on soil properties, crop yield and quality, as well as its interaction with existing soil microbiomes. Furthermore, studies should evaluate the economic feasibility and scalability of using insect excrement in different agricultural contexts. By addressing these research gaps, the agricultural sector can develop best practices for the effective use of insect excrement, ensuring they become a reliable and sustainable component of modern agricultural systems. In conclusion, the use of black soldier fly larvae in feed production and their excreta in sustainable agriculture represents a multifaceted approach to addressing global challenges in food security, waste management and environmental sustainability. The ability to transform organic waste into fertilizers rich in high-quality proteins and nutrients offers a circular solution that supports both animal and plant production systems. As the world moves towards more sustainable practices, the black soldier fly and its byproducts could play a critical role in shaping the future of agriculture and food production, fostering a more resilient and sustainable ecosystem.

6. References

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CHAPTER 3

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Mineral profile and heavy metals bioaccumulation in black soldier fly (*Hermetia illucens*, L.) larvae and frass across diverse organic substrates

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Abstract

Organic wastes from vegetable mix (V) and butchers (B) were used as substrates (V100%, V75%þB 25%, and V50%þB50%) for *Hermetia illucens* to assess the mineral profile of the larvae and frass, and the potential bioaccumulation of toxic elements (As, Cd, Pb, Hg). The V100 larvae had the highest concentrations of all minerals, except for Se and P, higher in the control group (poultry diet as substrate) (p<.01). The V50B50 and V75B25 larvae showed lower (p<.01) mineral concentrations than the other groups. The V50B50 group had the highest (p<.01) bioaccumulation factor (BAF) for Cd (8.52%), the V100 for Pb (p<.01). The equations to estimate the mineral content of larvae from the substrate were significant only for Ca and Mg (p<.05). The V100 frass had the highest (p<.01) levels of As, Cd, Pb, Fe, K and Mg, while the control group showed the highest (p<.01) levels of Se, Zn, Ca, P and K. Toxic elements can accumulate in black soldier fly larvae and frass, with the exception of Hg. However, organic wastes from V and B had a very low content of toxic elements and that strongly reduced the risk of excessive bioaccumulation in larvae. Indeed, the content of toxic elements in larvae is lower than the maximum values established for feed materials by EU regulations. In conclusion, organic substrates from vegetables or butchery wastes can be used for BSF larvae growth without the risk of accumulation of toxic elements.

1. Introduction

Over the last decade, interest in insect production as a way to address the shortage of protein resources for humans and animals has increased, due to the expected future increase in the world's population (FAO 2021; Secci et al. 2022). In addition, recent global crisis (COVID-19 pandemic, Russian-Ukrainian war) have drawn attention to the potential difficulties obtaining raw materials and energy, and increased the overall cost of human food and animal feed (Attia et al. 2022). Particular attention has been directed towards some bio-converting insects, in particular the black soldier fly (BSF, *Hermetia illucens*, L.), which is able to rapidly convert a wide range of organic wastes into a biomass (larvae) rich in both proteins and lipids (Scieuzo et al. 2023), as well as in bioactive compounds such as chitin, lauric acid and antibacterial peptides which might positively influence the intestinal morphometry and health of animals (Saviane et al. 2021; Candian et al. 2023). After harvesting of these larvae, the residual frass (containing insect exuviae and excrements, as well as residual substrate) could be used as a fertiliser, or to produce biogas or biofuel (Carroll et al. 2023; Wedwitschka et al. 2023; Mohan et al. 2023). In the literature, growth performance (Marono et al. 2017), chemical-nutritional characteristics (Marono et al. 2015), the potential use of insect products as a source of bioactive compounds (Borrelli et al. 2017, 2021), and the possible applications of insect frass (Carroll et al. 2023; Wedwitschka et al. 2023) have been investigated. However, there are very few research investigating the mineral content of insect larvae and frass in relation to the abundance of the corresponding minerals in the growth substrate (Proc et al. 2020) or in relation to the type of substrate. Today, when the introduction of insects into the food chain is being considered, it is vital to determine all the possible risks associated with their consumption, in particular when different growth substrates were used. In fact, in order to support the impetus to develop a circular economy, several researchers have focused their attention on the use of organic wastes for *H. illucens* larvae growth. The Regulation (EC) No 767/2009 prohibits the use of 'unsafe' rearing substrates such as manure, separate digestive tract contents, catering wastes and processed animal proteins (very often the substrates preferred by BSF adults for oviposition, according to Boafo et al. 2023) to reduce microbiological hazards, including prions. However, additional possible hazards have thus far been underestimated. Organic wastes often contain persistent pollutants such as heavy metals, which could accumulate in the larvae and frass entering the food chain and the environment. Heavy metals enter the waste stream in a variety of ways, whether through atmospheric emissions or improper disposal of waste containing heavy metal. The result is that terrestrial organisms may then ingest these contaminants resulting in greater environmental hazard (Diener et al. 2015).

Starting from the hypothesis that, in general, minerals can accumulate in BSF larvae in function of the correspondent amount in the substrate, the first aim of this study was to explore the possible relationship among mineral concentrations in larvae and frass and the associated substrate, none of which have been thoroughly examined previously. A further aim was to calculate the potential bio-accumulation of toxic elements in the larvae to identify and assess possible risks to animal and human health. For this purpose we tested substrates including wastes from vegetable and butchery markets, which are readily available throughout the year.

2. Material and methods

2.1 Substrates

Four substrates were used: broiler feed, as a control diet; a vegetable diet (V100); a diet comprising of 75% of vegetable diet þ25% of butchery wastes (V75B25); and a diet consisting of 50% of vegetable diet þ50% of butchery wastes (V50B50). The vegetable diet consisted of a mix of vegetable wastes containing 75% vegetables (broccoli 40%, celery 35%, cabbages 25%) and 25% fruits (50% oranges and 50% apples). The butchery wastes were obtained from butchers in the province of Napoli and mainly consisted of fat and meat resulting from the trimming of bovine carcases and cuts of meat. The collected vegetable wastes were stored for two days at room temperature to reduce the water content, while the butchery wastes were enclosed in dark bags and stored at ~ 2°C. Prior to use, the butchery waste was left at room temperature for approximately 2h. The vegetables and butchery wastes were also cut into small pieces and mixed to prepare the larvae substrate as described above. A batch feeding strategy was applied, meaning the substrates were placed in plastic containers (60cm x 40cm x 15cm) one day prior to placing the larvae. This allowed the substrates to heat up before the start of the experiment. The scheduled feeding rate (amount of substrate/ animal/day) was 0.075g of DM/larva/day.

2.2 Larvae and experimental groups

Five-day-old black soldier fly larvae (*Hermetia illucens*) were purchased from the commercial insect rearing company Smart Bug's (Treviso, Italy) in February 2020 and used in a growing trial. Up to 5days of age the larvae were fed a standard Gainseville diet. Each group (Control, V100, V50B50, and V75B25) consisted of 9 replicates, each placed in a plastic tray, resulting in a total of 36 trays. In each replicate, 10kg of substrate was placed. On the top of each substrate, 6000 larvae were transferred after weighing. The trays were covered with a perforated cap with a black nylon grid and then placed in a ventilated chamber (air flow around 2m/s) under controlled environmental conditions (T: 27±0.5°C; RH: 70±5%; L:D photoperiod: 16:8). More details concerning the experimental design are reported by Addeo et al. (2021). All the groups reached the end of the experiment (25% of prepupae) after 17d, when the larvae were 22d old.

At the end of the growing period, the larvae were separated from the frass and fasted for 24h. Samples of larvae and frass were then collected from each replicate, freeze-dried and stored at room temperature until analysis.

2.3 Chemical analysis

The samples of the substrates (*n*¼4), larvae (*n*¼9 per group) and frass (*n*¼9 per group), were analysed to

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determine their chemical composition in triplicate, according to the AOAC (2005) standard methods. For the larvae only, the calculation of the protein content was performed using with anitrogen-to-protein conversion ratio of 4.76, as indicated by Janssen et al. (2017). The samples of butchery waste were also analysed to determine water, protein and lipid contents.

2.4 Mineral profiles

For the mineral profile, before ICP-OES analysis, the sample (0.5*g*±0.02) was placed in a Teflon vessel with 7.0ml of 65% HNO_3 and 3.0mL of 30% H_2O_2 (Romil UpA). The vessel was sealed

and placed in a microwave assisted digestion system (Milestone, Bergamo, Italy). Microwave assisted digestion was performed using a mineralisation program for 50min at 200°C. The vessel was then cooled to 32°C, and the digestion mixture was transferred to a 50.0mL flask and the final volume was obtained by adding Milli-Q water (Ariano et al. 2019). Subsequently, the mineralised samples were subjected to a filtration process using 45mm SY25TF (PTFE) filters.
CETAC U5000AT (ThermoFisher Scientific, Waltham, MA, USA) and Perkin Elmer Optima 2100 DV (Perkin Elmer, Inc., Wellesley, MA, USA) instruments were used to detect the concentrations of trace elements (ICP- OES technique), while the purity of the chemicals was assessed by running two blanks and the calibration curve during each set of analyses. For quality control purposes, reference materials (BCR-422 cod muscle, IRMM Institute for Reference Materials and Measurements, DORM-2 fish protein, National Research Council, Canada), with values resulted all within certified limits, were included.
The Perkin Elmer ICP application study number 57's protocol (Barnard et al. 1993) was followed to assess instrumental detection limits, which were then expressed in wet weight.
Participation in interlaboratory studies, as organised by the Food Analysis Performance Assessment Scheme (Sand Hutton, UK) allowed the method's performance assessment.

2.5 Minerals bioaccumulation and rate of absorption

It is widely acknowledged that 'bioaccumulation' describes the accumulation and enrichment of contaminants in organisms, relative to those in the environment. Then, the bioaccumulation factor (BAF) for the toxic elements (As, Pb, Cd and Hg) in the studied larvae was calculated according to a dry mass basis using element concentrations determined by ICP analysis and defined according to the equation proposed by Walker (1990) as follows:

BAF¼concentrationoftheelementinthelarvae=concentrationoftheelementinthesubstrate*:*

2.6 Statistical analysis

Data were subjected to a one-way analysis of variance, using the procedure GLM of SAS (2002), according to the following model: Yij¼m þSiþeij, where Y is a single observation, m is the general mean and e is the error. The experimental unit was the replicate. To assess the differences among the means, Tukey's test was used (SAS 2002). The relationship among the mineral content of the larvae and frass and the substrate was studied using the PROC REG of SAS (2002), by comparing the average value of the 9 replicates for larvae and frass with the corresponding content in the substrate (*n*¼4). *p* values lower than .05 are considered significant, while *p* values ranging between .05 and 1 are considered as a tendency.

3. Results

The water contents of the vegetable and butchery wastes were 85.37±1.12% and 38.92±2.85%, respectively. The protein and lipid contents of the butchery waste on a dry matter basis were 7.12±0.65 and 42.23±1.33, respectively. The V100 substrate (Table 1) had the highest concentrations of heavy metals (As, Cd, Pb), and it was the only substrate containing traces of Hg. The V100 substrate was also the richest in Fe and P. The substrates including butchery wastes showed the lowest Mg values. The mineral profile of the BSF larvae (Table 2) showed that animals on the V100 had the highest concentrations of all the minerals studied, except for Se and P, which had the highest levels in the larvae from the control group (p<.01). In general, groups V50B50 and V75B25 showed lower (p<.01) mineral concentrations than the other groups.

The bio-accumulation factors for the toxic elements in BSF larvae as affected by the 4 tested substrates are reported in Table 3. Excluding Hg, the highest BAF was obtained for Cd in the V50B50 group (8.52), and the lowest (p<.01) for As in the V75B25 group (0.92). The V100 group had the highest BAF for Pb, followed by both the control group and the V50B50 and V75B25 groups combined (p<.01).

Of the heavy metals, As, Cd, and Pb showed a positive correlation with the respective content in the substrate, as did Fe, Ca, P and Mg while for Se, Zn and K the relationship with the substrate was negative (Table 4). However, the equation regression was significant only for Ca and Mg (p<.05) and the amount of the minerals in the substrate can explain the 97.83% and the 93.71% of the mineral content in the larvae, respectively.

In general, the frass obtained from V100 had the highest (p<.01) levels of the heavy metal elements (As, Cd, Pb) and of Fe, K and Mg, while the control group showed the highest (p<.01) levels of Se, Zn, Ca, P and K (Table 5).

Table 6shows the equations to estimate the content of a specific mineral in the frass, from the corresponding levels in the larvae and substrate. The estimations reached the significance (p<.05) only for Cd and Pb. However, the *r*2 values were higher than 98% for almost all the heavy metals studied, with the exception of the Zn.

Table 7shows the maximum levels set by the European Commission (2002) for As, Cd, Pb and Hg in feed, and compared them to the medium and maximum levels detected in our larvae samples. Compared to the maximum levels for heavy metals set by the EU Commission for feed materials, our samples were always resulted lower than the maximum values.

Table 1. Chemical characteristics and mineral profile of the 4 substrates used for black soldier fly larvae growing.

	Control	V100	V50B50	V75B25
Moisture, %	70.05	85.37	63.65	70.13
Ash, % DM	3.69	10.41	1.84	3.64
Lipids, % DM	5.12	3.98	49.76	36.97
Protein, % DM	22.69	19.11	14.88	16.15
As, lg/kg	0.51	1.95	0.45	1.00
Cd, lg/kg	1.01	3.53	0.35	0.45
Pb, lg/kg	1.25	6.45	1.95	3.35
Hg, lg/kg	0	0.21	0	0
Fe, lg/kg	2013	3512	1147	2069
Se, lg/kg	2.11	1.95	2.70	2.55
Zn, lg/kg	1025	764.50	353.50	1366
Ca, mg/kg	12.60	21.70	21.90	26.50
P, mg/kg	119	131.50	27.50	36.50
K, mg/kg	141	1125	131.5	158
Mg, mg/kg	56.10	45.60	8.05	14.20

V100: vegetable mix diet; V50B50: diet consisting of 50% of vegetable diet þ50% of butchery wastes; V75B25: diet consisting of 75% of vegetable diet þ25% of butchery wastes.

Table 2. Toxic, micro and macro elements of black soldier fly larvae raised on the 4 different substrates

	Control	V100	V50B50	V75B25	RMSE	*p*
Toxic elements						
As, lg/kg	1.19b	4.61a	0.72b	0.92b	0.53	<.0001
Cd, lg/kg	6.56b	7.20a	2.98c	3.20c	0.35	<.0001
Pb, lg/kg	3.63b	25.88a	3.24b	4.87b	2.42	<.0001
Hg, lg/kg	0b	0.12a	0b	0b	0.05	.0004
Micro and macro-elements						
Fe, lg/kg	2476b	11,861a	2823b	1356b	2139	<.0001
Se, lg/kg	11.45a	7.42b	2.88d	4.23c	0.61	<.0001
Zn, lg/kg	2132b	2746a	1438c	1097d	191.90	<.0001
Ca, mg/kg	135.70c	503.50a	87.33c	205.00b	42.11	<.0001
P, mg/kg	196.70a	158.60b	103.80c	102.30c	9.88	<.0001
K, mg/kg	257.30a	240.90a	161.20b	173.30b	13.43	<.0001
Mg, mg/kg	69.50a	67.59a	43.27b	39.10b	3.49	<.0001

a,b,c: $p<.01$; RMSE: root mean square error. V100: vegetable mix diet; V50B50: diet consisting of 50% of vegetable diet þ50% of butchery wastes; V75B25: diet consisting of 75% of vegetable diet þ25% of butchery wastes.

Table 3. Bioaccumulation factor for toxic elements in black soldier fly larvae raised on the 4 tested substrates.

	Control	V100	V50B50	V75B25	RMSE	*p*
As	2.83a	2.37a	1.59b	0.92c	0.30	<.0001
Cd	6.57b	2.03c	8.52a	7.11b	0.59	<.0001
Pb	2.90b	4.01a	1.66c	1.45c	0.55	<.0001
Hg	0b	0.62a	0b	0b	0.25	.0004

Table 4. Equations to estimate mineral concentration in black soldier fly larvae (Y) from that of substrates (X).

Y	X	Intercept	*r2*	SE	*p* value
Toxic elements					
As	þ0.35	þ0.32	0.86	0.83	.0711
Cd	þ0.54	−1.36	0.63	1.63	.2031
Pb	þ0.19	þ1.39	0.89	3.69	.0515
Micro and macro-elements					
Fe	þ0.17	þ1387	0.73	3089.80	.1451
Se	−0.07	þ2.82	0.66	2.71	.1861
Zn	−0.14	þ1133	0.05	873.10	.7590
Ca	þ0.50	−39.11	0.97	33.68	.0110
P	þ1.07	−73.32	0.81	24.01	.0901
K	−0.15	þ168.25	0.15	54.02	.6051
Mg	þ1.42	−47.36	0.93	4.88	.0320

r2: coefficient of determination; SE: standard error.

Table 5. Toxic, micro and macro elements measured in residual frass obtained at the end of growing period of black soldier fly larvae on the 4 tested substrates

	Control	V100	V50B50	V75B25	RMSE	*p*
Toxic elements						
As, lg/kg	1.64b	5.10a	0.72c	1.73b	0.39	<.0001
Cd, lg/kg	1.12b	1.52a	0.32c	0.41c	0.08	<.0001
Pb, lg/kg	6.86b	43.17a	5.33c	7.73b	2.56	<.0001
Hg, lg/kg	0	0.10	0	0	0.08	.1267
Micro and macro-elements						
Fe, lg/kg	3134b	23.57a	2033b	4937b	2163	<.0001
Se, lg/kg	11.20a	5.00b	2.57c	4.72b	0.88	<.0001
Zn, lg/kg	1926a	1358b	728.70c	1033c	74.09	<.0001
Ca, mg/kg	333.80a	155.80b	20.34d	58.70c	19.18	<.0001
P, mg/kg	192.40a	141.00b	57.42c	129.00b	11.43	<.0001
K, mg/kg	319.10b	1227a	127.40c	350.30b	23.15	<.0001
Mg, mg/kg	82.25b	95.83a	23.69d	51.82c	5.09	<.0001

a,b,c,d: $p<.01$; RMSE: root mean square error. V100: vegetable mix diet; V50B50: diet consisting of 50% of vegetable diet þ50% of butchery wastes; V75B25: diet consisting of 75% of vegetable diet þ25% of butchery wastes.

Table 6. Equations to estimate mineral content of residual frass (Y) fom that of substrate (X) and black soldier fly larvae (Z).

Y	X	Z	Intercept	*r2*	SE	*p* value
Toxic elements						
As	0.93	0.70	0.08	0.98	0.39	.1173
Cd	0.09	0.21	−0.32	0.99	0.03	.0311
Pb	2.81	1.53	−11.96	0.99	1.67	.0490
Micro and macro-elements						
Fe	4.19	1.31	−6790.60	0.98	2198.70	.1247
Se	5.36	1.33	−15.21	0.98	0.83	.1292
Zn	0.67	0.50	−255.10	0.64	532.40	.5999
Ca	−8.40	4.27	−198.00	0.70	130.50	.5387
P	−0.09	1.13	−21.95	0.72	50.21	.5217
K	−0.08	1.10	59.69	0.24	150.60	.8661
Mg	1.45	−0.32	35.94	0.81	23.73	.4250

r2: coefficient of determination; SE: standard error

Table 7. Comparison of the heavy metal levels of our samples with the maximum limits imposed by the European Commission in feed.

Heavy metal	Maximum content in lg/kg relative to a feed	Our results – medium levels (lg/kg)	Our results – higher levels (lg/kg)
As	2,000	2	6
Pb	5,000	11	30
Cd	500	5	8
Hg	100	0	0

4. Discussion

Black soldier fly larvae and frass are of considerable interest to the industry as the larvae can be used as either animal feed or food, and the frass to amendment for soil. In both contexts, the study of minerals accumulation from the growing substrate is a pivotal point. Considering macro and micro-minerals, it is important to determine if the insect products used for humans, animals or plants can be able to satisfy the mineral requirements. In addition, heavy metals are among the main contaminants of our food supply, and represent a serious issue for the ecosystem (Korish and Attia 2020; Mehar et al. 2023). Chronic exposure to toxic elements in human and animals can cause nephron, neuro, hepato, skin, and cardiovascular toxicity (Mitra et al. 2022). In addition, high levels of toxic elements in residual frass, used as plant amendment, can contribute to introduce these elements into the food chain, increasing the risk of chronic exposure for humans, and also having a direct effect on plants. In fact, heavy metal toxicity is one of the principal abiotic stressors on plants, affecting their growth and metabolism (Saxena and Shekhawat 2013).

The present research provides a unique contribution to the field, assessing the relationship among mineral concentrations in BSF larvae and frass and the associated substrate, which has not been thoroughly examined previously, to the best of our knowledge. Our research confirmed that the mineral profile of black soldier fly larvae is affected by the growing substrate, despite other factors also being involved in determining the bio-accumulation factor of the toxic elements.

Of the minerals most studied in insects, the toxic elements (Cd, As, Pb, Hg) play a pivotal role. In our trial, Cd showed the highest BAF, with an average value of 6.06, followed by Pb (2.51) and As (1.93). The Hg was not detected in any of the substrates used in the present trial and, when measured (as in V100 substrate), the BAF value in the corresponding larvae was less than 1, indicating no hazards were associated with possible accumulation, probably due to the very low levels contained in the V100 substrate. These results are in partial agreement with Proc et al. (2020) who found higher BAF values in BSF larvae for Cd than for Hg, despite the same authors founding no bio-accumulation for Pb and As. It has been reported that Cd has high toxicity for humans and animals, with long-term oral exposure having negative effects on several organs (kidney, liver, lung, bone, immune system, blood, and nervous system) and inducing the development of cancerous growths (Huff et al. 2007). Interestingly, the Cd levels found in our trial are lower than those detected in other studies examining edible insects (from 110 to 190lg/kg in the trials by Truzzi et al. 2019; Bessa et al. 2021): in the present trial the Cd concentration in the larvae ranged from 2.98 to 7.20lg/kg. This is probably associated with the very low level of this toxic element in the growing substrates. However, the BAFs calculated for Cd (ranging from 2.03 to 8.52) did align with those noted in other studies (Truzzi et al. 2019; Van der Fels-Klerx et al. 2020), confirming that Cd has a high level of bio-accumulation in BSF larvae and, in general, its accumulation showed a positive relationship with the level of Cd in the growing substrate (X coefficient þ0.5422, *r*2 ¼0.6349). In the case of some insects, like *H. illucens*, Cd can be absorbed by cells through Ca2þchannels due to its similarity to Ca2þ(Braeckman et al. 1999). In addition, the HSP70 protein family can be synthesised in dipterous cells as a consequence of the elevated Cd content. This protects other proteins from Cd interaction, and these mechanisms may explain the elevated Cd concentration in BSF larvae (Hu et al. 2023).

In disagreement with Proc et al. (2020), the bio- accumulation of lead occurred in all the tested groups, without a significant relationship between lead levels in the substrate and the larvae. This result can be attributed to the physiological metabolism of this element in some insects: in fact, Pb accumulates in the exoskeleton of the BSF larvae (Wang et al. 2018); so that the moulting could be considered a strategy to reduce the Pb concentration (Hu et al. 2023) in the insect body, as, unlike Cd, there are no active channels for Pb absorption. In our trial, the levels of lead detected in the BSF larvae were positively related to its content in the substrate with a *p* value tending towards significance (*r*2 ¼0.8906, *p*¼0.0515). In addition, the levels of lead in the residual frass were positively related to lead content in both the substrate and the larvae with a high determination coefficient (*r*2 ¼0.9937, *p*¼0.0490). Very interesting, even if V50B50 and V75B25 groups had different concentrations of lead in their growing substrates, the BAF coefficient was not different between the two groups. Looking at the growth performance of the larvae, as reported in previous research by Addeo et al. (2021), at the end of the trial, the larvae from the V75B25 group had a higher live weight than the V50B50 group (0.2163 vs 0.2047g, *p*<.01). Thus, our hypothesis is that the high growing rate of V75B25 group was responsible for the more effective elimination of the lead from the insect body. This hypothesis is further confirmed by the Pb levels in frass which are higher in the V75B25 than in V50B50 group.

The bio-accumulation of As occurred in almost all the groups with the exception of V75B25, the concentration of which in the larvae reflected that in the substrate (*r*2 ¼0.8626, *p*¼.0711), according to Biancarosa et al. (2018). However, the bio-accumulation of the arsenic in the BSF larvae was lower than that of other toxic elements. Lievens et al. (2021) reported that arsenic concentrations in BSF larvae decreased by starving, indicating that the measured As level could be linked to its presence in the gut contents and had not accumulated in the organism. This assertion suggests that probably As is unlikely to constitute a chemical hazard if the larvae are starved (Van Der Fels-Klerx et al. 2016). However, our results conflict with this finding as the larvae were starved at the end of the trial and a bio-accumulation of As occurred. This indicates that further study of As accumulation in BSF larvae is required.

In insects, Iron can be considered both an essential nutrient and a strong toxin; it can be stored in the insect body linked to the ferritin protein and is responsible for the larvae's colour (Janssen et al. 2019). Nairuti et al. (2022) observed that BSF larvae can accumulate high levels of Iron, relative to the level of this element in the growing substrate. However, considering our results suggest it is not only the levels of Fe in the substrate that regulate the concentration of Fe in BSF larvae, rather it is the form of Iron in the substrate that is crucial, as BSF larvae can only utilised some forms of Iron. In vegetables, Iron is in a non- haem form, and in general ferric ions that must be reduced by some mechanism in the mid-gut lumen of insects prior to uptake; in contrast, in substrates of animal origin Fe is in a haem form (Gorman 2023). In our trial, V100 larvae had the highest concentrations of Fe, however, the levels of iron in the frass obtained from V100 groups were more than double compared to that of other. The V75B25 group showed the second highest content of Iron, with its accumulation in the frass being 3.64 times greater than that in the larvae, suggesting different sources of the substrate can affect the utilisation of this element, even if, as indicated by Bonelli et al. (2020) a diet-dependent adaptation processes in the BSF mid-gut can ensure the exploitation of the substrates, in particular when there is a deficiency of a particular nutrient. In our trial, no significant relationship was observed between the growing substrate and larvae or among frass, larvae and substrates for iron.

Few studies are available in the literature that consider the concentration of Se in BSF larvae and frass. Selenium is an essential element, for both animals and humans (Attia et al. 2010; Adadi et al. 2019), as it is involved in the functioning of several enzyme systems (Constantinescu-Aruxandei et al. 2018). Ferrari et al. (2022), evaluated the possibility of producing BSF larvae fortified with Se, and observed that its accumulation in larvae was linked to the form of Se (organic or inorganic) in the substrate. They concluded that a diet enriched with inorganic selenite caused an increased Se levels in the BSF prepupae. Further investigations are mandatory to establish whether our results may be affected by the different form of Se in the different substrates.

Zn showed high concentrations in the BSF larvae. However, its levels in the substrates showed a trend (V75B25>control>V100>V50B50) that was completely different from that observed in larvae (V100>control>V50B50>V75B25) and frass (control>V100>V75B25>V50B50). This result agrees with the finding of Diener et al. (2015) which suggested active regulation of Zn absorption in the insect body. This element (Zn) is a mineral essential for insect metabolism, so when concentrations of this heavy metal in the substrate are low, the BSF larvae, similar to other insects, are able to increase their uptake from the substrate (Bohm et al. 2023). Looking at the estimation equation for Zn from substrate to larvae, even if the significance for the $R2$ value is not reached, the estimated coefficient for the larvae value is negative, reinforcing the negative relationship between substrate Zn and larvae Zn.

According to Proc et al. (2020) the concentration of all the minerals in the residual frass is higher than in the corresponding substrate and, in some cases, higher than that recorded in the larvae at the end of the growing period. The frass, which is the residue at the end of the larval growth period consists of residues of unused substrate, larvae faeces and exuviae. Therefore, we can observe three active mechanisms that regulate the amount of a single mineral in the frass: (1) the 'concentration' of a specific mineral due to the reduction of water and other nutrients; (2) the reduction of the mineral due to the accumulation in the insect body; (3) the increase of a specific mineral due to its elimination through insect excrements and exuviae. The balance among these three factors can explain the different behaviours of a specific mineral considering its concentration in feed, larvae and frass. With regard to the BSF larvae, there are contradictory results in the literature. For example, Romano et al. (2022) observed that when feeding BSF larvae on orange, banana and combinations of these, Ca was higher in the frass than in the BSF larvae due to the presence of the accumulated exuviae in the residual material. Conversely, Proc et al. (2020) observed higher contents of Ca in larvae than in frass when grown on fish feed. In our trial, Ca levels in the BFL larvae were positively and significantly (*r*2 ¼0.9783, *p*¼.0110) related to the growing substrate, while no significant relationship was noted among Ca levels in frass, larvae and substrate; even if in the estimation equation, the coefficient for the substrate was negative. Similar to Pb, the Ca is accumulated in the insect exoskeleton but the role of Ca differed completely from the toxic element. Paul et al. (2017) observed that an increase in the Ca concentration in the substrate was directly linked to increased Ca accumulation in the BSF larvae due to its future role in pupation.

In the case of Se and P, very few studies have focused on concentrations of these elements in BSF larvae. In our trial, the V100 substrate showed the highest level of P, while in both the larvae and the frass the highest concentration of this element was detected in the control group. This result can be explained by the different form of P in the substrates. In fact, in vegetables, the phosphorous has an inorganic form, which is in general linked to phytic acid and has low bioavailability for animals. The control group was also grown on a vegetable substrate (poultry feed), but it is normally supplemented with minerals, and in particular P to guarantee a high bioavailability for animals. In general, the amount of P in the frass is lower than that in the larvae and this is very interesting considering the possible applications of frass as a soil amendment. The relationship between P in the growing substrate and larvae is positive and tends to the significant (*r*2 ¼08164, *p*¼.0901).

According to the findings of other authors (Romano et al. 2022), the BSF larvae showed a very poor concentrations of K, which is confirmed as one of the few minerals whereby the concentration in frass is generally lower than in larvae.
The concentration of Mg in the BSF larvae showed a positive relationship with its content in the substrate (*r*2 ¼0.9371, *p*¼.032), suggesting it is possible to modulate the Mg content in BSF larvae and explaining the differences in Mg levels in different trials on BSF larvae.

5. Conclusions

The current study showed that toxic elements can be accumulated in black soldier fly larvae and frass, even if their concentrations are present in very small amount in the substrates, with the exception of Hg. The relationship between element contents in the larvae and the substrate was significant for only Ca and Mg and tended to a significance for As, Pb and P; this point needs further deep investigation by increasing the number of tested substrates. The same consideration can be applied to the relationship among frass, larvae and substrate which was significant only for Cd and Pb. However, the hazards in the use of organic wastes obtained from products intended for human consumption is very low due to the very low contents of toxic elements in these substrates, in compliance with EU regulations. Although further studies on a greater number of samples are needed for the assessment of heavy metals and other pollutants on the environment bio-accumulation. Concerning Pb, Cd, Hg and As concentrations, these preliminary results support the posibility to use these analysed samples for animal feed with no additional hazards.

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CHAPTER 4

New life for expired wet cat food through black soldier fly larvae bioconversion

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Abstract
The aim is to evaluate the growth and development of *Hermetia illucens* (black soldier fly) larvae using four different substrates, including three expired wet cat foods (beef, chicken, salmon) and a commercial broiler diet as a control, exploring the great potential of this insect in a bioconversion context. The expired cat foods, obtained from a local supplier, were dried to a moisture content of around 70% before being used. The trial included 1,200 larvae per tank, with each group having six replicate tanks. Larvae were monitored for growth, development, and prepupae formation over a 15-day period. Growth parameters, such as larval weight, length, mortality, substrate reduction, and waste reduction index, were measured. The larvae fed on salmon and the control diet reached prepupae at day 20, while the beef and chicken groups reached this stage at day 22. After the trial, larvae were freeze-dried and analyzed for chemical composition, including crude protein, lipids, and ash content. The study aimed to explore the feasibility of using expired cat food as a sustainable substrate for larval growth, contributing to waste reduction in the pet food industry.

1. Introduction

In Europe the global population of pets is estimated in 340 million animals with 26 % of dogs and 25 % of cats (Fediaf, 2023). The annual sales of petfood products are estimated around 10.9 million tons, with an average value growth rate of the pet food industry in the last 3 years of 5.1 % (Fediaf, 2023). According to the annual report of Assalco (2023), in Italy, a population of 8.76 ml of dogs and 10.23 ml of cats is estimated in 2022 and, in the same year, the market of dog and cat food developed a turnover of 2,759.5 million euros for a total of 673,449 tons sold, with further growth prospects for the coming years.

With a view to global market growth, it is reasonable to expect an increase in the waste from this industry, also in form of expired products. Standing our knowledge, no data is available in the literature regarding the quantity of pet food discarded because it has reached or exceeded the "best before date" shown on the packaging; however, these quantities could likely be quite high, becoming a waste. The disposal of expired dog food can be a problem for both manufactures and retail stores (Xu and Li, 2012). A possible re-utilisation and valorization of these wastes, particularly rich in protein and other macro and micro-nutrients, could be their use as a growing substrate for bio-converter insect larvae, such as *Hermetia illucens*.

The Regulation (EC) No 767/2009 prohibits the use of 'unsafe' rearing substrates for insect farming, including processed animal proteins that are largely used for pet food production and are, in general, among the substrates preferred by black soldier fly larvae (Boafo et al. 2023).

According to the European indications, the date of minimum durability ("best before") reported in the food packages indicates the end of the period under any stated storage conditions, during which the product will remain fully marketable and will retain any specific attributes for which tacit or express claims have been made (Soethoudt et al. 2013). However, beyond the best before date, the food may still be perfectly satisfactory under a nutritional and safety point of view.
Black soldier fly is a powerful bio-converting insect, able to use a wide range of substrates to produce a larval biomass rich not only in protein and lipids, but also in bioactive compounds such as chitin, lauric acid and antimicrobial peptides with possible uses in animal nutrition but also in different kinds of industry (Scieuzo et al., 2023; Addeo et al., 2024).
In order to contribute to the sustainability of pet food industry, and to explore new potential substrates for H. illucens growth, the aim of our research is to use wet cat food discarded from the market after the "best before" date as a substrate for H. illucens larvae growth. The wet food was chosen because it has the humidity content closest to the larval requirements. Our hypothesis is that a substrate particularly rich in macro and micronutrients, formulated to completely satisfy the nutritional requirements of a monogastric animal, can represent an optimal source of nourishment for the larvae of Hermetia illucens, determining rapid growth and good chemical-nutritional characteristics.

2. Materials and methods

2.1 Substrates

A total of 4 substrates were used in the trial. A commercial broiler diet was used for the control group. The other three substrates were represented by three wet complete food for adult cats named beef, chicken and salmon mousses. The cat foods were supplied by a local company (Farmina, Italy) and, at the time of use, had expired between 1 and 3 months. For each type of product (beef, chicken and salmon), 3 packages were chosen at random, and the moisture content was determined; for all types, the percentage of humidity ranged between 77.4 and 79.0. After measured the moisture, the other packages were open, and the water content of the substrates was reduced in a ventilated oven at low temperature (40 °C) until it reached values close to 70%. When the packages of cat foods were opened there were no anomalous odors or colors indicating a state of alteration of the product. A batch feeding strategy was applied, which means that the 4 substrates were placed in plastic containers (60 cm × 40 cm × 15 cm) one day prior to placing the larvae, at the beginning of the experiment. This allows the substrates to heat up until the start of the experiment. For each substrate, an amount of 1500 g ± 2.5 of dry matter was placed in each container, to ensure a substrate height of 10 cm. Each group had 6 replicate tanks. The chemical-nutritional characteristics of the substrates are reported in Table 1.

2.2 Larvae

Black soldier fly larvae were supplied by the start-up X-flies (Potenza, Italy). Larvae were sent by the company at 5 days of age, reached the facilities of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II at 6 days old and were placed into the respective substrates at 7 days old. During the first 6 days of life the larvae were fed a standard Gainsville diet.

At 7 days of age the larvae were divided in groups of 100 individuals, weighed and assigned to the different dietary treatments to have a similar average larval weigh/tank (0.174 g ± 0.016). A total of 1,200 larvae were introduced in each tank to have a feeding rate of 0.083 g/larva/d and estimating 15 days of growing period.

The tanks were covered with a perforated cap with a black nylon grid and placed in a ventilated chamber (air flow around 2 m/s) under controlled environmental conditions (T: 27 ± 0.5 °C; RH: 70 ± 5%; L:D photoperiod: 16:8).

At days 10, 12, 14, 16, 20, and 22, one hundred larvae per replicate were randomly selected weighed, and measured for length and thickness (measured at the middle of each larva) and were then returned to their respective tank.

Feeding of larvae was continued until more than 25% of the larvae in a tray had developed into prepupae. That happens at d 20 for the control and salmon groups and at day 22 for beef and chicken groups. The evaluation of the prepupae percentage has been undertaken by collecting exactly 100 g of substrate + larvae from each tank (in three replicates) and counting the number of larvae and prepupae contained in each replicate.

In addition, the following parameters were measured:

Larval mortality (LM), % = (ILN − (FLN + FPN)) ∗ 100/ILN

Growth rate (GR), % = (LFW, g − LIW, g)/d

Substrate reduction (SR), % = (AS, g − RS, g) ∗ 100/AS, g

Waste reduction index (WRI), % = (AS, g − RS, g) ∗ 100/AS, g/d

where ILN = initial larval number; FLN = final larval number; FPN = final prepupae number; LFW = larval final weight; LIW = larval initial weight; d = days of the trial; AS = administered substrate; RS = residual substrate.

All the weights are expressed on a dry matter basis.

At the end of the trial, the larvae were separated from the substrates by manual sieving, leave 24 hours in an empty tank to allow the digestive system to empty and then sieve a second time. Then, the larvae were freeze-dried using a Micromudulyo freeze drier (Thermo Electron Corporation, Thermo Fisher Scientific Inc., Whaltham, MA, USA) and analyzed for chemical composition. Dry matter (DM), ashes, and crude protein (CP) were analyzed according to AOAC (2002). In brief, for DM and ashes, around 2.5 g of sample were weighed into porcelain capsule and put in an electric oven at 103 °C until constant weight; then, the capsule was transferred to an electric stove at 550 °C for the whole night. The crude protein was determined using the Kjeldahl method; only for larvae, the nitrogen to crude protein conversion ratio was 4.76 according to Jansen et al. (2017). Total lipids were extracted from each sample according to the Folch et al. [22] method and gravimetrically quantified. The amount of carbohydrates (CHO) in the diets was calculated as follows: CHO, % DM = 100—Ash, % DM—CP, % DM—Lipids, % DM.

2.3 Statistical analysis

Data were processed by one-way ANOVA, using the GLM procedure of SAS (2002) according to the model:

$$Y_{ij} = m + S_i + e_{ij}$$

Where y is the single observation, m the general mean, S the effect of the substrate (i = control, beef, chicken or salmon), e the error. Differences among mean were evaluated by using the Tukey test (SAS, 2002).

Table 1 Chemical characteristics and fatty acid profile of the substrates utilised as feed for larvae growth.

	Control	Beef	Chicken	Salmon
Moisture, %	70.05	69.95	70.15	70.33
Crude protein, % DM	22.69	14.65	15.61	16.67
Lipids, % DM	5.12	9.55	9.22	10.11
Crude fibre, % DM	4.32	0.70	0.78	0.72
Ash, % DM	3.69	4.44	4.58	4.22
Carbohydrates, % DM	68.50	71.36	70.59	69.00
Fatty acid profile, g FA/100 g total FAME				
C10:0	0.00	0.060	0.049	0.076
C12:0	0.23	0.353	0.143	0.552
C14:0	0.21	1.219	1.072	1.666
C14:1n-5	0.04	0.177	0.160	0.171
iso-C15:0	0.03	0.041	0.027	0.037
anteiso-C15:0	0.01	0.044	0.028	0.027
C15:0	0.11	0.157	0.128	0.168
iso-C16:0	0.00	0.023	0.012	0.008
C16:0	19.45	24.236	24.675	22.849
C16:1n-9	0.11	0.412	0.463	0.426
C16:1n-7	0.34	4.209	4.228	3.846
C17:0	0.15	0.257	0.208	0.261
C17:1?	0.01	0.017	0.017	0.013
C18:0	2.68	9.438	9.737	9.350
C18:1n-9(cis+trans)	29.00	33.179	34.524	32.344
C18:1n-7	1.29	2.112	2.167	2.167
C18:2n-6cis	40.11	18.298	16.358	16.404
C18:3n-6	0.03	0.101	0.123	0.100
C18:3n-4	N.D.	0.030	0.031	0.056
C18:3n-3	1.50	2.170	2.018	2.331
C18:4n-3	N.D.	0.032	0.062	0.111
C20:0	0.46	0.105	0.108	0.147

C20:1n-11	0.09	0.062	0.059	0.105
C20:1n-9	0.82	0.369	0.439	0.900
C20:1n-7	N.D.	0.011	0.025	0.032
C20:2n-6	0.18	0.152	0.133	0.215
C20:3n-6	N.D.	0.305	0.163	0.165
C20:4n-6	0.01	1.179	1.716	2.159
C20:3n-3	0.02	0.035	0.035	0.098
C20:4n-3	0.02	0.102	0.018	0.121
C20:5n-3	0.03	0.223	0.160	0.540
C22:0	0.32	0.051	0.042	0.063
C22:1n-11	0.86	0.010	0.044	0.531
C22:1n-9	1.10	0.034	0.048	0.112
C22:4n-6	N.D.	0.152	0.142	0.187
C22:5n-6	0.01	0.028	0.030	0.069
C22:5n-3	N.D.	0.335	0.195	0.306
C24:0	0.41	0.068	0.067	0.083
C22:6n-3	N.D.	0.172	0.284	1.086
C24:1n-9	N.D.	0.042	0.064	0.117
SFA	24.12	36.05	36.30	35.28
MUFA	33.66	40.63	42.24	40.76
n-6 PUFA	40.40	20.21	18.66	19.30
n-3 PUFA	1.57	3.069	2.772	4.594
n-6/n-3	25.73	6.58	6.74	4.20

Beef: Chicken, beef (5%), herring, chicken liver, hydrolised animal protein, rice, vitamins, minerals. Nutritional additives: Vitamin A 4000IU, Vitamin D 400IU, Vitamin E 130mg, Taurine 800mg/kg.
Chicken: Chicken, herring, chicken liver, hydrolised animal protein, rice, vitamins, minerals. Nutritional additives: Vitamin A 4000IU, Vitamin D 400IU, Vitamin E 130mg, Taurine 800mg/kg.
Salmon: chicken, salmon (5%), herring, chicken liver, hydrolised fish protein, rice, vitamins, minerals. Nutritional additives: Vitamin A 4000IU, Vitamin D 400IU, Vitamin E 130mg, Taurine 800mg/kg.

3. Results

Table 2 shows the live weight of the BSF larvae according to the diverse growing substrates. Until 12 days of age, no differences were observed among the groups, then the larvae of the Salmon group had the highest ($P < 0.01$) live weight at 14 and 16 d; at 20 days the live weight of larvae on control and salmon substrates was not different and higher ($P < 0.01$) that that of beef and chicken groups. At 22 days of age, larvae on chicken substrate had a higher ($P < 0.05$) live weight than that of beef.

Table 2 Live weight (g) of BSF larvae along the trial

	Control	Beef	Chicken	Salmon	RMSE	P value
D7	0.085	0.086	0.087	0.086	0.006	0.8254
D10	0.143	0.146	0.141	0.143	0.01	0.7329
D12	0.173	0.175	0.175	0.173	0.017	0.9852
D14	0.197^{B}	0.185^{B}	0.199^{B}	0.220^{A}	0.015	<.0001
D16	0.226^{B}	0.207^{C}	0.226^{B}	0.250^{A}	0.013	<.0001
D20	0.253^{A}	0.225^{B}	0.229^{B}	0.267^{A}	0.017	<.0001
D22	-	0.227^{b}	0.245^{a}	-	0.018	0.0334

A, B: $P < 0.01$; a, b: $P < 0.05$; RMSE: root mean square error

Body length of the larvae according to the growing substrate is reported in Table 2. Starting from 14 days of age, differences ($P < 0.0$) appear among the groups. In particular, at day 14 salmon and control group showed a higher larval length than the beef and chicken groups, while at day 20 chicken group had a higher larval length than that of control and beef group. At day 22, the larval length of chicken group was higher ($P < 0.01$) than that of beef group.

Table 3 Body length (cm) of BSF larvae along the trial

	Control	Beef	Chicken	Salmon	RMSE	P value
D7	1.26	1.25	1.26	1.25	0.09	0.8329
D10	1.49	1.43	1.43	1.52	0.10	0.6214
D12	1.71	1.65	1.64	1.70	0.106	0.4067
D14	1.94^{A}	1.74^{B}	1.80^{B}	1.94^{A}	0.095	<.0001
D16	1.94	1.93	1.98	1.96	0.086	0.5730
D20	1.93^{B}	1.96^{B}	2.06^{A}	2.00^{AB}	0.051	0.0002
D22	-	1.98^{B}	2.07^{A}	-	0.040	0.0002

A, B: P < 0.01; RMSE: root mean square error

The body thickness of the larvae (Table 4) at day 16 was higher (P < 0.05) in the salmon than in beef group. At 20 d salmon group showed larvae with a body thickness higher (P < 0.01) than that of the control and beef groups, while at 22 days no differences were recorded between the two remaining groups (beef and chicken).

Table 4 Body thickness (cm) of BSF larvae along the trial

	Control	Beef	Chicken	Salmon	RMSE	P value
D7	0.39	0.39	0.38	0.39	0.01	0.6896
D10	0.42	0.41	0.42	0.43	0.03	0.2569
D12	0.44	0.44	0.43	0.44	0.015	0.1931
D14	0.45	0.44	0.47	0.47	0.019	0.0681
D16	0.48^{ab}	0.47^{b}	0.49^{ab}	0.50^{a}	0.020	0.0301
D20	0.49^{B}	0.49^{B}	0.50^{AB}	0.51^{A}	0.011	<.0001
D22	-	0.49	0.50	-	0.009	0.2289

A, B: P < 0.01; a, b: P < 0.05; RMSE: root mean square error

Table 5 reports body shape index of the black soldier fly larvae. At day 14 the beef and chicken groups showed a higher value than the control; at 20 d the salmon group had a higher (P < 0.01) body shape index than the chicken group and at 22 days beef group showed a higher value (P < 0.05) than the chicken group.

Table 5 Body shape index (cm/cm) of BSF larvae along the trial

	Control	Beef	Chicken	Salmon	RMSE	P value
D7	30.59	31.20	30.16	31.21	0.325	0.6512
D10	28.19	28.67	29.37	28.29	0.278	0.7423
D12	25.02	26.46	26.02	25.68	2.06	0.8517
D14	23.26^{B}	25.43^{A}	26.01^{A}	24.72^{AB}	1.30	0.003
D16	24.74	24.50	24.97	25.38	1.12	0.3450
D20	25.01^{AB}	24.82^{AB}	24.28^{B}	25.71^{A}	0.77	0.0024
D22	-	24.67^{a}	23.84^{b}	-	0.728	0.0194

A, B: P < 0.01; a, b: P < 0.05; RMSE: root mean square error

Table 6 summarizes the growth indexes of the black soldier fly larvae according to the diverse growing substrates. Salmon group had a higher (P < 0.01) growth rate than the beef and chicken groups; beef group had a lower growth rate than control and salmon groups. The control group showed a mortality rate higher than chicken and salmon groups, while the beef group had a mortality rate higher than that of salmon group. The substrate reduction index was the highest (P < 0.01) in the salmon and chicken groups, followed by beef and then the control group. The waste reduction index reached the highest (P < 0.01) value in the control group and the lowest in the beef group.

Table 6 Growth performance of BSF larvae at the end of the trial

	Control	Beef	Chicken	Salmon	RMSE	P value
Growth rate, g/d	0.009^{AB}	0.005^{C}	0.006^{BC}	0.010^{A}	0.003	<.0001
Mortality, %	7.78^{A}	7.18^{AB}	6.61^{BC}	6.25^{C}	0.730	0.0002
SR	78.30^{C}	86.90^{B}	88.68^{A}	89.90^{A}	1.43	<.0001
WRI	8.70^{A}	7.90^{C}	8.03^{B}	8.17^{B}	0.13	<.0001

The chemical-nutritional characteristics and the fatty acid profile of the lipids of the black soldier fly larvae are presented in Table 7. The salmon substrate produced larvae with a crude protein content higher (P < 0.05) than the beef substrate. Beef and chicken larvae had a higher (P < 0.01) ash content than control and salmon larvae and the beef larvae had the lowest (P < 0.05) percentage of total lipids. Larvae on salmon substrate had a higher (P < 0.05) content of cholesterol than beef and control group. In the letter group the cholesterol was not detected.

The lauric acid (C12:0) content was the highest (P < 0.01) in the control larvae, followed by chicken (-21.0 %), salmon (-25.0 %) and beef larvae (-30.0 %). The same trend was observed for saturated fatty acids. Beef larvae had a higher (P < 0.01) percentage of MUFA than the other groups and the control group had a lower MUFA content than the other groups. N-3 content of salmon and control larvae was higher (P < 0.01) than that of chicken and beef groups. The n6/n3 ratio was the highest (P < 0.01) for chicken larvae, followed by beef, control and salmon larvae.

Table 7 Proximate composition, cholesterol content and fatty acid profile of *Hermetia illucens* freeze-dried larvae.

	Control	Beef	Chicken	Salmon	RMSE	P value
Moisture, %	66.33	68.25	65.67	66.80	4.78	0.2569
Protein, % DM	44.63[ab]	43.12[b]	44.29[ab]	45.64[a]	1.09	0.0112
Ashes (% on DM)	7.45[b]	10.84[a]	10.22[a]	7.82[b]	0.59	<.0001
Total lipids (% DM)	23.23[a]	16.98[b]	23.17[a]	24.65[a]	3.89	0.039
Cholesterol (mg/100 g DM)	0.00[c]	56.02[b]	58.97[ab]	82.23[a]	17.92	0.0001
Fatty acids profile, g/100 g total fatty acid methyl esters						
C12:0	60.34[a]	42.18[d]	47.63[b]	45.23[c]	1.51	<.0001
C14:0	9.05[a]	6.36[b]	6.93[b]	6.51[b]	0.19	<.0001
C16:0	8.31[c]	15.20[a]	14.13[b]	13.88[b]	0.47	<.0001
C16:1n-9	0.21[d]	1.03[c]	1.11[b]	1.19[a]	0.04	<.0001
C16:1n-7	2.18[c]	5.27[a]	4.54[b]	4.40[b]	0.14	<.0001
C18:0	1.19[b]	1.68[a]	1.28[b]	1.58[a]	0.12	<.0001
C18:1n-9	7.09[c]	17.69[a]	14.96[b]	15.96[b]	0.83	<.0001
C18:1n-7	0.60[b]	1.32[a]	0.72[b]	1.19[a]	0.21	0.0003
C18:2n-6cis	7.67[a]	5.71[b]	5.89[b]	6.25[b]	0.65	0.0024
C18:4n-3	1.44[a]	0.83[b]	0.61[c]	0.73[bc]	0.16	<.0001
SFA	79.71[a]	66.05[d]	70.40[b]	67.79[c]	0.90	<.0001
MUFA	10.44[c]	25.69[a]	21.67[b]	23.13[b]	1.17	<.0001
n-6 PUFA	7.72	6.60	6.62	6.84	0.70	0.1082
n-3 PUFA	2.00[a]	1.42[b]	1.21[b]	2.10[a]	0.21	<.0001
n-6/n-3	3.92[c]	4.67[b]	5.46[a]	3.26[d]	0.28	<0.0001

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The following FAs (found in percentage below 1% of total FAME) were utilised for calculating the Σ classes of FAs but they are not listed in the table: C13:0, C14:1n-5, C15:0, anteisoC15:0, isoC15:0, isoC16:0, C16:2n-4, C17:0, C17:1, C18:2n-4, C18:3n-6, C18:3n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:1n-9, C20:0, C20:4n-6, C20:5n-3, C21:5n-3, C22:0, C22:1n-11, C22:4n-6.

RMSE: root mean square error.

$p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

ns: not significant ($p > 0.05$)

The fatty acid profile of the neutral lipid fraction (Table 8) indicated a higher ($P < 0.01$) lauric acid content in the larvae obtained on the control substrate than the other groups and chicken group had a higher value than both beef and salmon groups. A similar trend was observed for the total SFA. Salmon larvae had the highest ($P < 0.01$) n-3 content and the lowest n-6/n-3 ratio in comparison to the other groups.

Table 8 Fatty acids of the neutral lipid fraction (g/100 g total fatty acid methyl esters)

	Control	Beef	Chicken	Salmon	RMSE	P value
C10:0	1.45[a]	1.09[b]	1.13[b]	1.20[ab]	0.06	<.0001
C12:0	62.11[a]	44.34[c]	47.69[b]	44.98[c]	1.40	<.0001
C16:0	8.18[c]	14.99[a]	14.40[a]	14.19[b]	0.43	<.0001
C16:1n-9	0.19[c]	1.00[c]	1.08[b]	1.17[a]	0.05	<.0001
C16:1n-7	2.06[c]	5.03[a]	4.34[b]	4.24[b]	0.21	<.0001
C18:0	1.12[b]	1.63[a]	1.28[b]	1.65[a]	0.11	<.0001
C18:1n-9	6.33[c]	16.31[a]	14.25[b]	15.31[ab]	0.91	<.0001
C18:1n-7	0.56[b]	1.25[a]	0.70[b]	1.17[a]	0.20	0.0003
C18:2n-6	6.87[a]	5.29[b]	5.55[b]	5.96[ab]	0.67	0.0192
SFA	82.90[a]	69.21[c]	71.99[b]	69.25[c]	1.11	<.0001
MUFA	9.51[c]	23.89[a]	20.69[b]	22.23[ab]	1.27	<.0001
n-6 PUFA	6.97	6.28	6.69	6.88	0.60	0.3476
n-3 PUFA	0.51[b]	0.48[b]	0.56[b]	1.50[a]	0.20	<.0001
n-6/n-3	13.73[a]	13.49[a]	12.20[a]	4.76[b]	1.77	<.0001

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The following FAs (found in percentage below 1% of total FAME) were utilised for calculating the Σ classes of FAs but they are not listed in the table:: C13:0, isoC14:0, C14:1n-5, C15:0, anteisoC15:0, isoC15:0, C15:0, isoC16:0, C16:2n-4, C17:0, C17:1, C18:2n-4, C18:3n-6, C18:3n-3, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-9, C20:4n-6, C20:5n-3, C22:1n-9, C22:1n-7, C22:5n-6

RMSE: root mean square error.

p < 0.05 (*); p < 0.01 (**); p < 0.001 (***).

ns: not significant (p > 0.05)

Regarding the fatty acid profile of the polar lipid fraction, no differences were observed among the groups for the content of lauric acid and total SFA. The control group had the lowest ($P < 0.01$) value of MUFA and the highest ($P < 0.01$) of n6-PUFA in comparison to the other groups. The n-3 PUFA showed the highest ($P < 0.01$) value in the salmon group, followed by the control and, together, beef and chicken groups. The salmon group had the lowest n6-n-3 ratio.

Table 9 Fatty acids of the polar lipid fraction (g/100 g total fatty acid methyl esters)

	Control	Beef	Chicken	Salmon	RMSE	P value
C12:0	28.87	30.87	31.52	28.54	6.66	0.8838
C14:0	4.89	4.69	4.71	4.41	0.98	0.9199
C16:0	12.05	13.77	13.21	13.61	1.01	0.1035
C16:1n-7	2.51[c]	4.44[a]	3.81[b]	3.70[b]	0.22	<.0001
C18:0	4.50	3.37	3.28	3.92	0.68	0.0637
C18:1n-9	20.31	24.22	23.85	25.93	3.37	0.1647
C18:1n-7	0.74[a]	1.09[b]	0.69[c]	1.11[b]	0.17	0.0023
C18:2n-6	14.92[a]	8.50[b]	9.87[b]	9.91[b]	2.07	0.0025
C20:0	1.36	1.11	1.20	1.28	0.34	0.7137
C22:0	1.37	1.25	1.32	1.38	0.37	0.952
C22:5n-6	3.60[a]	1.56[b]	1.64[b]	0.29[b]	1.19	0.0117
SFA	54.99	56.36	56.39	54.47	6.04	0.9497
MUFA	24.32[b]	30.83[a]	29.46[a]	32.02[a]	3.53	0.0363
n-6 PUFA	18.71[a]	11.55[b]	12.97[b]	11.47[b]	2.63	0.0041
n-3 PUFA	1.16[b]	0.82[c]	0.82[c]	1.61[a]	0.17	<.0001
n-6/n-3	16.14[a]	14.17[a]	15.69[a]	7.12[b]	1.47	<0.0001

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The following FAs (found in percentage below 1% of total FAME) were utilised for calculating the Σ classes of FAs but they are not listed in the table:: C10:0, isoC14:0, C14:1n-5, anteisoC15:0, isoC15:0, C15:0, isoC16:0, C16:1n-9, C16:2n-4, C17:0, C17:1, C18:3n-6, C18:3n-3, C18:3n-4, C18:4n-3, C18:4n-1, C20:1n-11, C20:1n-9, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:1n-9, C22:1n-6, C22:5n-3, C24:0, C22:6n-3.

RMSE: root mean square error.

$p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

ns: not significant ($p > 0.05$)

4. Discussion

The waste accumulation and its consequent management is a global concern and, among the diverse strategies to alleviate the problem, there is the possibility to reuse waste for strategic production (UN Environment Programme, 2024). In a contest of circular economy, organic waste can gain a new life if used as substrate for insect larvae growing. When the reuse is focused on wet cat food exceeded the "best before date" reported in the packaging, it seems that only the salmon diet was able to give similar black soldier fly larvae performance than the control group, feeding a control, entirely vegetable, broiler diet. In fact, compared to the other two cat foods (based on beef and chicken, respectively), the larvae on salmon diet had a short life cycle (20 vs. 22 days), and a higher final weight (+14.98 and +8.24 % than beef and chicken larvae, respectively). However, compared to the control one, larvae of salmon group had a lower (-19.7 %) mortality rate. These results suggest that wet cat food can be successfully used by H. illucens larvae with satisfactory results in terms of in vivo perfomance, but not all the products, despite very similar chemical-nutritional characteristics can give the same results. Looking at the ingredients reported on the packaging of the cat foods used as substrates, the main difference among the 3 diverse types is the inclusion, at low percentage, of a specific animal protein from chicken, beef or salmon. Thus, in our opinion, the differences among the larvae growing on the diverse cat foods can be ascribed to a different possibility of the larvae to utilize the beef, chicken or salmon sources. Bonelli et al. (2020) observed that midgut of H. illucens larvae is able to adapt to diets with different nutrient content and the result of this adaptation is the ability of these insects to grow on a variety of feeding substrates. However, the changes of the hindgut were studied in substrates with a poor nutritional quality and no research is available to understand if the hindgut changes can have a similar efficiency when the growing diets are able to supply adequate amount of nutrients.

As expected, the diverse growing substrates affected the chemical-nutritional characteristics of the resulted larvae. The decrease of larval protein content due to use of beef substrate is in line with the finding of Addeo et al. (2021) who observed lower percentage of protein in larvae grown on butchery waste, consisting of meat from the trimming of bovine carcasses and cuts of meat, in comparison to larvae grown on a vegetable diet. However, the amount of protein content in larvae ranged from 43.12 to 45.64 %

Despite the lipid content in the control substrate is more/less half than that of the other substrates, the lipid content of the larvae on the diverse substrates was not different and only larvae on beef had a lower lipid content. That because, as for other insect species, the lipid synthesis in H. illucens is from carbohydrates (Bailey, 1975) representing a major component of dietary substrates also in our trial. However, despite the similar carbohydrate content than the other substrates, larvae on beef cat food showed a lower amount of lipids stored in the body. The lipid accumulation in insects can be affected by different condition such as genetic and environmental factors, in particular temperature and diet (Boatta et al., 2023). In our trial the only difference among the group is the growing substrate, so it is logical to deduce that larval diet is the main responsible for these differences. The results suggest that BSF larvae are able to use all the tested substrates giving interesting performance, but not all the growing substrates can be used in the same way. During the larval stage in the insects there is an accumulation of storage lipids in the droplet cells as a reserve for further uses during other phases of the insect live such as metamorphosis, flight, reproduction, etc. Adult flies with higher lipid reserves are able to give better performance in terms of lifespan and reproductive activity in comparison to adult insect with a lower amount of storage lipids (Downer & Matthews, 1976; Arrese & Soulages, 2010; Ziegler & Van Antwerpen, 2006). If the low level of lipid reserves is not good in the perspective of a future adult insect, in terms of feed industry it could be an interesting result because the high level of lipid in insect larvae can be a problem for the use in the formulation of animal diets (Motte et al., 2019) and very often a total or partial defatting of the insect meal is required before its use, increasing the costs of production.

The cholesterol was detected only in larvae on cat food diets. This result is not surprising because insects are not able to synthesize the cholesterol (Spindles et al., 2009) due to the deficiency of enzymes for cellular synthesis, thus it is possible only an assimilation from the substrates during the feeding phase, in our case the larval stage. Insects require dietary cholesterol to meet their cellular, physiological, developmental, and reproductive needs (Haas et al., 2022). Thus, an increased content of cholesterol in BSF larvae could be suggestive for a better development of the insects. Unfortunalety it was not possible to measure the cholesterol content in the substrates; however, it is logical to suppose a similar trend than in insect larvae.

Not only the amount, but also the composition of the lipids is important in insects, particularly the presence of polyunsaturated lipids, as these are precursors for sex pheromones and play a crucial role in the cell membrane in maintaining homeoviscosity at different temperatures (Boatta et al.,

2023). In our trial, the control and salmon group had a higher content of total PUFA than the other two groups (9.72, 8.02, 7.83, an 8.94, respectively for control, beef, chicken and salmon group). Looking at a possible use of H. illucens larvae in human nutrition, it is very interesting to note that the use of the salmon-based substrate significantly increased the PUFA n-3 content in the diet and, as a consequence, improve the n6/n3 ratio, confirming that the fatty acid profile of BSF larvae can be modulate according to the fatty acid profile of the substrates.
The lauric acid was always the most abundant fatty acid in the lipid of black soldier fly larvae, irrespective of its content in the feeding substrates. However, it is possible to detect some important differences among group with the highest content in the control larvae and the lowest in beef larvae. BSF larvae are able to synthetize LA from the carbohydrates of the diet and several studies showed an increased level of LA with the increase of CHO content in the diet (Ewald et al., 2020; Hoc et al., 2020; Eggink et al., 2022). However, in the present trial the amount of CHO is very similar among the different substrates, thus it is logical to suppose that not only the amount but also the kind of carbohydrate can have a role in the de novo synthesis of lauric acid. On this regard, the control diet is based on vegetable ingredients and part of its carbohydrates are structural carbohydrates, measured as crude fiber. In the tested cat food the carbohydrates are partly of vegetable origine (from rice) and partly of animal origin (glycogen); it was not possible in this trial to differentiate the different kinds of carbohydrates but our results are strongly suggestive for a different use of carbohydrate sources by Hermetia illucens to produce lauric acid. This point is very interesting and needs further research also considering the potential application of the insect larvae and their products in an industrial context. Lauric acid shows a lot of interesting properties: it is able to modulate blood cholesterol (Decker, 1996) and exerts antimicrobial properties in vivo and in vitro (Dayrit, 2015).

5. Conclusions

Black soldier fly larvae can use a wide range of substrates and expired wet can food can be a valuable growing substrate, giving growing performance competitive with the control group. However, the diets with a large amount of animal product can affect the larvae chemical composition, thus, the use of these substrates can be evaluated also taking into account the potential use of the larvae. In fact, if the aim is to produce adults for reproduction, the wet cat foods seem to be interesting because the accumulation of cholesterol in the body of the larvae. However, the beef base substrate is the less suitable for this purpose due to a lower accumulation of lipids. For industrial applications, the low levels of lauric acid can be considered a negative aspect, while under a nutritional point of view, the increase of PUFA n-3 content and the improvement of n6/n3 ratio in salmon larvae is a positive aspect. Thus, expired wet cat food could be a promising growing substrate for Hermetia illucens larvae, with no concerns about safety.

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CONSIDERATIONS

This work reflected on the progress and challenges of large-scale industrial farming of *Hermetia illucens* (black soldier fly) as a sustainable protein source. The research highlights the ability of these larvae to grow on various organic substrates, showing great potential as a key tool for sustainable production within the framework of the circular economy. A central idea emerging from the study is the *Hermetia illucens* larvae's capacity to convert organic waste into high-quality biomass, offering significant environmental benefits by reducing the impacts of traditional animal protein production. The larvae were reared on different types of substrates, including vegetable and butchery waste, and the findings demonstrate that the nutritional profile of the larvae, such as protein and fatty acid content, is closely linked to the type of substrate used. Notably, larvae reared on vegetable-based substrates exhibited favorable nutritional profiles, with low saturated fat levels and high polyunsaturated fatty acids. This suggests that substrate choice can be a tool to modulate the nutritional composition of the larvae, depending on the desired product outcomes.

Another key aspect explored in the thesis is food safety and the potential bioaccumulation of toxic elements, particularly heavy metals, in the larvae. The research shows that, although toxic elements are present in the substrates, the risk of accumulation in the larvae is low, remaining well below the limits set by European regulations for animal feed. This finding highlights the need for further research and regulatory oversight, but it also suggests that farming *Hermetia illucens* on organic waste can be safe if properly monitored. One of the main barriers to large-scale development of the insect farming industry is the lack of a clear and unified international regulatory framework. While the European Union has made significant progress by allowing insect-derived proteins in aquaculture and poultry feed, regulatory gaps remain, especially regarding the use of insects in other sectors, including human food. There is a pressing need for more political commitment to develop legislation that facilitates the full integration of the insect farming industry into the global food and feed supply chains. Finally, the economic and social implications of transitioning to insect-based proteins, such as those from *Hermetia illucens*, are promising. In addition to reducing the environmental impact of animal protein production, the insect industry could create new job opportunities and improve food security, especially in lower-income regions. The low production cost, rapid reproduction rate, and adaptability of insects to various climates make *Hermetia illucens* farming an attractive prospect for the future. In conclusion, farming *Hermetia illucens* offers promising solutions to global challenges related to protein production, organic waste management, and reducing environmental impact. However, to fully harness the potential of this species, greater collaboration between scientists, policymakers, and industries is required, along with a robust regulatory framework to support the development of a sustainable and safe insect farming sector.