Spent mushroom substrates as feed for black soldier fly larvae: Opportunities and constraints

Cumulative Dissertation

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen "Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis" in carrying out the investigations described in the dissertation.

Date, Place Signature

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Abstract

The consumption of insects as a source of food and feed has gained significant attention over the past few decades. This trend is driven by the urgent need to identify alternative protein sources for both humans and animals. Among various insect species, the black soldier fly (BSF; *Hermetia illucens*) has emerged as a particularly prominent option due to its remarkable ability to thrive on organic substrates, including those with low nutritional value. Utilizing BSF larvae reared on organic waste as a feed or feed ingredient for livestock and aquaculture supports the principles of a circular economy, contributing to sustainable resource utilization and waste reduction.

Rising awareness of environmental pollution from the disposal or burning of organic waste has driven researchers to investigate its potential as a feed source for BSF larvae. Numerous studies have been conducted to identify suitable organic waste combinations for rearing BSF larvae; however, comparisons among these studies remain challenging due to variations in test parameters and inconsistent research methodologies. Despite these challenges, efforts have been made to compare specific parameters and assess larval performance under different conditions. Key parameters for larvae include feeding rate, larval density, feeding substrate, depth, aeration, temperature, moisture, and pH. For adult flies, factors such as cage size, fly density, light, ambient temperature, and relative humidity are considered. An overview of tested substrates emphasizes the advantages of aeration for managing high-moisture substrates, as well as the optimal substrate depth and temperature conditions. This comparative analysis of biotic and abiotic factors provides insights into effective BSF rearing practices and identifies promising organic waste streams for use as feed.

Spent mushroom substrate (SMS), a side stream of mushroom harvesting has been evaluated as a potential substitute for chicken feed (CF) in feeding BSF larvae. SMS from *Pleurotus eryngii* and *Lentinula edodes* was used to replace CF at varying proportions (0–100%) while feeding 200–1000 BSF larvae. A fixed amount of 100 g dry matter (DM) per box with 60% substrate moisture was provided. Key parameters considering larval growth and development and substrate degradation were assessed. Results indicated that SMS had no negative impact on larval survival. However, when the proportion of CF was substituted with SMS beyond 20%, there was a decrease in individual larval weight, total harvested biomass, larval growth rate (LGR), feed conversion ratio (FCR), substrate reduction, and waste reduction index (WRI). Notably, the highest larval weight was achieved with no SMS substitution, and the lowest density of 200 larvae per box. The substitution of 20% SMS and a density of 250 larvae per box showed no significant difference in total biomass, LGR, and FCR. Chemical analyses were performed on SMS, CF feed, larvae, and resulting frass for the 250 larval density per box. This study offers a comprehensive comparison of SMS as a feed for BSF larvae, which has not been previously reported.

In addition, a 50% mixture of *Pleurotus eryngii* and *Lentinula edodes* SMS and 50% CF was investigated as feed for BSF larvae. Here, 250 larvae were fed with a 100 g DM-formulated feed. Key parameters such as substrate moisture, larval density, and experimental scale were examined to evaluate their impact on larval performance. The moisture levels of 65%, 70%, and 75% were tested, with results showing that higher moisture content improved larval performance. Additionally, densities of 300 and 350 larvae per 100 g DM were tested at 70% and 75% moisture to assess feed utilization. In the final experiment, the scale ranged from 10–2500 g of feed and 25–6500 larvae, with a consistent feeding rate across all scales. Based on all the findings, a substrate moisture of 75%, a larval density of 250 larvae per 100 g DM, and a scale containing approximately 2 larvae per cm^2 are recommended for optimal BSF rearing conditions while using equal amounts of CF and SMS.

To conclude, the experiments highlight the ability of BSF larvae to efficiently utilize SMS as a feed source. Furthermore, the findings emphasize the need for conceptualization and execution of trials to optimize feed formulations based on substrate type, climatic conditions, and larval requirements. Further studies on various organic wastes could enable more effective utilization of these materials, maximizing their potential as sustainable feed sources.

Zusammenfassung

Der Verzehr von Insekten als Nahrungs- und Futtermittel hat in den letzten Jahrzehnten erheblich an Aufmerksamkeit gewonnen. Dieser Trend wird durch die dringende Notwendigkeit vorangetrieben alternative Proteinquellen für Menschen und Tiere zu identifizieren. Unter den verschiedenen Insektenarten hat sich die Schwarze Soldatenfliege (BSF; *Hermetia illucens*) als besonders vielversprechende Option hervorgetan, da sie bemerkenswert gut auf organischen Substraten – einschließlich solcher mit geringem Nährwert – wächst. Der Einsatz von BSF-Larven, die auf organischen Seitenströmen als Futtermittel für Nutztiere und Aquakulturen gezüchtet werden, unterstützt die Prinzipien der Kreislaufwirtschaft und trägt zu einer nachhaltigen Ressourcennutzung und Abfallreduzierung bei.

Das zunehmende Bewusstsein für Umweltverschmutzung durch die Entsorgung oder Verbrennung organischer Abfälle hat Forscher dazu veranlasst, deren Potenzial als Futterquelle für BSF-Larven zu untersuchen. Zahlreiche Studien wurden durchgeführt, um geeignete Kombinationen organischer Seitenströme für die Aufzucht von BSF-Larven zu identifizieren. Erkenntnisse bisheriger Studien mit ihren unterschiedlichen Testparametern und Forschungsmethoden wurden verglichen und spezifische Parameter im Hinblick auf die Leistung der Larven unter verschiedenen Bedingungen bewertet. Wichtige Parameter für Larven umfassen Fütterungsrate, Larvendichte, Futtersubstrat, Substrattiefe, Belüftung, Temperatur, Feuchtigkeit und pH-Wert. Für adulte Fliegen werden Faktoren wie Käfiggröße, Fliegendichte, Licht, Umgebungstemperatur und relative Luftfeuchtigkeit berücksichtigt. Ein Überblick über getestete Substrate hebt die Vorteile der Belüftung zur Handhabung feuchter Substrate sowie optimale Substrattiefen und Temperaturbedingungen hervor. Diese vergleichende Analyse biotischer und abiotischer Faktoren liefert Einblicke in effektive BSF-Aufzuchtpraktiken und identifiziert vielversprechende organische Abfallströme für den Einsatz als Futter.

Abgetragenes Pilzsubstrat (*spent mushroom substrate*: SMS), ein Seitenstrom der Pilzernte, wurde als potenzieller Ersatz für Hühnerfutter (CF) bei der Fütterung von BSF-Larven untersucht. SMS von *Pleurotus eryngii* und *Lentinula edodes* wurde in unterschiedlichen Anteilen (0–100%) als Ersatz für CF verwendet, wobei 200–1.000 BSF-Larven gefüttert wurden. Pro Box wurde eine feste Menge von 100 g Trockensubstanz (TS) mit 60% Substratfeuchtigkeit verwendet. Wichtige Parameter für das Larvenwachstum, die Entwicklung und den Substratabbau wurden bewertet. Die Ergebnisse zeigten, dass SMS keinen negativen Einfluss auf das Überleben der Larven hatte. Allerdings führte ein SMS-Anteil von mehr als 20% zu einem Rückgang des individuellen Larvengewichts, der gesamten geernteten Biomasse, der Larvenwachstumsrate (LGR), des Futterverwertungsverhältnisses (FCR), der Substratreduktion und des Abfallreduktionsindex (WRI). Bemerkenswerterweise wurde das höchste Larvengewicht ohne SMS-Ersatz und bei der niedrigsten Dichte von 200 Larven pro Box erreicht. Ein SMS-Anteil von 20% und eine Dichte von 250 Larven pro Box zeigten jedoch keinen signifikanten

Unterschied in der Gesamtbiomasse, der LGR und der FCR. Chemische Analysen wurden an SMS, CF-Futter, Larven und dem daraus resultierenden Frass für die Dichte von 250 Larven pro Box durchgeführt. Diese Studie bietet einen umfassenden Vergleich von SMS als Futter für BSF-Larven, der bisher nicht berichtet wurde.

Zusätzlich wurde eine spezielle 100 g TS-Mischung aus 50% *Pleurotus eryngii* und *Lentinula edodes* und 50% CF auf die Eignung als Futter an 250 BSF-Larven getestet. Wichtige Parameter wie Substratfeuchtigkeit, Larvendichte und Versuchsskala wurden untersucht, um ihren Einfluss auf die Leistung der Larven zu bewerten. Feuchtigkeitsstufen von 65, 70, und 75% wurden getestet, wobei die Ergebnisse zeigten, dass ein höherer Feuchtigkeitsgehalt die Leistung der Larven verbesserte. Darüber hinaus wurden Larvendichten von 300 und 350 pro 100 g TS bei 70% und 75% Feuchtigkeit getestet, um die Futterverwertung zu bewerten. Die Versuchsskala reichte von 10–2500 g Futter und 25–6500 Larven, wobei die Fütterungsrate über alle Skalen hinweg konstant blieb. Basierend auf den Ergebnissen wird eine Substratfeuchtigkeit von 75%, eine Larvendichte von 250 Larven pro 100 g TS und eine Skala mit etwa 2 Larven pro cm² für optimale BSF-Aufzuchtbedingungen empfohlen.

Die Versuchsergebnisse zeigen, dass SMS eine effiziente Futterquelle für BSF-Larven darstellen kann. Darüber hinaus unterstreichen die Ergebnisse die Notwendigkeit Versuche zur Optimierung der Futterformulierungen auf der Grundlage der Substratart, der klimatischen Bedingungen und der Anforderungen der Larven zu konzipieren und durchzuführen. Weitere solcher Studien zu verschiedenen organischen Seitenströme könnten eine effektivere Nutzung dieser Materialien ermöglichen und ihr Potenzial als nachhaltige Futterquellen maximieren.

Peer-reviewed publications

1. Nayak A, Rühl M, and Klüber P. *Hermetia illucens* (Diptera: Stratiomyidae): Need, potentiality, and performance measures. Agriculture. 2024;14: 8.

2. Nayak A, Rühl M, and Klüber P. Bioconversion efficiency and chemical composition of *Hermetia illucens* larvae fed spent mushroom substrates. AMB Express. 2024;14: 133.

3. Nayak, A and Klüber P. The hidden drivers: Unraveling the impact of density, moisture, and scale on *Hermetia illucens* rearing. PloSONE 2025; 20(1): e0317049.

Conference contributions

1. Nayak A, Oonincx DGAB, van Loon JJA, and Bosch G. Development of housefly larvae on manure from herbivores, omnivores, and carnivores. 72nd Annual Meeting of the European Federation of Animal Science, 30.08.2021-03.09.2021, online poster presentation.

2. Nayak A and Rühl M. Optimizing the production of *Hermetia illucens* larvae. 14th GGL Annual Conference, 29.-30.09.2021, power point presentation.

Award: Second best talk

3. Nayak A and Rühl M. Larval production or substrate degradation: *Hermetia illucens* density test on spent mushroom substrate diet. 15th GGL Annual conference, 14.-15.09.2022, power point presentation.

Award: Second best talk

4. Nayak A and Rühl M. Overcoming the challenges in *Hermetia illucens* protein production fed with agricultural byproducts. 16th GGL Annual Conference, 20.-21.09.2023, poster presentation.

Award: Second best poster presentation

List of abbreviations and acronyms

AFFIA	Asia Food and Feed Insects Associations
BSE	Bovine Spongiform Encephalopathy
BSF	Black Soldier Fly
DM	Dry Matter
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
GHG	Greenhouse Gas
IPAA	Insect Protein Association of Australia
IPIFF	International Platform of Insects for Food and Feed
NACIA	North American Coalition for Insect Agriculture
PAP	Processed Animal Proteins
SMS	Spent Mushroom Substrates

1 Synopsis

1.1 The challenges of sustainable food and feed production

The challenges of sustainable food and feed production stem from the need to balance environmental, economic, and social factors while meeting the growing global demand for nutrition. On planet earth, agriculture including pastures uses around 40% of the arable land (Foley et al., 2005). The agricultural expansion results in biodiversity loss due to the destruction of forests, greenhouse gas (GHG) emissions, land degradation, environmental pollution, climate change, overfishing, and depletion of ecosystem services while adding relatively little percentage to the global food production (Foley et al., 2011; Gibbs et al., 2010). It highlights the difficulty in meeting the demands of an ever-growing population with their increased income (Henchion et al., 2014). The resulting improved living standards increase the demand for food, feed, fuel, and several other end-products, which exploit the resources to a level above the recovery line (Ghisellini et al., 2016; Maina et al., 2017). It is therefore already challenging to produce enough for everyone; aiming to make it sustainable needs tremendous effort and thinking.

The trend in meat consumption over the years gives an idea of the potential problems. The consumption of meat increased by around 60% between 1990 and 2009 (Henchion et al., 2014). The per capita meat consumption in developing countries between 2005/2007 to 2050 is estimated to increase from 28 kg to 42 kg (Alexandratos & Bruinsma, 2012). The livestock sector occupies 70% of the agricultural land (Steinfeld et al., 2006) and releases 18% of total anthropogenic GHG emissions, mainly due to land use and conversion of forest covers to agricultural lands. Feed production and processing, enteric fermentation from ruminants, manure storage, and manure processing are major sources of GHGs (Gerber et al., 2013; Steinfeld et al., 2006). Besides, the competition for limited water and land resources is continuing between different agricultural sectors focusing on food or feed (Godfray et al., 2010; Steinfeld et al., 2006). This pressurizes us to utilize livestock for food and feed production in a sustainable and thoughtful manner.

Due to the fact that sustainable production of food and feed is intertwined with a circular economy approach, it is important to be aware of resource wastage and its minimization. The global municipal solid waste accumulation is approximately 1.3 billion tons per year, and it releases approximately 3.3 billion tons of CO_2 equivalent of GHGs per year (FAO, 2013). A large component of municipal solid waste comes from food residues, kitchen scraps, leftovers from restaurants, and markets (Campuzano & González-Martínez, 2016). The use of agricultural side streams is hence essential to not only reduce resource utilization but also to minimize the resulting environmental issues. Moreover, exploring other possibilities beyond conventionally bred species, such as insects, lab-grown meat, microalgae, and seaweed, can create opportunities for more sustainable and resource-efficient food and feed production within a circular economy framework.

1.2 Insects in food and feed

Although insect protein is considered a novel food within the European Union (EU), around two billion people eat insects regularly (Van Huis, 2013). The number of species that are listed in the edible insects group is 2,111 (Jongema, 2017). The consumption of insects is called entomophagy and is more common in the tropics; the year-round availability, the larger size of insects, and the close bonding between people and nature unlike in the Western world are the main reasons (Van Huis et al., 2013). Nevertheless, the concept of insects as food and feed has been gaining more attention worldwide for a couple of decades. This is crucial for all the benefits that could be achieved by considering insects as a source of protein instead of discarding them because of the yuck factor.

The additional advantages of using insects are (Bondari & Sheppard, 1981; Van Huis, 2013) i) their rich nutritional profile that meets the amino acid requirements for humans (Rumpold & Schlüter, 2013), fishes (Bondari & Sheppard, 1981), and terrestrial animals (Veldkamp & Bosch, 2015), ii) lower GHG production (Oonincx et al., 2010), iii) less land requirement because of vertical farming, iv) shorter generation time, v) less water consumption (Van Huis et al., 2013), vi) higher feed conversion efficiency, and vii) their ability to thrive on side streams (Surendra et al., 2016).

Varieties of insects such as palm weevils (*Rhyncophorous ferrugineus*; Coleoptera: Curculionidae) in Africa, crickets (*Gryllus* spp.; Orthoptera: Gryllidae) in South East Asia, and chapulines or grasshoppers (*Sphenarium purpurascens*; Orthoptera: Pyrgomorphidae) in Mexico are consumed not just because of the above-mentioned benefits but because they are considered a delicacy (Van Huis et al., 2013). Edible insects are not only a source of nutrition but a source of income for many in these countries. Water bugs, honey bees, weaver ants, termites, varieties of beetles, and caterpillars are other popularly known edible insects that are wild-harvested for domestic or market purposes in most parts of the world. Most insects are seasonal commodities as they depend on specific host plants that are seasonally available. For the same reason, the varieties of harvested insects and their quantity differ throughout the year. In certain areas overharvesting has led to the threatened population of several species such as water boatman (*Graptocorixa abdominalis*; Hemiptera: Corixidae), leaf-footed bug (*Thasus gigas*; Hemiptera: Coreidae), ant (*Liometopum apiculatum*; Hymenoptera: Formicidae) in Mexico (Ramos-Elorduy, 2006) and mopane worm (*Imbasia belina*; Lepidoptera: Saturniidae; (Gondo et al., 2010). Farming insects on large scales is therefore a suitable way in tropical countries to ensure a regular supply (Van Huis & Tomberlin, 2017).

In the EU, despite the stigma surrounding insect consumption, several insect species are permitted for consumption under the novel food legislation and following safety assessments by the European Food Safety Authority (EFSA). It refers to legal frameworks and guidelines that regulate the approval, marketing, and consumption of food or food ingredients that have not been significantly consumed by humans before May 15, 1997 (*Regulation (EU) No 2015/2283*). These include yellow meal worm

(*Tenebrio molitor*; Coleoptera: Tenebrionidae), house cricket (*Acheta domesticus*; Orthoptera: Gryllidae), migratory locust (*Locusta migratoria*, Orthoptera: Gryllidae), and lesser / buffalo mealworm (*Alphitobius diaperinus*; Coleoptera: Tenebrionidae) each in frozen, dried, or powdered forms.

Further species of insects can now be used as animal feed following the lifting (or easing) of the EU's "feed ban" regulations. Although the restrictions on producing feed are less strict than those for food, the process remains challenging. The EU regulation 2017/893 addresses the use of insects as a source of protein for livestock and aquaculture (*Regulation (EU) No 2017/893*). The EU Regulation 2021/1372, which was published on 17 August 2021 (*Regulation (EU) 2021/1372*), amends the EC Regulation 999/2001 concerning the feeding of non-ruminant farmed animals. Specifically, it allows processed animal proteins (PAPs) derived from insects to be used in the feed for poultry and pigs (*Regulation (EC) No 999/2001*). The insects allowed as feed in the EU include all insects permitted as novel food and black soldier fly (BSF; *Hermetia illucens*; Diptera: Stratiomyidae), common housefly (*Musca domestica*; Diptera: Muscidae), banded cricket (*Gryllodes sigillatus*; Orthoptera: Gryllidae), field cricket (*Gryllus assimilis*; Orthoptera: Gryllidae), and silkworm (*Bombyx mori*; Lepidoptera: Bombycidae).

1.3 The business of bugs

The growing acceptance of insects as food and feed in the modern world is evident in the increasing number of production companies and associations dedicated to advancing this concept globally. The International Platform of Insects for Food and Feed (IPIFF) was founded in 2012 as an alliance of key players in the insect industry to represent their interests and promote the use of insects as a sustainable source of protein for human consumption and animal feed. It was formally established as a structured organization in 2015 in Brussels, Belgium. The organization includes over 70 members, primarily small and medium-sized enterprises, as well as research institutions and other stakeholders in the insect production chain. Its mission is to promote the use of insects for food and feed and to support the development of favorable EU legislative frameworks for the insects as food and feed sector (*IPIFF, n.d.*). Besides, the North American Coalition for Insect Agriculture (NACIA), the Asia Food and Feed Insects Associations (AFFIA), and the Insect Protein Association of Australia (IPAA) are some of the other major associations promoting awareness and support for sustainable insect farming practices (*AFFIA*, n.d.; *IPAA*, n.d.; *NACIA*, n.d.). These organizations are instrumental in promoting the adoption and integration of insects into global food systems, as they bring together various stakeholders, including insect-producing companies, policymakers, researchers, and academic institutions. Their collaborative efforts help drive progress in the field, fostering both innovation and regulatory alignment across different sectors.

Insect-producing companies adopt eco-friendly methods to cultivate insects and process them into high-quality protein and nutrient-rich products. These products are utilized across diverse sectors, including

aquaculture, livestock feed, pet nutrition, and even for direct human consumption. Some of the insect production companies are Aspire food group, Ÿnsect, Protix, Innovafeed, Enterra feed corporation, Entomo farms, Entofood, Illucens GmbH, Hermetia Baruth GmbH (**Table 1**). Other companies dealing with BSF include EnviroFlight from USA, Farminsect GmbH and Made by made from Germany, and Hexafly from Ireland.

Table 1: Comprehensive description of the major insect companies across the globe focusing on food, feed, and fertilizer.

Aspire Food Group (https://aspirefg.com/)	
Species Crickets **Location** Canada **Market target** Food, feed, and fertilizer	Aspire Food Group was founded by a team of five students who participated and won the 2013 Hult Prize. Today, the company has grown into one of the largest food-grade cricket processing facilities, with an anticipated production capacity of up to 12,000 metric tons annually. The facility itself spans 150,000 square feet and stands 11 stories tall (Rowley, 2023). The company processes crickets into protein powder and snacks for human consumption, as well as pet food. In addition, the frass (insect exuviae and feed residue) is marketed and sold as a biofertilizer.
Ÿnsect (https://www.ynsect.com/)	
Species Mealworms **Location** France/USA **Market target** Food, feed, and fertilizer	Ÿnsect production company was founded by a team of four scientists and environmental activists. With research centers in both France and the Netherlands, it operates production facilities in France and the United States and is planning to expand with new sites on every continent (Ÿnsect, 2023). Yellow mealworms are sold either live or processed into defatted mealworm powder containing up to 70% protein, ground frozen mealworms, dry and liquid protein hydrolysates, oil extracted from mealworms, water-soluble extracts, whole dry mealworms with up to 53% protein content and frass.
Protix (https://protix.com/)	
Species Black soldier fly **Location** The Netherlands **Market target** Feed and fertilizer	Protix is known for being the first industrial-scale insect farm, which began production in 2019. The Protix facility spans 15,000 square feet and uses advanced robotics and artificial intelligence for breeding, rearing, and processing insects. It is processing over 70,000 tons of food waste annually using the larvae (Fantom, 2022). Protein and fat-based feed targeting fish, livestock, and pet animals are sold through this company. Besides, products like fertilizers and black soldier fly eggs for insect breeding are also sold.
Innovafeed (https://innovafeed.com/en/)	
Species Black soldier fly **Location** France/USA **Market target** Feed and fertilizer	Innovafeed, founded in 2016, has grown rapidly and is now one of the leaders in the insect production industry, specifically using BSF. Innovafeed has raised substantial funding to fuel its growth, including a €250 million Series D round in 2022, bringing its total financing to €450 million. The company joined hands with multinational corporation Cargill to provide new ingredients to the animal nutrition industry (Moore et al., 2022) BSF larvae are processed as dry feed, protein powder, and oil for aquaculture feed, livestock feed, and pet food. Insect frass is sold as plant fertilizer.

Enterra Feed Corporation (https://www.enterra.com/)	
Species Black soldier fly **Location** Canada **Market target** Feed and fertilizer	Enterra Feed Corporation, established in 2007, is a trailblazer in Canada's insect farming industry. The company operates a state-of-the-art 188,000-square-foot facility where it repurposes recycled food waste from local farms, grocery stores, and food production facilities to feed its insects. The facility is capable of recycling over 130 tonnes of food waste daily (*Agriculture and Agri-Food Canada*, 2021). The products include black soldier flies dried larvae, protein meal, fertilizer, and fat**.**
Entomo farms (https://entomofarms.com/)	
Species Crickets **Location** Canada/USA **Market target** Food, feed, and fertilizer	Entomo Farms, established in 2014, operates a 60,000-square-foot production facility in Canada. The company received funding from Maple Leaf Foods, Canada's largest protein consumer packaged goods company, to support its growth and innovation efforts. Entomo Farms specializes in supplying cricket-based ingredients to over 50 companies across eight countries, which are used to create a variety of food and pet food products (Goldin, 2018). Insects are processed into protein powder and snacks for human consumption, as well as pet food.
Entofood (https://www.entofood.com/)	
Species Black soldier fly **Location** Malaysia **Market target** Feed and fertilizer	Entofood, based in Malaysia, produces insect protein, primarily for aquaculture, using black soldier fly larvae fed on organic waste. In 2017, it partnered with Veolia to build a bioconversion plant, significantly increasing its production capacity while maintaining high biosecurity and quality standards. The products include black soldier fly larvae protein powder, oil, and frass.
Illucens GmbH (https://www.illucens.com/)	
Species Black soldier fly **Location** Germany **Market target** Feed and fertilizer	Illucens GmbH was established in 2009 in Ahaus by an entrepreneur alongside two food industry specialists. According to the company's website, it focuses on black soldier fly larvae-based products such as protein, lipids, whole dried larvae, larvae puree, and fertilizer, utilizing a fully automated technology. While the website highlights a zero-waste production system, specific details regarding production capacity are not publicly disclosed.
Hermetia Baruth GmbH (https://www.hermetia.de/)	
Species Black soldier fly **Location** Germany **Market target** Feed and fertilizer	Hermetia Baruth GmbH, founded in 2006 in Germany, was a pioneer in successfully mass-breeding BSF larvae in Europe. It is a sister company of Katz Biotech AG that produces insects as biocontrol agents. The company produces protein, fats, and fertilizers, primarily for animal feed and agriculture. The company's large-scale facility produces thousands of tons annually. It has also gained support for projects like developing bio-based lubricants from insect fats, funded by Germany's Federal Ministry of Education and Research (*BioLube*, n.d.) Black soldier fly larvae are sold as whole dried larvae, protein powder, and fat as animal feed including pet food. In addition, the frass is sold as a fertilizer.

1.4 Challenges faced by insect production companies

Insect-producing companies are typically organized into two primary units: the breeding unit and the rearing unit. The breeding unit focuses on maintaining and developing insect colonies, while the rearing unit involves nurturing the insects under controlled conditions to meet their basic needs. Although rearing has traditionally entailed simple maintenance practices, larger and more advanced companies have integrated sophisticated technologies and innovative methods to optimize production efficiency. However, many of these proprietary advancements remain undisclosed, as companies consider them a critical competitive advantage (Cadinu et al., 2020; Halloran et al., 2018). Efficient insect production facilities require the maintenance of precise environmental conditions, particularly optimal temperature and humidity conditions. Even minor climatic variations can profoundly impact farming outcomes (Cadinu et al., 2020; Dossey et al., 2012). Climate control systems, including heating in winter and cooling in summer, are energy-intensive and expensive, particularly in temperate climate zones. Besides, the cost of feed for insects can be high, largely due to regulations governing the types of feed permitted for insect rearing. These regulations also play a critical role in determining the sustainability of insect production. Moreover, the insect diet significantly impacts their health, growth performance, and quality as a feed source (Cadinu et al., 2020). Maintaining hygiene standards is another critical aspect of insect farming. The uncontrolled growth of pathogenic microbes poses risks to insect health and can compromise the safety of the end products. Potential sources of microbial contamination include the insects themselves, their feeding substrates, and the overall production environment (Gałęcki et al., 2023). Adhering to rigorous hygiene protocols is essential not only for maintaining the health of the insects but also for obtaining regulatory approval. Once hygienic standards are achieved, the authorization of insects as food and feed products is facilitated. The EFSA conducts comprehensive risk assessments to either approve or reject specific insects or insect-derived products under the framework of novel foods (Sogari et al., 2023). Presently, regulatory frameworks governing insects as food and feed are relatively lenient. However, only a limited number of insect species have received approval for commercial use. This is in stark contrast to the immense diversity of insect species available globally. Expanding the range of approved insect species will require collaborative efforts involving scientists, industry stakeholders, entrepreneurs, and policymakers to address regulatory, technical, and societal challenges (Cadinu et al., 2020). Another major obstacle to the widespread adoption of insect-based food and feed is consumer acceptance, particularly in Western societies. A general sense of disgust and unfamiliarity with insect-derived products often hinders their integration into mainstream diets. Overcoming this barrier requires targeted efforts to raise public awareness, provide education on the benefits of insect-based foods, and encourage greater engagement with the concept (Cadinu et al., 2020). Therefore, raising public awareness and fostering engagement is crucial as the industry continues to evolve. Moreover, intensive insect farming may elevate the risk of pathogen accumulation, which could potentially lead to the transmission of diseases to other animals or humans,

resulting in zoonotic infections. Despite the growing interest in large-scale insect production, the associated risks of disease transmission and zoonoses remain underexplored (Lange & Nakamura, 2021; Maciel-Vergara et al., 2021). To advance the insect-based food and feed sector, significant improvements in production processes are essential. Currently, these processes rely heavily on manual labor, which limits scalability and efficiency. Enhancing automation in production systems, optimizing the separation of insects from frass, and developing advanced processing technologies to extract high-quality ingredients are crucial for expanding the applications of insect-based products (Cadinu et al., 2020). These advancements are expected to address current limitations, reduce costs, and promote the sustainable growth of this emerging industry

1.5 What fuels insects?

Insects such as mealworms, black soldier flies, houseflies, and grasshoppers are gaining attention as alternative protein sources for animal feed (Rumpold & Schlüter, 2013; Van Huis et al., 2013), human consumption (Oonincx et al., 2010; Van Huis et al., 2013), and even biofuels (Jung et al., 2022; Rumpold & Schlüter, 2013). As previously mentioned, large-scale insect farming requires meticulous management of their diet to maximize growth rates, nutritional value, and overall productivity. The selection of feed plays a crucial role in determining the economic feasibility and environmental sustainability of insect farming. However, in the EU, the choice of feed is influenced by factors such as location, season, and most importantly, regulatory guidelines. For instance, Regulation (EU) 2017/893 governs the use of insects in animal feed (*Regulation (EU) 2017/893*). It establishes standards for the processing and application of insects in feed, including restrictions on the types of feed that can be provided to insects raised for this purpose. The regulation ensures that these feed materials comply with specific health and safety standards to prevent the transmission of diseases or contaminants to the insects, and, in turn, to the animals consuming the insect-based feed. Insects are categorized as farmed animals and therefore their feeding is subject to the same laws as conventional livestock. The regulation prohibits feeding insects mammalian by-products, ruminant materials, and certain side streams (such as manure, catering waste, and products containing meat or fish) to prevent diseases like Bovine Spongiform Encephalopathy (BSE). Insects must not be fed cooked animal proteins from ruminants (such as cows, sheep, or goats) to avoid the risk of prion diseases. On the other hand, vegetal matter and category 3 animal products, such as fishmeal, non-ruminant blood products, animal-derived di- and tricalcium phosphate, hydrolyzed proteins from non-ruminants, collagen, gelatin, ruminant hides and skins, dairy products, honey, rendered fats, and eggs, are allowed as feed for insects (*Regulation (EU) 2017/893*).

Despite certain restrictions, numerous feed trials are conducted to determine the most effective or optimal diets for insects. The diets provided to insects vary depending on the species, life stage, and several other factors, such as the company's location, feed availability, cost, and regulatory considerations. Generally, insects are fed organic materials that support their overall health and

development. Below is an overview of the feeds used for the most commercially popular insects. Additionally, the potential feeds suitable for these insects are also briefly discussed.

Mealworms: The larvae of mealworms are renowned for their high protein and fat content, making them a valuable source of food and feed (Oonincx & De Boer, 2012; Ramos-Elorduy et al., 2002; Van Peer et al., 2021). Producing one kilogram of mealworm protein requires significantly less land and emits fewer GHGs compared to milk, chicken, pork, and beef protein (Oonincx & De Boer, 2012). Mealworms are predominantly fed wheat bran, which is a common and efficient feed substrate. Their ability to thrive on a wide range of organic substrates enhances their potential as an eco-friendly protein source (Langston et al., 2023).

The type of feed provided plays a crucial role in mealworm growth, nutrient profile, and production efficiency. For instance, mealworms reared on wheat bran yielded the highest biomass compared to other substrates such as wheat flour, maize flour, lucerne, and oats (Langston et al., 2023). Research has also explored various side streams as potential mealworm feeds, including vegetable and garden waste (Harsányi et al., 2020), maize hulls, oat bran, rice flour (Kröncke & Benning, 2022), brewer's spent grain (Kim et al., 2016), and spent mushroom substrates (Li et al., 2020). Although mealworm feed is typically dry, it can include carrots, potato peels, and other vegetables, which serve as a source of moisture and additional essential nutrients (Fasce et al., 2022). Utilizing such alternative feeds has the potential to reduce input costs in mealworm production.

Crickets: Crickets are widely valued in the food and feed industries for their high protein content (Halloran et al., 2018; Van Huis et al., 2013). They are commonly reared on diets that include grains and wheat bran, which support their growth and development. Compared to conventional meat products, cricket protein production requires significantly less land, water, and feed, making it an environmentally friendly alternative (Oonincx et al., 2010). To ensure cricket protein remains a sustainable resource, optimizing feed efficiency and utilizing low-cost, locally available substrates are essential strategies (Van Peer et al., 2021). Research has demonstrated that crickets can thrive on various organic materials, including vegetable waste (Oonincx et al., 2015), fruit peels (Romano et al., 2023), spent grains (Oonincx et al., 2015), and side streams from the food industry, such as potato protein and barley mash (Sorjonen et al., 2019). They have also been successfully reared on food waste and crop residues (Lundy & Parrella, 2015), further contributing to the promotion of a circular economy.

However, large-scale adoption of organic waste as feed for crickets is still limited. One key challenge is the potential risk of disease spread associated with using waste materials, which is particularly concerning in mass-rearing systems (Maciel-Vergara et al., 2021). Further research and innovation are needed to explore other easily accessible plant-based substrates that can enhance sustainability while minimizing risks, ensuring the long-term viability of cricket protein production.

Black soldier flies: The larvae stage of the BSF is particularly fascinating, as it marks the period when they become voracious feeders. The larvae can be efficiently reared on a variety of side streams due to their unique midgut anatomy, diverse digestive enzymes, and specialized gut microbiome, all of which contribute to their rapid metabolism (Kim et al., 2011; Klammsteiner et al., 2020). The digestive enzymes such as trypsin and chymotrypsin play a crucial role in protein digestion. These serine proteases are primarily active in the posterior midgut and facilitate the breakdown of proteins into amino acids (Bonelli et al., 2020). Carbohydrate digestion is highly adaptable to dietary variations, with α-amylase and β-amylase levels fluctuating based on the feed composition (Bonelli et al., 2020). Notably, α-amylase activity is highest in the anterior midgut (Bonelli et al., 2019). Lipid metabolism is driven by lipase, an enzyme responsible for breaking down fats. Additionally, the nutritional composition of the feed directly influences the gut microbiota, including bacterial and fungal loads, which in turn affect digestive enzyme activity (Chen et al., 2023). Despite variations in diet, a core microbial community remains stable within the larval gut, underscoring the larvae's ability to efficiently process organic substrates (Klammsteiner et al., 2020). The robustness of BSF gut to dietary changes and pathogens has gained significant attention over the past decade, establishing them as an effective bioconversion tool capable of transforming low- to high-quality waste streams into valuable biomass (Lievens et al., 2023). Thus, BSF larvae can consume a wide range of organic waste, including vegetable peels and pulp waste (Broeckx et al., 2021; Lindberg et al., 2022; Rahmi et al., 2020), fruit peels and pulp waste (Lindberg et al., 2022; Rahmi et al., 2020), fish offal (St-Hilaire et al., 2007), restaurant pre- and post-consumer wastes (Broeckx et al., 2021; Zheng et al., 2012), bakery wastes (Magee et al., 2021; Oonincx et al., 2015), seed press cakes (Tegtmeier et al., 2021), brewer's spent grain (Chia et al., 2018; Shumo et al., 2019), and manure (Shumo et al., 2019). This adaptability is a significant advantage, as it allows for the utilization of a variety of waste materials that would otherwise be discarded.

Black soldier fly larvae can also be fed with side streams from various plant-based industries, offering an additional sustainable feed source that further enhances their role in waste management and bioconversion (Palma et al., 2018). Utilizing such feeds helps reduce industrial waste, supports a circular economy, and minimizes environmental impact. Additionally, the nutritional composition of these side streams remains consistent when sourced from specific industries. Examples of plant-based side streams suitable for black soldier fly larvae include residues from the soybean industry, mushroom production, and fruit and vegetable processing industries. However, while many small-scale experiments have been conducted using these side streams, their application at an industrial scale remains limited. In the EU, most BSF producers commonly use chicken or pig feed for rearing the larvae. However, utilizing local agro-industrial side streams as feed offers a more sustainable alternative and could lead to significant long-term savings in input costs (Bava et al., 2019; Galassi et al., 2021). As research continues and regulatory frameworks evolve, the potential to incorporate diverse waste

streams into BSF production will play a pivotal role in advancing the industry and contributing to a circular economy.

1.6 The mushroom boom

The mushroom industry has experienced significant global growth over the years, driven by its appeal as a delicious and nutritious food rich in dietary fiber, vitamins, and other essential nutrients (De Cianni et al., 2023; Okuda, 2022). Mushrooms are abundant in bioactive compounds, naturally occurring chemical substances in plants and animals, that promote health by influencing biological processes. As a result, mushroom consumption is widely encouraged and promoted for disease prevention and fostering a healthy lifestyle (Antonelli & Donelli, 2023; Ba et al., 2021). Packed with essential vitamins, minerals, and antioxidants, mushrooms are a low-calorie food suitable for various dietary preferences. They are an excellent source of β-glucans and some vitamins, including riboflavin, niacin, thiamine, and pantothenic acid, which are crucial for energy production and metabolism. In addition, mushrooms provide vital minerals such as potassium, selenium, and copper, which contribute to overall health by supporting immune function and cardiovascular well-being (You et al., 2022). The growing consumption of mushrooms can be attributed to multiple factors, including increased awareness of their health benefits, the rising popularity of plant-based diets, and the demand for sustainable agricultural practices.

The most widely cultivated mushrooms globally, in terms of production volume, are shiitake mushrooms (*Lentinula edodes*), followed by oyster mushrooms (*Pleurotus* spp.), wood ear mushrooms (*Auricularia* spp.), and button mushrooms (*Agaricus bisporus*) (Royse et al., 2017). Mushroom production is predominantly concentrated in China, which accounts for approximately 93% of global output, producing 40 million tons in 2020 (De Cianni et al., 2023; Okuda, 2022). Other leading producers include Japan, with an annual production of 471,810 tons, the United States with 370,280 tons, the Netherlands with 260,000 tons, India with 201,000 tons, and Poland with 182,900 tons (De Cianni et al., 2023). Despite the limited number of major production countries, mushrooms are consumed worldwide due to their culinary versatility and nutritional value. Additionally, the practice of wild mushroom harvesting remains prevalent in various regions. In Europe, the primary importers of mushrooms are the United Kingdom and Germany, reflecting strong consumer demand in these markets (De Cianni et al., 2023). Despite the increasing popularity of mushrooms, the production process faces significant challenges, particularly in achieving sustainability within the industry. A key aspect of this challenge lies in fostering local production and effectively managing side streams generated by the mushroom industry. Addressing these issues requires both scientific knowledge and practical expertise.

Mushroom production holds great potential to contribute to a circular economy, as mushrooms can be cultivated on a diverse range of organic waste materials, including straw, wood, paper, and even manure (De Cianni et al., 2023; Geng et al., 2024). This approach facilitates the recycling of nutrients and the

efficient management of organic waste, underscoring the environmental benefits of mushroom farming. The widespread availability of various organic waste materials further suggests that mushroom production has the capacity to expand globally, adapting to local waste resources. However, since 2012, a stagnation or decline in mushroom production has been observed worldwide, except for China, which continues to demonstrate enormous growth in this sector (De Cianni et al., 2023; Okuda, 2022). This trend underscores the need to promote mushroom cultivation practices that utilize organic waste, aligning production with sustainability goals. In addition, the industry has yet to fully exploit the vast diversity of mushroom species. Out of approximately 3,000 known edible mushroom species, only around a dozen are commercially cultivated (De Cianni et al., 2023). Expanding the range of cultivated species could enhance the industry's growth and provide new opportunities for innovation in mushroom production.

One of the most pressing challenges in the mushroom industry is the effective management of side streams, particularly spent mushroom substrate (SMS). It refers to the residual material left after mushroom harvesting and, despite its potential applications, it is often incinerated to generate energy and facilitate disposal. This practice not only squanders valuable resources but also has adverse environmental consequences. SMS has demonstrated utility in various applications, including energy production, soil amendment, reuse as a mushroom substrate, packaging material, and animal feed (Grimm & Wösten, 2018; Leong et al., 2022; Martín et al., 2023). As a soil amendment, SMS improves soil structure by enhancing organic matter content, water retention capacity, soil temperature, and microbial activity (Grimm & Wösten, 2018). Despite these benefits, its underutilization remains a concern, particularly given that approximately 5 kilograms of SMS are generated for every kilogram of mushroom production (Williams et al., 2001). The degradation efficiency of SMS varies significantly depending on the mushroom species. For instance, the degradation efficiency for substrates used in the production of shiitake mushrooms and oyster mushrooms ranges between 40% and 80% (Rodriguez Estrada et al., 2009). SMS typically consists of fungal mycelium, lignocellulosic residues (e.g., sawdust, corn cobs, seed hulls, and husks), livestock manure, wood chips, and amendments such as gypsum, peat, and lime, along with other nutrients (Najafi et al., 2019). However, the exact composition of SMS is highly variable and depends on factors such as the type of mushroom cultivated, the ingredients used in the substrate preparation, and regional practices (Leong et al., 2022; Li & Wang, 2023). For example, paddy straw-based SMS has been analyzed to contain 38–46% dry matter as well as 7–8% crude protein, 1–1.5% ether extract, 20–23% crude fiber, and 22–25.5% total ash each n a dry matter (DM) basis (Ghosh et al., 2024). Given the significant volume of SMS generated and its diverse composition, further research and innovation are essential to develop sustainable strategies for its utilization. This would not only mitigate environmental concerns but also enhance the circularity and resource efficiency of the mushroom industry.

The utilization of insect protein and their rearing on agricultural side streams have emerged as a promising trend to valorize these low-value substrates. SMS, while supported by evidence demonstrating its potential as animal feed (Baptista et al., 2023; Grimm & Wösten, 2018), remains predominantly theoretical with limited practical application. Insects, known for their voracious feeding behavior, can efficiently utilize the nutrients in SMS, thereby contributing to a more sustainable approach in food and feed production systems. However, research on the use of SMS as insect feed is still in its nascent stages, with only a few studies reporting its application for specific species such as *Protaetia brevitarsis* larvae (Wei et al., 2020), mealworms (Li et al., 2020), crickets (Ventura et al., 2023), and BSF larvae (Li et al., 2021; Mao et al., 2023). Further research is essential to explore the potential of SMS as a feed source for insects and to expand its practical applications.

1.7 Research objectives

The rising global population and the increasing demand for sustainable, cost-effective protein sources have posed significant challenges to modern food production systems. Conventional protein sources, such as fishmeal and soybean-derived proteins, face growing scrutiny due to their substantial environmental impacts. To address challenges in food security, agricultural efficiency, and environmental stability innovative and alternative solutions are necessary. Among emerging solutions, the black soldier fly (BSF; *Hermetia illucens*) stands out as a promising candidate for the bioconversion of organic wastes into protein. The BSF's remarkable efficiency in converting underutilized organic waste into nutrient-dense biomass positions it as a viable candidate to reduce the environmental footprint of food production. BSF larvae have demonstrated exceptional potential in valorizing a wide range of organic substrates, producing biomass suitable for animal feed. This versatility has contributed to the increasing global popularity of BSF-based systems. The primary objectives of this study were to evaluate the necessity of BSF larvae as a tool for sustainable bioconversion, to explore their potential to utilize various organic side streams effectively and to assess their performance on diverse organic substrates. Furthermore, the study compared critical parameters influencing BSF rearing and breeding across existing literature.

Over the past two decades, numerous feed trials have investigated BSF larvae's performance on various substrates, including agricultural side streams and manure. Despite these advances, practical application remains limited due to the lack of a comprehensive understanding of the biological, ecological, and operational parameters governing BSF rearing and breeding. This paper aims to bridge critical knowledge gaps in BSF breeding and rearing, emphasizing both larval productivity and adult reproductive success. Key objectives include evaluating the suitability of diverse organic waste streams as larval feed, identifying optimal environmental conditions for rearing, and exploring diet formulations that enhance larval growth and development. Additionally, the study investigates the reproductive performance of BSF adults, including factors such as mating behavior, oviposition rates, and egg viability, under varying environmental and lighting conditions. Special emphasis is placed on rearing

parameters, including substrate type, depth, temperature, moisture, pH, feeding rates, and larval density, and their collective impact on bioconversion efficiency. The findings contribute to the development of innovative strategies that maximize the economic and ecological benefits of BSF, positioning it as a cornerstone of sustainable protein production systems in agriculture. Detailed information on the BSF life cycle, parameters influencing its growth and are presented in this publication:

Nayak A, Rühl M, Klüber P. *Hermetia illucens* (Diptera: Stratiomyidae): Need, Potentiality, and Performance Measures. Agriculture. 2024;14: 8.

The exploration of ideal feed or feed ingredients for BSF larvae has led to the investigation of various substrates. While regulatory frameworks impose restrictions on permissible feed materials, extensive research has been conducted to identify optimal feeding options. Such options should not only promote the growth and development of larvae but also utilize substrates that are readily available, cost-effective, and derived as side streams. One such side stream is the residue that remains after harvesting mushrooms, also known as spent mushroom substrates (SMS). Mushroom cultivation generates substantial amounts of SMS, which, despite its nutrient content, often lacks efficient recycling pathways. This study aimed to assess the suitability of SMS as a larval feed, examining its effects on BSF growth and performance and the resulting chemical composition of larval biomass. By systematically analyzing these parameters, the study sought to identify key factors influencing the nutritional and practical viability of SMS-based diets for BSF larvae.

To achieve this, BSF larvae were cultivated on varying proportions of SMS derived from two types of mushrooms under controlled conditions. The bioconversion efficiency was assessed using parameters, including the substrate reduction rate, biomass yield, and waste reduction index. Furthermore, the chemical composition of the larvae, encompassing protein, fat, amino acids, and fatty acids, was analyzed to evaluate the nutritional quality of the resulting biomass. The research also explored the influence of substrate properties, such as nutrient content, on larval performance and bioconversion outcomes. The findings of this study contribute to advancing the understanding of BSF larval feeding systems. By integrating waste reduction with sustainable protein production, the results offer a scalable model for agricultural waste management and the circular bioeconomy. Detailed methodologies and performance metrics of BSF larvae reared on SMS are presented in the following publication:

Nayak, A., Rühl, M. & Klüber, P. Bioconversion efficiency and chemical composition of *Hermetia illucens* larvae fed spent mushroom substrates. AMB Expr 14. 2024; 133.

The application of SMS as a feed ingredient for BSF larvae remains insufficiently explored. For optimal larval production, SMS must be evaluated across multiple parameters. Key factors such as moisture content, larval density, and production scale significantly impact larval yield, feed conversion efficiency, and substrate reduction. The moisture content of the feed is influenced by substrate type and its water-holding capacity, while larval density plays a critical role in maintaining appropriate

temperatures within the rearing environment and minimizing competition among larvae. Moreover, the experimental scale is crucial for extrapolating laboratory findings to larger production systems, ensuring their relevance to industrial applications. This study investigated the effects of three moisture levels and three larval density rates on larval yield to determine optimal conditions. The best-performing moisture level and density combination were subsequently tested across five rearing scales. These scales ranged from laboratory setups using SMS and chicken feed-based diets. The outcomes contribute to a deeper understanding of the potential for customizing BSF diets using diverse side streams, thereby optimizing larval yield and enhancing the valorization of agricultural residues. This approach not only supports sustainable waste management but also provides actionable insights for industries seeking to integrate SMS or other side streams into BSF rearing systems. A detailed examination of all investigated parameters, methodologies, and results is presented in the subsequent publication.

Nayak, A and Klüber P. The hidden drivers: unraveling the impact of density, moisture, and scale on *Hermetia illucens* rearing. PloSONE 2025 20(1): e0317049

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2 Peer reviewed articles

2.1 Peer reviewed article 1 – *Hermetia illucens* (Diptera: Stratiomyidae): Need, potentiality, and performance measures.

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Review

Hermetia illucens (Diptera: Stratiomyidae): Need, Potentiality, and Performance Measures

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Abstract: The research on black soldier fly (BSF; *Hermetia illucens* L.; Diptera: Stratiomyidae) rearing is on the rise. The larval ability to grow on organic substances makes it an ideal candidate for the bioconversion of agricultural and other organic side streams. While there are several publications on the variables influencing the growth and development of different stages of BSF, juxtaposing the results could be amiss. This is because of the different experimental approaches and units used by the researchers. A few publications also lack information that might be necessary for comparing the results when using similar substrate and rearing conditions. In this review, we have analyzed the studies on rearing variables such as the type of feeding substrate, substrate depth and aeration, substrate temperature, substrate moisture, pH, feeding rate, and larval density mainly, but not exclusively, for the larvae. For the adults, factors such as the cage size, fly density, light, ambient temperature, and relative humidity are considered. In addition, larval performance when fed with side streams is encapsulated. This provides a backbone for future researchers to identify the already assessed variables along with their range and encourages them to define and use standardized rearing practices for a better comparison of the results.

Keywords: black soldier fly; *Hermetia illucens*; insect rearing; rearing variables; side streams; circular economy; sustainability; insects as feed; alternative protein; waste reduction

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1. Introduction

In mimicking nature, insects can be deployed to convert low-value side streams into protein-rich insect-derived feed for animals [1]. Many industrial side streams are inedible by humans and cannot be applied as feed for conventional farmed animals but could be a source of feed for rearing insects [2]. The agricultural side streams have the potential to be used as feed for insects creating a sustainable circular economy [2,3]. The insects can then be directly fed or processed and fed to animals [4,5]. Insects are a valuable source of protein and fat for both humans and animals [1,6,7]. The additional advantages of using insects are [1,4] their rich nutritional profile, which meets the amino acid requirements for humans [8], fishes [4], and terrestrial animals [9], their lower GHG production [10], a lesser land requirement because of vertical farming, a shorter generation time, a lesser water consumption [6], a higher feed conversion efficiency, and their ability to thrive on side streams [11].

In the EU, insects have been allowed as protein sources for aquaculture since 2017 [12], and for poultry and pigs since 2021 [13]. The insects include the black soldier fly (BSF; *Hermetia illucens*; Diptera: Stratiomyidae), yellow mealworm (*Tenebrio molitor*; Coleoptera: Tenebrionidae), common housefly (*Musca domestica*; Diptera: Muscidae), lesser mealworm (*Alphitobius diaperinus*; Coleoptera: Tenebrionidae), house cricket (*Acheta domesticus*; Orthoptera: Gryllidae), banded cricket (*Gryllodes sigillatus*; Orthoptera: Gryllidae), field cricket (*Gryllus assimilis*; Orthoptera: Gryllidae), and silkworm (*Bombyx mori*; Lepidoptera:

Bombycidae). Insects are categorized as farmed animals and their feeding is subject to the same laws as conventional livestock. In the EU, some of these insects have also been proposed for human food. Albeit stringent regulation, the EU approved four of these insects; in the order of approval, the yellow mealworm, house cricket, migratory locust (*Locusta migrotaria;* Orthoptera: Acrididae), and more recently, the lesser mealworm.

In this review, the BSF is considered a potential insect for mass production. Through better knowledge of feed composition and the resulting larval composition, the nutrient profile of the larvae could be modified according to the requirements of feed production [14]. BSF is one of the seven insects for which larvae have been proposed to the European Commission for use in human food, although it has not yet been approved for such use. BSF is a polyphagous insect. Additionally, BSF larvae are not as susceptible to pathogens compared to other insects used for industrial rearing [15]. All the above-mentioned traits make BSF larvae the best candidate insect for industrial production.

The BSF industry plays an important role in feed safety, animal husbandry, garbage disposal, and environmental protection [1]. However, the mass production of insects, as a relatively new industry, has some challenges both in breeding and producing high-quality insects at low cost. The major problems that still exist are upscaling, unknown diet-specific feeding rate to reduce internal competition, lack of precise information on juvenile and later instar densities to maintain optimum temperature range for larval development, and preventing any disease outbreaks. For mass production, the major setbacks are automation and digitalization, the regulations that limit the use of certain feeds for insects, and their restricted sales [16]. Although protocols are available for industrial BSF breeding and rearing [11,17], there is a lack of information on handling the possible changes in the insect behavior on different feeding substrates. Despite scale (dimension) being an important factor that interacts with and influences the variables, the reliability of the BSF industry on the published laboratory scale studies can give unexpected results. The use of a variety of substrates that tremendously vary in terms of their properties, such as surface area to volume ratio, moisture, salinity, pH, or nutrients, makes it difficult to conclude on the best diet for the BSF larvae. In addition, the differences in the development time, fecundity, behavior towards specific environmental conditions, the yield, and the protein and fat production due to the insect strain are not well known for BSF [18]. The use of agricultural side streams, although sustainable, does not promise consistency in terms of product quality and nutritional value. This risk is higher if the agricultural side stream is seasonal and collected from several suppliers. Even with all the challenges, some progress has been made in understanding the biology of the BSF. Some of the information that is known on breeding and rearing BSF can be found in the life cycle section.

There are a variety of side streams available and tested as a feed material to rear insects and, in particular, BSF. However, an assessment of factors influencing larval production and a comparison of larval growth and development on side streams has not been summarized up to date. Therefore, this manuscript focuses on the abiotic variables essential for the optimum maintenance of all life stages. Additionally, a brief overview of the animal- and plant-derived substrates used as feed is given. The variables such as the type of feeding substrate, substrate depth and aeration, substrate temperature, substrate moisture, and pH are not applicable to adult flies in the majority of cases since they do not rely classically on feed but on their energy reserves to survive and reproduce successfully [17]. However, comprehensive information was gathered from the available literature. In addition, the volumetric factors, such as the cage size and fly density, are known to affect the fecundity, hence they are also listed in detail. Due to the overwhelming amount of BSF publications, the references cited are the most important representative studies and do not include all the data from every literature available so far. This work paves the way for the process of making BSF one of the mainstream feed sources by identifying insect feeds that are economical and environmentally friendly while highlighting different experimental approaches by researchers.

2. The Life Cycle of BSF

Adult flies, once emerged, live for approximately 9–11 days (d) when provided with no food, approximately 21 d when provided with water, and up to 50–73 d when provided with sugar water [19]. Besides the size, the sex of adult BSF can be determined by external dimorphisms, including antennal and abdominal structures (Figure 1i–m). The flies also need abundant sunlight or artificial lighting with a temperature range of 25–30 °C for mating and subsequent oviposition. The mating of the flies was observed at temperatures of 27 °C and above accompanied by bright sunlight conditions [20].

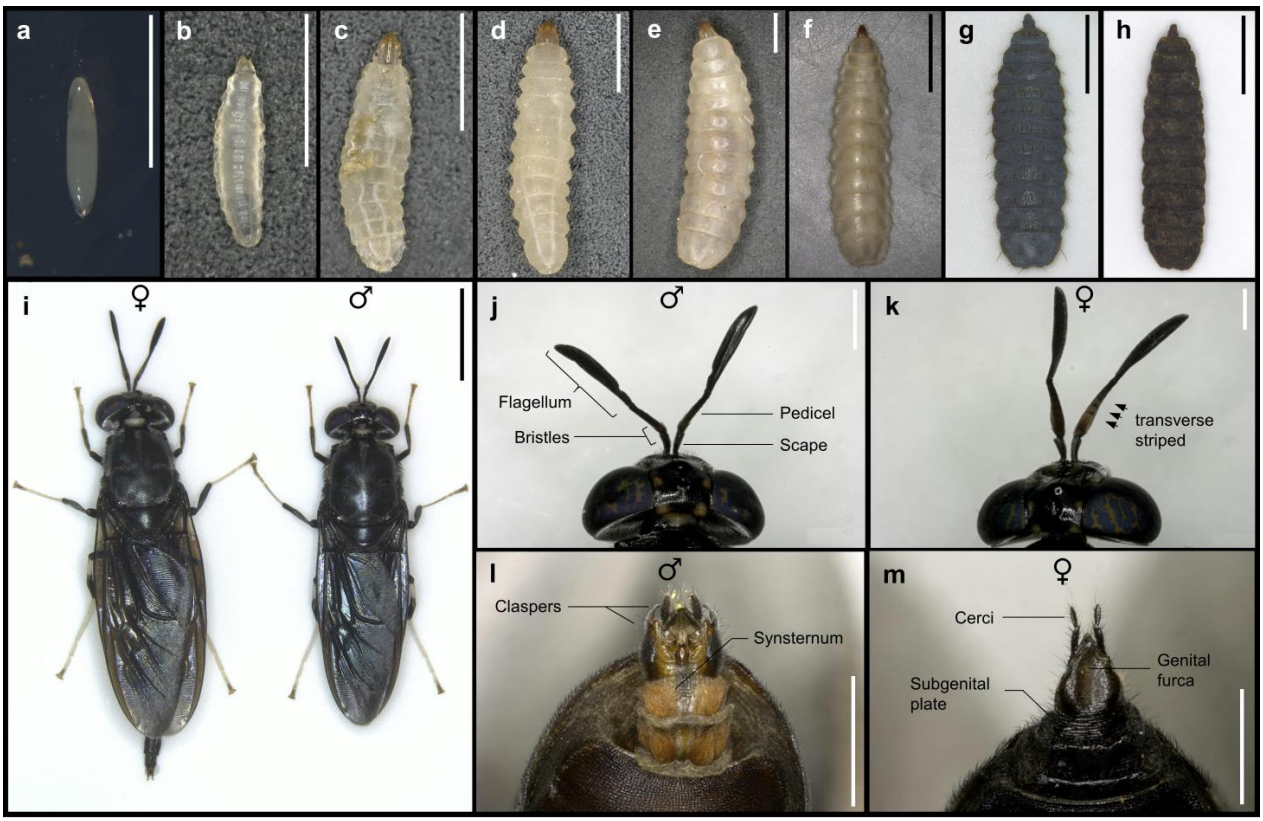

Figure 1. Life cycle and sexual dimorphisms of the antennae and abdominal genitalia of adult BSF. (**a**) Single deposited egg; (**b**–**g**) temporal sequence of larval instars L1–L6 (prepupa); (**h**) pupa; (**i**) full body view of adult female (left) and male (right) BSF; (**j**,**k**) dorsocranial view of male and female antennae; (**l**,**m**) ventrocaudal view of male and female genital structures (scale bars: white = 1 mm, black = 5 mm).

Mating occurs 2 d after fly emergence and up to 70% oviposition happens after 2 d of mating. Females lay approximately 120–820 eggs in clutches in dry spaces near decomposing matter using their ovipositor (Figure 1m) [21,22]. Up to 86.5% of oviposition was restricted to the noon hours (h) between 12:00 and 15:00, with a temperature range of 30–33 °C. The volatile compounds from decomposing materials [20,23], fresh fruit and vegetable waste [21,24], millet porridge mash [25], and the Gainesville diet [17] stimulate oviposition. Egg traps are used for the easy collection of eggs. The eggs turn white 12 h after laying (Figure 1a) and take 4–5 d to hatch at 24–27 °C and 70–90% relative humidity (RH) [20,26,27]. There are six larval instars (L1–L6) with the prepupa being the final one (Figure 1b–g). The first two instars are very fragile and the chances of mortality are higher during handling. Larval growth takes approximately 14–20 d under ideal climatic conditions with a nutritious diet [26,28]. Due to the larval mouthparts, ingestion is facilitated in a moist substrate with a smaller particle size. The fifth instar represents the reservoir of the highest nutrients and hence the desirable stage of harvest for animal

feed. Furthermore, larvae reach their highest biomass in the fifth instar. At the prepupae stage, the feeding stops and they move out of the moist substrate to pupate in a dry and safe space. Prepupae are dark brown to dark grey (Figure 1g). The longevity of the flies is also influenced by the caloric content of prepupae, which is diet-dependent. Higher caloric content in the prepupae extends the life span of the resulting adults by 4 d, as observed in wild BSF populations [26]. The pupation period is usually approximately two [29] to three [23] weeks under ideal conditions (Figure 1h).

3. Variables Affecting BSF Rearing

3.1. Type of Feeding Substrates and Corresponding Challenges

Saprophagous insects, particularly the BSF, can consume a diverse group of organic wastes [30]. Hence, BSF can be reared on a range of substrates, such as agricultural wastes [3,31–33], animal and human remains [34,35], fish wastes [36,37], food or kitchen waste [38–43], municipal organic wastes [44–47], compost leachates [30] as well as human [48,49] and livestock feces [29,50–54], and restaurant wastes [33,55] transforming them into a protein-rich biomass and residual frass that can act as a potent biofertilizer. Despite all the available side streams, there are some limitations. All types of organic materials are not allowed by the EU legislation to prevent potential dangers like the spread of zoonotic diseases. For example, side streams such as manure, catering wastes, slaughterhouse wastes, and unsold products from supermarkets and food industries (containing meat and fish) are prohibited as feeding substrates for insects in the European Union [12]. The main reason is the transmission of zoonotic diseases, especially by prions. The restrictions in the EU began due to bovine spongiform encephalopathy, which is a neurodegenerative disease in cattle [56]. Substrates currently being considered for insect feeding are dependent on availability, regulatory frameworks, and their feasibility of use [57]. To date, BSF producers in Europe are using poultry feed or pig feed as the main feed for BSF rearing. The increased demand for feed in recent decades is compensated by the import of feed ingredients such as soybeans. To meet export demand, forests and agricultural lands in developing countries are being converted to grow more feed crops [58]. Therefore, using these high-cost feeds raises the insect production price while competing with conventional feedstock. Moreover, using high-quality feed for insects, although they can grow perfectly well on low nutrient-based feed, is a waste of resources. Identifying a sustainable feed that is nutritionally ideal for the larvae and permitted by the legislation of the respective country can therefore be challenging. Feed for insects is often conceptualized based on the observed habits and habitat behaviors for that particular species in nature or in fields, rather than considering the nutrient requirements of the diet. Although observations of their natural behaviors in nature, for example, in feed consumption, give an idea of their preferred feed, nutritional composition would assist in optimizing growth and development [59]. The substrate selection for industrial-scale insect production must be performed based on its local availability, its suitability for larval growth and development, and its positive impact on waste reduction by utilizing substrates that are usually discarded. Preferentially, using side streams as feed for BSF larvae can be profitable for the industries [60]. Substrate selection also relies on its properties and how it can be best prepared to feed insects. For instance, the substrates can be blended [61], heat treated [62], steamed [41], fermented [63–65], sterilized [66], or pretreated with probiotic microbes [67–69] before usage as feed for the larvae. Furthermore, substrate properties such as particle size, pH, and moisture retention are also to be considered to make the substrate ideal for larval growth and development. Another challenge is separating the larval biomass from the feeding substrate; this is discussed in more detail in the chapter on substrate moisture. This leads to the exploration of factors that are crucial in rearing insects and how larval and adult performance responds to relevant substrate modifications.

3.2. Substrate Depth

Substrate depth is the height of the feed in which larvae live until harvest. Substrate depth is considered to influence larval development as it can affect larval movements, oxygen availability, and intraspecific competition. Larvae fed on mixed organic waste were studied for three weeks on the effect of feed depth and larval aggregation temperature [61]. A homogenized feed with a 5 cm depth had 60% of the prepupae, including the highest weighing larvae (approximately 270 mg FM) and a waste reduction of approximately 63% dry mass (DM) in comparison to all other feed depths (10, 15, and 20 cm). A feed depth of >10 cm prevented access to all the provided feed. In addition, the larval survival rate also decreased to lower than 80% as the feed depth increased [61]. Lopes et al. (2023) tested four substrate depths (1.0–6.5 cm) and also concluded that a substrate depth of higher than 5 cm impairs material reduction and bioconversion efficiency [70]. Generally, larvae accumulate in masses resulting in an increased temperature (due to metabolism and movement) known as the aggregation temperature. This temperature was highest for the homogenized feed with a depth of 15 cm. According to Brits (2017) and Lopes et al. (2023), to obtain a maximum waste reduction and higher larval biomass, the feed has to be homogenized and fed to the larvae at a 5 cm depth [61,70]. However, a depth of 5 cm is not a good option for upscaling production because of the space needed and the huge surface, which would lead to higher evaporation. Therefore, it is recommended to adjust the feed depths to <10 cm [61]. Peng et al. (2022) concluded that for the examined substrate depths of 10–20 cm, the bioconversion efficiency of pig manure is higher at the lower depths of 10–15 cm [71]. A depth of 3 cm is suggested to be the best in terms of biomass conversion efficiency and larval yield [70]. In a study by Lalander et al. (2020), the substrate depth was between 3.2 and 8.0 cm for substrates with a moisture content of 76% and ~98%, respectively. The corresponding larval survival rates were 19% and 97%; which might be the result of synergistic interactions between depth, aeration, and substrate moisture [72].

Substrate depth could also apply to neonates [21] and pupae [73,74]. A pupation substrate is not mandatory [75] but having wood shavings [3,22,73], sand or soil [22], vermiculite [74,76], or wood chips [74] prevents desiccation and was reported to support adult emergence. For instance, the percentage of fly emergence was only approximately 88% without the use of pupation substrate and increased to approximately 97% with the use of sand, topsoil, wood shavings, and potting soil. The duration from the prepupae stage to adult emergence took 17 d without any pupation substrate and sand in comparison to approximately 15 d for topsoil, wood shavings, and potting soil [22].

The limited literature makes it difficult to identify an ideal substrate depth, especially since studies usually examine specific depth ranges under different rearing circumstances. In addition, interaction with other variables such as the moisture content, homogenization level, and aeration might play a role.

3.3. Substrate Aeration

The performance of larvae could be influenced by the aeration within the substrate which in turn depends on the stickiness [21], viscosity [77], particle size [28,78], and physical properties of the feed, such as its fibrous nature [79]. The particle size range described in the literature includes 0.1–1.5 mm [75], ~0.4 mm [33], ≤1–15 mm [80], ~1 mm [63], ~5 mm [81], 4.00–6.35 mm [78], 1–2 cm [23], and ≤3.8 cm [82]. According to Dortmans et al. (2017), the substrate has to be shredded to a particle size of at least less than 1–2 cm in diameter [23]. Larval weights were slightly higher at a 6.35 mm (25 mg DM) particle size than at 4.00 mm (~23 mg DM), when fed almond hulls for two weeks [78]. The authors explain that the increased access of the feed to microbes and reduced oxygen transport are assumed to be reasons for lower larval weight with a smaller particle size [78]. Yakti et al. (2023) found that the larval weight reduced from ~125 mg FM to ~75 mg FM as the straw particle size decreased from >3 mm to ≤1 mm [80]. Usage of a blended substrate could result in less accessibility of feed for the larvae due to high feed compactness and lower oxygen levels at the bottom of the rearing box forming anaerobic zones harboring corresponding

microbiota [61]. Thus, the optimal substrate depth correlates with its density. The inclusion of low compactness components such as sawdust and wood chips as matrix elements during larval rearing may increase porosity and absorption of excess water in the feed [82]. Using pupation substrates of lower compact density (e.g., potting soil and wood shavings) is recommended in comparison to substrates such as sand with high natural compaction [22].

Dried sugar beet pulp and a mixture of middlings and distillers' dried grains adjusted to a moisture content of approximately 73% were considered for studying the BSF larvae performance. According to the authors, the higher aeration in dried sugar beet pulp compared to the mixture of middlings and distillers dried grain was due to different substrate physical properties. Especially the higher fiber content in dried sugar beet pulp resulted in a higher survival rate of ~78% than in the middling mixture (~56%) and dried distillers' grains (~28%) [79]. In contrast to the former study [79], the larvae were found to prefer a coffee pulp bed that is dense and homogenous [77]. Larval dry weight and overall yield were increased by three and five times, respectively, with increasing aeration from the 0.04–0.36 mL/g feed DM/min of substrate. However, higher aeration rates of $\geq$0.95 mL/g feed DM/min of substrate demonstrated no significant difference in the larval weight or the overall yield. According to Palma and colleagues, higher aeration possibly benefitted the microorganisms competing for resources. The reduced yield in the low aeration approaches is presumably due to the competition for the already scarce oxygen between larvae and microbes. Larval growth and development are influenced by the depth of the feed due to the formation of zones and varied aeration. It is therefore important to consider the factors of aeration and microbial activity during BSF production [83]. The medium-range aeration of 0.19–0.26 mL/g feed DM/min was used by Palma et al. (2019) without highlighting its effect on larval consumption or growth rate. In addition, the room ventilation of 1.22–1.39 m^3/kg feed FM/h by channel fans has been used for the substrates with a moisture content of 90–97.5% [78]. Ventilation can help in reducing the substrate depth build-up and obtaining a drier residue that is important for the easy separation of larvae during harvest. Although water removal through active ventilation is possible, substrate moisture of more than 90% would demand higher resources in terms of energy, space, and workload for a smaller change in outcome. Substrates with less than 80% moisture content are predicted to need less than 1 mL/g feed DM/min ventilation to obtain a frass of approximately 50% moisture [72]. The aeration within the substrate can also differ based on the types of lids used. Most [21,72,84] but not all studies [15] use an open lid with or without mesh to preserve the substrate moisture.

Several substrates are used to stimulate directional oviposition but mostly as a source of volatile compounds and not considering the substrate depth preferred by the females if allowed to lay eggs directly on those substrates. In the case of direct inoculation, eggs are usually placed on the surface of the substrate. Hence, the substrate depth here is not an applicable variable.

Active aeration is usually not a regular practice in rearing BSF. Although, there are several advantages such as avoiding the formation of anaerobic zones or easy separation of larvae during harvest, the energy consumption and substrate desiccation might increase disproportionate to the benefits.

3.4. Substrate Temperature

The temperature ranges found in the literature were between 23–30 °C for L1–L5 instars, ~22–33 °C for larvae and prepupae, and 10–30 °C for all larvae, prepupae, and pupae. These developmental periods are categorized by the description from the papers. The temperature of the feeding substrate plays an important role in the growth and development of BSF (Table 1). As an example, larvae took 1.7 and 5.2 d longer, respectively, to reach a prepupal stage at 30 °C and 36 °C in comparison to 27 °C. The 36 °C group did not support the transformation of prepupae to pupae [28]. The larval growth period in cow manure was almost two months at a substrate temperature of 22 °C [36]. The total weight gain by BSF larvae was higher (4.7, 4.3, and 11.1 g FM) at 27 °C in comparison to 23 and

32 °C for chicken, cow, and hog manure, respectively. There was no significant difference in weight gain for hog manure between 28 °C and 32 °C [85].

Table 1. Effect of temperature on BSF weight gain on a grain-based diet.

Temperature (°C)	Individual Larval Weight (mg FM)	References
24.9	125	[86]
27.0	150 [1]	[28]
27.6	175	[86]
30.0	138 [1]	[28]
32.2	125	[86]

[1] Individual prepupa weight (mg FM).

Substrate temperature is directly related to the ambient temperature. On the other hand, it is also clear that there is some interaction between feed, the substrate temperature, and larval aggregation temperature resulting in a varied larval performance for the same diet (Table 1). This indicates that it is essential to rear larvae at different temperatures for the same diet to determine the effect of temperature on the preferred larval trait. However, the substrate temperature does not remain constant during the feeding trials. At the beginning of the trials with early instar larvae, the substrate temperature is more crucial and is dependent on the ambient temperature, while the larvae generate a higher amount of excess heat as they gradually develop. Temperatures can rise to 42 °C at a room temperature of 23 °C with a high larval density [21,87]. The temperature during the decomposition of organic wastes by the larvae also differs. The highest temperature of 39 °C was recorded during the first days of an experiment for the mixture of vegetable and fruit waste, while the lowest temperature was recorded for vegetable waste alone after 20 d (28 °C) [45]. None of the larvae survived at temperatures of 10 and 42 °C. Furthermore, prepupae and pupae are vulnerable even to 40 °C. At 30 °C, the pupal development was the shortest with 8–10 d depending on the diet. The period from hatching to fly emergence can be as low as 28–31 d at 30 °C to approximately 182 d at 15 °C. The temperature range of 30–35 °C revealed the highest survival in most life stages [88]. Although the slower developmental times are not beneficial for insect mass production, it is important to study the threshold temperatures that slow down the rapid metabolism but are not life-threatening to the insect.

Hence, to obtain optimum larval, prepupal, and pupal development, the temperature is a key regulator. Furthermore, the consideration of other rearing factors, in particular larval density and substrate moisture, is necessary as they might affect the substrate temperature.

3.5. Substrate Moisture Content

Ambient or relative humidity affects substrate moisture, which is the quantity of water in the given substrate. The amount of water could be provided at once at the beginning of a feed trial, also known as initial moistening [73,79,89], or at different intervals during the experiment [50]. Initial substrate moisture in rearing BSF larvae was studied within a range between 20 and 90% by various experimenters. The optimum substrate moisture for BSF development was found to be approximately 40–60%. Substrate moisture of at least 40% is essential for growth and development [50]. A 56% increase in larval yield was observed as the substrate moisture content increased from 48 to 68% [83]. Cammack and Tomberlin (2017) postulate that optimum moisture not only affected the yield but also shortened developmental times, and increased pupation and emergence rates. The larval development was a week faster and required 25–50% less feed at 70% moisture content in comparison to 55%. There was up to a 3% higher fly emergence at 70% moisture compared to the 55% diet [76].

Ewusie et al. (2018) used substrates with an initial moisture ranging between 61 and 91% [45]. In another experiment, pre-consumer (vegetable trimmings, spent coffee and tea residues, no meat) and post-consumer (leftovers with meat) food wastes, with substrate moisture contents of 70, 75, and 80% were considered [90]. Here, the differences in wet larval weight were negligible between the different moisture levels. Larval weights at the moisture levels tested varied between 119 and 125 mg FM for pre-consumer and 143 and 161 mg FM for post-consumer waste. In contrast, the post-consumer diet produced heavier larvae (~153 mg FM) compared to the pre-consumer (~122 mg FM) diet due to the higher crude protein (CP) content in the former and not because of the difference in moisture levels. The larval growth was found to be 3–5 d faster in 80% substrate moisture compared to 70 and 75%. However, the survival rate was not affected by the moisture content [90]. Although early instar larvae are more robust than eggs (Figure 1a–c), lower percentages of moisture in the pre- and post-consumer treatments resulted in the drying of substrates, disabling larvae from obtaining enough nutrients, and thus reducing growth and development [90]. Nguyen et al. (2013) examined substrates with moisture contents between 75.2 and 96.5% but the effect of moisture on larval performance is not specified [39]. A broader moisture content range between 20 and 90% studied at 10% intervals by Fatchurochim et al. (1989) revealed differences in survival rates on moistened poultry manure [50]. The BSF survival was best and similar for the substrate moisture ranging between 40 and 60% in comparison to lower (20 and 30%) and higher ranges of moisture (70, 80, and 90%). As opposed to other publications, the 70% moisture reduced the BSF survival rate to 38.8% but the weight of the adult flies was the highest (4.4 mg DM) [50]. The survival rates between different life stages can vary depending on other factors besides rearing in the moist substrate [15]. A few studies also use structuring compounds in the feed such as sawdust [91], or wheat bran and brewers' spent grain [92]. Generally, pupae are able to survive and eclose without any pupation substrate. In spite, a study testing pupation substrates of varying moisture (dry to 150%) concluded that its use does affect prepupae mortality and formation of pupae. According to the authors, moistened substrates reduced the prepupal mortality by $\geq$88%, whereas dry pupation substrates enhanced the pupation rate by up to 9%, compared to the groups without pupation substrates [74].

Similarly, the use of substrate for the egg stage is limited unless the eggs are immediately inoculated onto the substrates [3,28,36]. The direct inoculation of eggs on substrates with 60–70% [17,28,91] may lead to reduced hatchability [17]. Providing water for flies is recommended by most studies. This is performed by either spraying water on the cages [17,45] or by keeping a water source like a wet cotton wick [76].

Substrate moisture is a crucial factor not only for BSF growth and development but also for larval harvest, especially in an industrial setting. In conventional bioconversion studies, organic wastes have been used as such without adjusting the water content to the requirements of the BSF [46]. Although this could save time, separating residues at approximately 80% moisture is considered difficult to impossible. Feeding larvae daily reduces the sieving efficiency as the FM feed is mostly moist [72]. Thus, reducing the substrate decomposition rate resulting in the emission of a foul smell because of incomplete substrate degradation. Larvae also tend to escape from the moist substrate. Usually, the substrate moisture decreases with ongoing rearing because of larval movement, mixing of the substrate, evaporation, and assimilation of nutrients. Increasing moisture content is crucial for some substrates, such as almond hulls, for breaking down the particles by larvae and associated microorganisms [83]. BSF larvae can ingest the nutrients dissolved in water with ease when the substrate moisture is maintained at approximately 70% [45].

Although substrates with higher moisture content could be fed to the larvae, the use of substrates with less than 80% moisture is recommended for proper larval development and is favorable for separating larvae from frass [72]. Egg hatching does not require a moist substrate, albeit few studies inoculate the eggs directly onto it. Therefore, an optimal moisture content cannot be suggested based on the current studies.

3.6. Substrate pH

There are only a few studies on substrate pH and the corresponding effects on larval growth and development, and almost none on the effects on hatching when eggs are placed immediately onto substrates. Therefore, the influence of pH on BSF growth has not been fully elucidated, although available studies have included pH values ranging from 2.0 to 10.0. Ma et al. (2018) showed that the performance of BSF larvae is pH-dependent [93]. While the pH of the diets differed considerably at the beginning of feeding, it was observed that pH values settled between 8.9 and 9.4 at the end of feeding (Table 2).

Table 2. Change in pH and corresponding individual larval weight on different diets.

Feed	Initial pH	Duration (d)	Final pH	Individual Larval Weight (mg FM)	References
Gainesville diet	4.0–9.5	9.7	8.9–9.4	140–150	[94]
Coffee pulp	7.6	13.0	8.9	147	[95]
Chicken feed	~5.0	22.3	7.2	240	[75]
Cottonseed press cake	~6.0	23.7	8.9	140	[75]
Dairy manure	8.2	21.0	7.3	-	[96]

Both acidic and alkaline pH values were well-regulated and resisted by BSF larvae [94]. The increase in pH may be due to the release of ammonia in substrates rich in nitrogen [85,97]. The pH seems to be affected by the feeding rates or the feed amount because the measured pH of the substrates (vegetable wastes) at lower feeding rates of 60 mg FM/larva/d remains more or less constant between 7 and 8. However, higher feeding rates such as 200 mg FM/larva/d possibly create an anaerobic condition pulling the pH to acidic values ranging between 4 and 5. The acidity was assumed to have lowered the growth performance of the larvae [43]. Interestingly, two nutritionally differing diets with pH values in the neutral (6.8) and slightly acidic (4.5) range showed no difference in pH levels within the larval gut. The pH values in the anterior, middle, and posterior midgut of larvae on both diets were approximately 5.5, 2.0, and 8.5, respectively [81].

The pH of fresh dairy manure was 8.2 [96]. Conversely, the initial pH of pig manure was 6.0–6.2, whereas that of chicken manure ranged between 7.4 and 8.2 [85]. Here, the harvested larval weight was 2.4-fold higher in hog manure than in chicken manure at 27 °C. According to the authors, the difference in pH probably affected some antimicrobial peptides influencing larval growth [85]. The pH of a diet consisting of chicken manure and dairy manure in the 2:3 ratio used by Ur Rehman et al. (2019) was 7.6 [67]. The pH of the fresh pig manure used in a choice test against a plant-based side stream diet ranged between 6 and 7, while the plant-by-product was between 3 and 4. It is unknown if the larval choice for pig manure was based on their aversion to the sensed acidity from the other feed [98].

There are several studies giving initial pH values but without further relationships between pH and life-history traits of BSF larvae [67,96,98]. Understanding the changing pH during larval rearing might help to optimize the dietary composition and feeding rates.

3.7. Feeding Rate

The feeding rate is defined as the amount of feed provided to the larvae or adult flies at a given time to support their growth, development, and maintenance. There are numerous studies focused on feeding, but only a few that address feeding rates for the successful production of BSF larvae. Larval feeding could either be performed initially in one batch [48,93,94] or at regular intervals during the experimental duration. The continuous feeding interval varies across the studies from daily feeding [76,94] to feeding once a week [84] or in other intervals [41,48,72]. Studies using continuous feeding either replace the feed completely at defined periods [84,99] or add a new feed on top of the remaining feed [48,53]. The feeding

aspect also differs in terms of quantity. The feed could be provided at defined feeding rates [53,89] or ad libitum [15] and sometimes with no information on the total amount of feed [2,86]. The feeding rates included in the studies ranged from 12.5 mg FM/larva/d to approximately 1000 mg FM/larva/d (Table 3).

Table 3. Larval feeding rate in various studies.

Feeding Rate (mg FM/Larva)	Duration (d)	References
60	144–215	[53]
350	15	[79]
960	12	[94]
1000	19–21	[67]
1667	21–29	[93]
13–200 [1]	10–36	[100]
13–200 [1]	38–45	[32]
50–200 [1]	15	[61]
70–170 [1]	20	[89]
90–230 [1]	~25–30	[52]
100 [1]	29–43	[99]
100–1000 [1]	12	[48]
200 [1]	12–19	[101]
~286 [1]	-	[84]
~40–73 [1]	-	[39,40]

[1] Feeding rate in mg FM/larva/d.

For enhanced prepupal growth, the frequency of adding feed is more impactful than the amount of feed itself [48]. If the final product is a protein with the desired amino acid profile and fat, a feeding rate of 125 mg FM/larva/d or higher was recommended by Brits (2017). For a faster yield that results in early prepupae formation, i.e., 60% of prepupae within 21 d after hatching, the feeding rate can be raised to 200 mg FM/larva/d. Larval biomass did not significantly differ between both feeding rates. However, the efficiency of the conversion of digested feed is highest at the 125 mg FM/larva/d (42.2%) in comparison to ~32% for the 200 mg FM/larva/d feeding rate [61]. In a study by Myers et al. (2008), larval performance varied when cow manure was fed at different feeding rates of 90–230 mg FM/larva/d, with the highest feeding rate resulting in the greatest larval weight (~178 mg FM) and the highest prepupal fresh weight (~137 mg FM), faster larval development (~25 d), and the highest adult fresh weight (~55 mg FM) [52]. In contrast, the lowest feeding rate resulted in ~142 mg FM of larval and ~89 mg FM of prepupae, ~30 d for larval development, and ~37 mg FM of adult weight. A feeding rate of 100 mg FM/larva/d chicken feed resulted in prepupae of 48 mg FM with a substrate degradation of ~42%. The feeding rate of 200 mg FM/larva/d produced 63 mg FM prepupae with a substrate degradation of ~26% [100]. In another study, 200 larvae were fed with homogenized rice straw powder at rates of 12.5, 25, 50, 100, and 200 mg FM/larva/d. In the latter feeding rate, the prepupae weight was highest with 13.6 mg DM after 38 d of rearing compared to ~2 mg DM in the lowest feeding rate after 54 d. At 200 mg FM/larva/d, the substrate consumption was merely ~10% in comparison to the feeding rate of 12.5 mg FM with 30% substrate consumption harvested at 50% prepupal formation. This confirms that, independent of the feeding rates, the harvested weights are very small and not suitable [32]. According to Parra-Paz et al. (2015), feeding up to 163 mg DM/larva/d and maintaining a density of two larvae/cm^2 is considered ideal based on the predictions from modelling in terms of biomass gain (59 g DM/m^2/d) and waste reduction index (2.6%/d) without decreasing the pH to acidic levels [43]. Klammsteiner et al. (2021) fed larvae with oil waste at 70 mg FM/larva/d and food waste at 170 mg FM/larva/d to obtain the same organic matter as in 100 mg FM chicken feed per larva [89].

After the larvae have been fed for a certain period, insects must be harvested or separated from the remaining frass. This procedure is performed either by handpick-

ing them [53] or sieving [79], usually depending on the substrate moisture at the end of the experiment. In addition, the harvesting also varies in terms of the developmental stage. For example, harvesting was conducted at the L5 stage [102] when the first prepupae were found [103], or at 10–100% prepupae formation [82,84,104], or at the pupae stage [105]. However, other studies predefine a harvest date that could range between 8 d [80] to 20 d [89] or allow the prepupae to self-harvest by actively crawling out of the substrate [24,96]. The emerged flies are not fed in the conventional sense but are commonly offered water to drink. However, providing substances like sucrose solution [73,106,107], moistened sugar cubes [19], honey, D-Glucose, *Spirulina* or *Chlorella* powder [108], milk powder, or bacteriological peptone [107] has a positive effect on fly longevity [106] and fecundity [107]. An experiment by Bertinetti et al. (2019) with no feed, drinking water, agar with sugar water, and a mixture of sugar, bacteriological peptone, and milk powder resulted in increased oviposition and egg weight with a protein availability of 2.5% and 9%, respectively, for the agar and milk diet. The adult longevity was less than 10 d without feeding. Giving water increased the longevity by 3 d in male and 2 d in female flies. The provision of agar or milk did not significantly change the longevity in males, whereas female longevity increased to 13 and 15 d, respectively [107]. The oviposition period was 10 d in the agar diet and 17 d in the milk-based diet. The total egg mass obtained in the no diet (532 mg FM) and water diet (~556 mg FM) were lower than in the agar (~931 mg FM) and milk (~1552 mg FM) treatments. The egg hatchability was ~82% and differed significantly from the other three treatments with approximately 75% [107]. Similar to Bertinetti et al. (2019), Nakamura et al. (2016) reported an adult life span of 9–11 d without feeding the flies. However, they observed a tremendously increased longevity of approximately 21 d for both males and females when provided water. The longevity increased to approximately 48 d in females and 73 d in males with a sugar diet [19]. Besides sugar and water, the life history traits of adults were examined by a feeding experiment with protein-rich (0.05–0.5%), carbohydrate-rich (5–50%), and a 0.5% saline solution [108]. Here, the feeding rate was 2 mL/pair/d of the corresponding solutions. The adult longevity was approximately 6 d with no feeding, 18–21 d with tap water, 16–19 d with distilled water, and 10–13 d with 0.5% NaCl. The females failed to lay eggs if they did not receive water. Feeding 5% honey increased the total egg yield to ~490 mg compared to ~321 mg in tap water, while 5% glucose led to a reduction of longevity (14 d) and egg yield (241 mg). When fed with the lowest concentrations of both microalgae powders, adult longevity decreased to 14–16 d, whereas the total egg yield did not differ compared to tap water [108].

It can be concluded that for higher adult longevity and egg mass, providing an energy source such as carbohydrates and protein is recommended rather than starving or offering only water at the adult stage.

3.8. Larval and Adult Density

It is important to ensure a consistent number of larvae per container depending on the amount of feed. The amount of feed one larva could consume varies between developmental stages (Figure 1b–f). The larvae have to be provided with enough feed to minimize intraspecific competition. However, too many larvae in a container can increase the substrate temperature to an uncomfortable level, which might result in larval escape and lower larval weight gain. The number of larvae used per unit area, and their stages as well as the container dimensions differ greatly between studies (Table 4). The lack of standardization makes the comparability of data considerably difficult.

Table 4. Density of BSF juveniles and adults in various studies.

BSF Density	Container Details	References
~0.03 larvae/cm^3	30 × 30 × 6.5 cm	[40]
~0.04 larvae/cm^3	76.5 × 56.5 × 30.5 cm	[24]
~0.08 larvae/cm^3	17.8 × 11.4 × 6.5 cm	[53]

Table 4. *Cont.*

BSF Density	Container Details	References
0.11 larvae/cm^3	21 × 27 × 16 cm	[2]
0.22 larvae/cm^3	60 × 40 × 30 cm	[79]
~0.11 larvae/cm^3	1.89 L	[82]
0.18 larvae/cm^3	5.5 L	[101]
0.2 larvae/cm^3	100 L	[82]
~1.7 larvae/cm^3	0.5 L	[22]
1.2 larvae/cm^2	-	[89]
1.45 larvae/cm^2	23 × 15 cm	[84]
2 larvae/cm^2	21 × 17 × 11 cm	[72]
6 larvae/cm^2	60 × 40 × 12 cm	[72]
2–6 larvae/cm^2	-	[43]
4.2 and 6.3 larvae/cm^2	~194–2060 cm^2	[109]
4–10 kg prepupae	1.0 × 1.0 × 2.5 m and 2.5 × 2.5 × 2.5 m	[110]
~111 flies/m^3	1.5 × 1.5 × 3.0 m	[111]
500–8500 flies/m^3	45 × 45 × 45 cm	[24]
740–3704 flies/m^3	30 × 30 × 30 cm	[112]
~741 flies/m^3	30 × 30 × 30 cm	[106]
~5080 flies/m^3	27 × 27 × 27 cm	[19]

Moreover, the age of inoculated larvae varies greatly. For example, Miranda et al. (2019) used neonates (<12 h after hatching), while other studies inoculated <1 d [53], 2 d [2], 3 d [17,99], 4 d [22,40], 5 d [45,72,78,94,113], 6 d [33,67], several day-old larvae [80,85,98,101,109,114] or even larvae older than 2 weeks after hatching [48]. It is only known that eggs and early instars are more fragile compared to later instars (Figure 1a–g) [17]. However, no recommendation on the ideal larval age for inoculation onto a feed is reported in the literature. In general, it can be stated that the influence of a feeding regime of interest is more difficult to trace with increasing age, as larvae may have been previously cultivated under different circumstances and diets.

Larval densities are reported either based on egg weight [15,63,91], larval numbers calculated based on the average weight of a given number of individuals [72,79,96], or by manually counting larvae [3,45,98]. Most publications only state the number of larvae or eggs that are inoculated onto the substrate [22,40] but not whether larvae were manually counted or weighed. When using eggs, the egg weights differed from 10 mg in 9.0 × 9.0 cm Petri dish containers [27] to 150–200 mg in 19.5 × 16.5 × 9.5 cm containers [15]. It is important to be aware of the differences in hatching rate depending on the egg clutches inoculated and the resulting stocking density.

Adult density and cage size are crucial variables because of their role in egg production [24,110]. The fly densities in various experiments ranged from ~111 [111] to 8500 [24] flies/m^3 in cages of different dimensions (Table 4). Based on the fly densities used for *M. domestica* [115], a density of ≥13,000 flies/m^3 is possible [24]. In a study examining densities of 500–8500 flies, the oviposition period was significantly shorter (11 d) at 500 flies/m^3 density compared to all the other densities (~15.9 d) [24]. The total egg mass obtained increased as the fly density per cage increased, i.e., 0.4 g at 500 flies/m^3 to 7.8 g at 8500 flies/m^3. The egg mass found in female-ratio-dominant cages was always higher but not significantly different for the densities of 500, 2500, and 4500 flies/m^3. In contrast, at 6500 flies/m^3 and 8500 flies/m^3, the egg mass collected from female-ratio-dominant cages was significantly higher (7.6 and 9.0 g) than the male-dominant cages (5.2 and 6.6 g), respectively. The egg hatching rate reduced although not significant from 97.5% at 500 flies/m^3 to 92.1% at 8500 flies/m^3. Authors recommend >6500 flies/m^3 in 45 × 45 × 45 cm cages [24]. The total number of eggs obtained (based on the egg weight of 100 eggs) were 8366, 20,772, and 42,633, respectively, for 740, 1852, and 3704 flies/m^3. The density of flies in this study did not affect adult survival [112]. According to Park et al. (2016), it is recommended to use higher densities of 8–10 kg prepupae in cages ranging between 1.0 × 1.0 × 2.5 m and

2.5 × 2.5 × 2.5 m to increase the number of eggs and their mass, instead of using lower densities (4 kg prepupae) [110].

It is hence noticeable that there is a considerable discrepancy between the studies, particularly in the implementation and information provided, and that no standard density of larvae or flies could be determined to achieve optimum yield.

3.9. Ambient Temperature and Relative Humidity

Ambient temperature is the temperature of the chamber or immediate environment, where the insects are reared at a relative humidity (RH) that is often controllable. The BSF thrive well in tropical climatic conditions at most of their life stages due to their habitat origin [116], which refers to rearing temperatures above 25 °C and a comparatively high RH. However, providing a tropical climate throughout the year in the northern hemisphere is expensive. That makes it important to know the exact conditions required at different life stages in BSF production. The ambient temperature for the larval stages corresponds to the substrate temperature (besides larval aggregation temperature) and hence most of it is mentioned in the substrate temperature chapter. In the case of other life stages, temperature is an essential variable as well. The range of temperatures tested or maintained for adult longevity, mating, oviposition, and egg hatchability was between 10 and 42 °C [20,73,88]. According to Holmes et al. (2016), at 12 °C (and 70% RH), eggs fail to hatch, while at 16 °C, the time taken for egg eclosion is 7 d longer than at 19 °C (approximately 8 d). Also, the eclosion rate at an ambient temperature of 16 °C was found to be only approximately 12% compared to 75% at 19 °C. The early instar larvae from the 16 °C ambient temperature died within 3 d. In the 19 °C conditions, the rate of adult emergence was approximately 32%, indicating that the minimum threshold temperature in rearing BSF is 19 °C [117]. The pre-oviposition time was as long as 16 d at 20 °C in contrast to just 5 d at 35 °C. However, the highest egg numbers were obtained at 30 °C. No oviposition was observed at temperatures less than 12 °C [21]. The egg viability was lower than 11% at 10, 37, 40, and 42 °C. The egg eclosion time was shorter as the temperature increased from 15 to 40 °C. The eclosion was 11.4 d faster at 35 °C than at 15 °C. The highest egg eclosion rates were at 30 and 35 °C with an average of 75% [88]. Another experiment with a temperature range of 20.5–39.8 °C observed up to 99.6% of the oviposition between the temperature ranges of 27.5–37.5 °C [20].

In general, temperatures of approximately 26–30 °C and 60–70% RH are maintained in the majority of publications. A RH of 59–82% was recorded during the bioconversion of feed by BSF larvae [45]. Holmes et al. (2012) studied a RH range between 25–70% for egg eclosion and adult emergence. The prepupal development took the longest (10.4 d) at 25% RH and shortest (9.5 d) at 70% RH. The pupal and adult mortality were 65, 23, and 2% and 84, 41, and 7%, respectively, for increasing RH of 25, 50 and 70%. The adult life span was shortest (~5 d) at 25% RH and longest (~8 d) at 70% RH [118]. Under high ambient temperatures, maintaining a higher RH range of 60–70% RH can minimize evaporation from eggs or moist feeding substrates. Up to 75% of eggs hatched in just 2.6 d at 35 °C ambient temperature and 70% RH. However, the larval development time at this temperature took 16 d, whereas the shortest larval developmental time of 13 d was achieved at 30 °C (70% RH) [88]. The extent to which RH affects larval growth performance is limited based on the few publications that focus on this factor.

The RH in different rearing setups and experiments includes a range of 25–90% for both flies and eggs. Jucker et al. (2017) maintained the flies at 25% RH [3]. According to Sheppard et al. (2002), mating and oviposition can be observed at a wide range of 30–90% RH [17]. There are no studies that explicitly examine the effect of varying RH on fly performance or egg hatching, except Holmes et al. (2012). Here, the egg eclosion was approximately 7, 20, 38, 73, and 38%, respectively, at a RH of 25, 40, 50, 60, and 70%. The time taken for egg eclosion was the longest (~131 d) at 25% RH in comparison to 71 d at 60% RH and 84 d at 70% RH [118]. An adequate RH is important to prevent egg desiccation; spraying water [75,119] or using wet tissues [21] are some of the commonly used techniques.

In particular, understanding the specifics of RH could facilitate the transport of live animals and improve the safety and efficiency of regular supplies of young larvae to small, decentralized insect farmers. Based on the results of the above-mentioned studies, an ambient temperature of approximately 28 °C and a RH of approximately 70% seems to be ideal for BSF production.

3.10. Light

Light, or photoperiod, is the number of hours in a day in which an organism receives illumination. Although the influence of photoperiod on BSF larvae is unknown, studies are maintaining specific photoperiods in larval rearing. The photoperiod tested ranges between zero [63,81,98], 10 h [107], 12 h [2,45,53,73,86,99], 14 h [22,76,117] and 16 h [27], or as natural sunlight through a greenhouse [82]. The egg hatching is not affected by light color temperature [120], nor by light intensity [24]. The photoperiod does not affect egg hatching as well [24]. Nevertheless, in some studies, eggs were light exposed for 12 h [118], 14 h [22,26,76,94], 16 h [27,52] to 24 h [39]. The reason for this is probably that the different developmental stages do not have to be spatially separated, thus saving money and space. The mating of BSF is most efficient under natural sunlight [111,121]. Illumination via artificial sources was shown to stimulate reproduction in adult BSF as well, offering a way for commercial year-round production [27,119,122,123]. For artificial illumination, various systems have been examined, including fluorescence lamps [106,119,122], quartz–iodine lamps [121], light-emitting diodes (LEDs, [106,119,122,123]), halogen lamps [119,122], and metal halide lamps [121]. The wavelengths studied were 300–885 nm [122], 332–535 nm [106], 350–450 nm and 350–2500 nm [121], 380–780 nm [24], and 400–700 nm [121] range. The light intensity range included 700–3700 lux [27], 3–800 μmol/m^2/s [111], 40 μmol/m^2/s [24], ~50–200 μmol/m^2/s [121], 59 μmol/m^2/s [119], and 300 μmol/m^2/s [108]. Liu et al. (2020) tested four artificial light sources for mating success and egg clutches. They used a 50 W halogen lamp, a combined white LED and compact fluorescent lamp of 50 W each, a 400 W metal halide lamp, and a 20 W LED lamp that matched the visual spectral sensitivity of adult BSF. From each light source, wavelengths of 300–885 nm were maintained. The highest mating success (90%) and proportion of fertile egg clutches (91.4%) were achieved from the 20 W LED lamp. No fertile egg clutches were found for the metal halide light [122]. A 500 W quartz-iodine lamp (350–2500 nm) and a 450 W rare-earth lamp (350–450 nm) were used to study mating and oviposition in BSF. The number of mating pairs observed was 70, 40, and zero for sunlight, quartz-iodine, and rare-earth lamp, respectively [121]. Artificial light rich in ~440–540 nm is recommended for enhanced mating [123]. Ultraviolet, blue, and green light between the wavelengths 332 and 535 nm are perceived by the BSF adults, influencing their mating behavior [106]. However, Zhang et al. (2010) suggest a wavelength spectrum of 450–700 nm. The mating peak was observed at an irradiance of 110 μmol/m^2/s [121]. Tomberlin and Sheppard (2002) recommend maintaining a minimum light intensity of 63 μmol/m^2/s, and, for optimum mating, an intensity of >200 μmol/m^2/s [111]. The mating rate increased from 23 to 70% as the irradiance increased from 0.92 W/m^2 to 431 W/m^2 [123]. The oviposition peak was on the 17th and 13th d for sunlight versus the quartz-iodine lamp and both took 4 d for egg hatching [121]. The day of oviposition was (~16 d) with a LED of low intensity (~14 W/m^2), whereas it was approximately 13, 11, and 10 d, respectively, for an LED of high intensity (~24 W/m^2), a fluorescent tube of low intensity (~5 W/m^2), and a fluorescent tube of high intensity (~23 W/m^2) [106]. Here, the authors conclude that the higher light intensity in the cages decreases longevity because of higher energy loss due to increased flying activity. LED illumination is considered to support a higher egg hatchability within the same number of egg clutches in comparison to fluorescent tubes [106].

The photoperiod differs between the studies and was shown to have an effect on fly performance. The oviposition period is light dependent [19,24,119,121]. The experimental or rearing photoperiod ranged between 2–16 h [19], 6–18 h [24], 8–16 h [112], 9 h [121], 10 h [107], 12 h [20,61,88,106,109], 14 h [93], 15 h [73], and 16 h [119]. According to Hoc et al. (2019), the illumination periods of 2–6 h and 12–18 h had oviposition peaks at 5 and 3 d,

respectively. The lowest light duration in this study yielded the lowest egg mass (4.1 g). Although the egg hatching was not influenced by photoperiod, increasing light duration from 6 to 18 h reduced the oviposition period by 3 d [24].

Neither egg hatching, nor the development of juvenile stages of BSF has been shown to be affected by light exposure, thus saving energy. In contrast, different variables of lighting, including wavelength spectrum, photoperiod, intensity, and color temperature are crucial modulators for reproductive success.

4. Assessment of BSF Larvae on Various Feeding Substrates

Although BSF larvae are popularly known as voracious feeders of biodegradable substances, their performance in terms of survival rate, developmental time, biomass, and capacity to reduce waste differs depending on the nutritional composition of the substrate. The compilation of larval growth and development on various biodegradable materials helps to obtain an overall picture of what could be more suitable for the larvae and how they can be used as converters of biodegradable substances based on their specific needs. Although the current EU regulations restrict the use of certain side streams as feed, there is a huge potential in utilizing available side streams of animal or plant origin. These include livestock side streams such as manure, slaughterhouse waste, dairy side streams, as well as food waste or agricultural side streams consisting of vegetable and fruit production and processing side streams, seed press cakes, brewer's grain, fisheries side streams, and seed husks.

The comprehensive chapter on manure is intended to provide insight into its use as feed for BSF larvae. Other agricultural side streams and food wastes could be fed to other livestock animals [124] but manure remains unused except for its use as compost or fertilizer. Therefore, the use of manure as feed for BSF larvae can minimize the competition between different animals for feed availability. Hence, a compilation of manure studies on BSF performance is gathered in the next chapter. In addition, aquaculture (and fisheries) side streams like fish rendering and fish offal, meat and bone meal, feathers, and bedding materials are also categorized as side streams. These can be a potential source of feed for the insects.

4.1. Manure

BSF larvae are found to thrive on different manure-based substrates [40,67]. Among the feed trials on manure, the ones that are used widely are chicken manure, pig manure, and cow manure. The properties of manure seem to influence larval survival. Miranda et al. (2019) found that the BSF pupation rate was 60–80% higher in fresh chicken manure compared to 2 and 4 d aged chicken manure. In this experiment, the pupation was not observed when the BSF larvae were added to the 6 and 8 d old manure [103].

In general, feed trials sometimes include manure with additives, such as specific microbes or chabazite, a zeolite that reduces unpleasant odor by absorbing volatile compounds such as ammonia (Table 5). It can be summarized that on chicken manure survival of larvae becomes challenging if the substrate moisture is too high or if the manure is aged. The larval development time is predominantly longer when feeding manure in comparison to high-quality standard diets, as shown by Oonincx et al. (2015), where the development took 20 d on a chicken feed diet [53]. In addition, the developmental time to reach the prepupal stage in fresh chicken manure was shorter (16 d; [103]). This discrepancy could be explained either by the drying and remoistening process [53] or changes in the manure-associated microbial community and changes in the nutritional composition due to the ongoing degradation process. In most cases, larvae need approximately 20–25 d of developmental time on fresh chicken manure.

Table 5. Survival rate (%), developmental duration (d), and individual BSF weight (mg FM) on chicken manure-based diets.

Feed	Survival Rate (%)	Duration (d)	BSF Stage	BSF Weight (mg FM)	References
ChM (air-dried and remoistened to 80–90%)	0.0	NA	Larva	NA	[50]
ChM (dried and remoistened to ~66%)	82.0	144	Larva	57.0	[53]
ChM + chabazite + water	86.0	-	Prepupa	90.0	[125]
ChM + *B. subtilis* strain S19	99.3	-	Prepupa	87.0	[68]
ChM + *B. subtilis* strain S15	98.7	-	Prepupa	95.0	[68]
ChM (frozen and thawed)	98.0	-	Prepupa	78.0	[68]
ChM: Cow manure 3:2	95.0	21	Larva	90.5	[67]
ChM: Cow manure 3:2 + *Bacillus* sp. strain MRO_2	99.1	19	Larva	112.5	[67]
ChM (fresh)	-	26	Prepupa	225.0	[113]
ChM (fresh)	-	13	Larva	80.0	[69]
ChM (fresh) + *B. subtilis* strain BSF-CL	-	13	Larva	93.0	[69]

ChM—chicken manure.

The larval weight was 116 mg FM in chicken feed but only 57, 69, and 74 mg FM in chicken, pig, and cow manure, respectively. Here it should be noted that the provided feed amount was rather low (60 mg/larva). The larvae were harvested when the first prepupa was observed [53]. The use of fresh poultry manure resulted in the prepupae weighing almost 225 mg FM [113]. In contrast, the prepupae weight in the fresh chicken manure was only 53 mg FM [103]. The larvae of 93 mg FM were obtained on chicken manure and inoculated with the *Bacillus subtilis* strain BSF-CL at 1×10^9 CFU/mL (1 L of bacterial inoculation to 1000 kg manure). Without bacterial inoculation, the weight of the larva was only 80 mg FM [69]. Chicken manure yielded higher larval mass in 14 d when inoculated with *Kocurina marina*, *Proteus mirabilis*, and *Bacillus subtilis* (each had 22 mg DM) in comparison to the chicken manure without any inoculation (18 mg DM). In this study, the bacterial strains were inoculated at 1% (*v*/*w*) proportion onto 500 g chicken manure at a concentration of 1×10^8 CFU/mL [126]. These data show that microbes considered for a co-digestion process seem to be beneficial for BSF larvae (Table 5). Furthermore, BSF larvae are capable of reducing the bacterial load of manure-based substrates, as shown by Erickson et al. (2004) [85]. Here, the concentration of inoculated *E. coli* O157:H7 (10^7 CFU/g) in chicken manure was reduced to approximately 10^1 CFU/g within 3 d [85]. In contrast, no reduction of *Enterococcus* spp. was reported throughout the rearing cycle [85,127,128]. Larvae exposed to contaminated manure still contained viable *Salmonella enterica* Serovar Enteritidis after 6 d in their gut [85]. In addition, foodborne pathogens like *Bacillus cereus* could also be found in the BSF larval gut. This emphasizes proper decontamination before use as food or feed [129].

Sheppard et al. (1994) found a substrate reduction rate of up to 50% in their chicken manure management system. The amount of manure used is not specified by the authors [29]. In other studies, a total reduction rate of 75% of 300 g chicken manure [125], 35.8% of 1000 kg chicken manure [69], and 40.5% from 1000 kg chicken manure inoculated with the *Bacillus subtilis* strain BSF-CL were found [69]. The substrate reduction rate was better (54%) in *Bacillus subtilis*-inoculated chicken manure than in the control diet (49%) without bacterial inoculation [126]. Another study with *Bacillus* sp. strain MRO_2 inoculation had comparable results of 48% waste reduction from a chicken and dairy manure mix, whereas the control diet without bacterial inoculation was reduced by 42% [67].

These results indicate that the chicken manure can be managed very well using BSF. However, the substrate moisture, manure age, microbiome, and quality are some of the factors to be considered. Interestingly, no study so far highlights the effect of inoculating helpful microbes on the pathogens present in the manure.

The other widely tested manure is cow manure. The weight of larvae reared on cow manure and cow manure-based diet varies greatly (Table 6).

Table 6. Survival rate (%), developmental duration (d), stage of harvest, individual BSF weight (mg FM), and feeding rate on cow manure-based diets.

Feed	Survival Rate (%)	Duration (d)	BSF Stage	BSF Weight (mg FM)	Feeding Rate (mg FM/larva)	References
CoM	85.0	-	Prepupa	137	133	[52]
CoM	71.0	-	Prepupa	179	233	[52]
CoM	91.0	24	Larva	63	1000 [1]	[130]
CoM: SCR 1:4	98.8	21	Larva	123	1000 [1]	[130]
CoM: SCR 2:3	98.5	21	Larva	117	1000 [1]	[130]
CoM: SCR 3:2	98.4	21	Larva	112	1000 [1]	[130]
CoM (dried and remoistened)	87.8	214	Larva	~74	60	[53]
CoM	-	21	Prepupa	100	150	[36]
CoM: FO 9:1	-	21	Prepupa	140	150	[36]
CoM: FO 1:1	-	21	Prepupa	150	150	[36]

CoM—Cow manure; SCR—Soybean curd residue; FO—Fish offal. [1] Feeding rate in mg FM/larva/d.

The substrate reduction in cow manure was 26% [130] and 22% [96] on a dry matter basis. The percent waste reduction increased as the soybean curd residue replaced the cow manure [130]. According to the authors, BSF larvae reared on cow manure can be used to produce clean energy coupled with manure management since they produced approximately 16 g of BSF oil in 10 d from 1200 larvae [96]. In cow manure, the *E. coli* O157:H7 abundance was similar (~10^7 CFU/g) with or without larvae and at all three temperatures and feed regimes examined [85]. Contrastingly, the presence of 15 d old BSF larvae significantly reduced the *E. coli* O157:H7 concentration in cow manure from approximately 10^7 CFU/g to 10^1 CFU/g in 3 d [127].

Pig manure was also a preferred substrate in the BSF larvae feed trials. Larvae of all ages were observed to prefer pig manure over a plant-based side stream diet, irrespective of the feed they were previously fed with. The preference for pig manure increased as the larval age increased. Admittedly, this study only conducted a preference behavior but information regarding larval development was not examined [98]. The larval survival rate was 97% in dried and remoistened pig manure with a developmental time of 144 d [53]. A study conducted by El-Dakar et al. (2021) harvested larvae of approximately 202 mg FM from fresh pig manure in 36 d of development [113]. In contrast, Veldkamp et al. (2021) yielded larvae weighing just 37 mg FM at the time of harvest, when 10% prepupae were formed. The lower weights could be due to the use of 7 d old manure in the experiment, which was stored at 4 °C until use. Additionally, some general rearing issues might explain the lower weight obtained for all dietary groups including the chicken feed control (69 mg FM) [104]. The *E. coli* O157:H7 population increased slightly in the presence of larvae in the pig manure unlike in chicken manure. However, the total biomass obtained was still higher (11 g) in pig manure than in chicken manure (5 g) [85]. In the manure management experiment using BSF, the on-farm reduction in pig manure mass was 56% DM in 14 d [54]. The waste reduction index for pig manure was 3 g DM/d [104].

The larval performance on manures from the same animal species might differ based on the feed and health condition of that particular animal. The variations in survival rate, growth, and development of BSF larvae fed similar substrates could be due to the different experimental setups, including manure age and storage, BSF strains used, time to harvest, feeding rates, and general differences in rearing conditions. More research is necessary on the use of manure and its effects not only on larval performance but also on its further use as an animal feed with a standardized rearing protocol [131]. For example, varying factors such as the initial larval age, experimental duration, climatic conditions, and stage of harvest do not give a comparable larval yield even on a similar substrate. A publication by Bosch et al. (2019), which proposes a protocol for conducting a feed trial for larval production can be used as a template. They suggest using a standard

rearing diet in addition to experimental diets [131]. There is no standardization protocol for trials with adults or parameter-specific studies on the BSF. However, providing detailed information on the experiment could help understand any existing differences in the results. Since manure is considered to have a high microbial load and studies postulate ambivalent data on pathogen reduction or accumulation after BSF treatment, further experiments are recommended. However, high larval survival rates indicate a species-appropriate rearing when manure is used as feed.

4.2. Food Waste and Agriculture Side Streams

Food wastes and agricultural side streams encompass kitchen, household, canteen, and restaurant leftovers, vegetable and fruit scraps, as well as side streams from plant- and animal-based industries. Generally, these side streams are highly heterogeneous making it hard to standardize a consistent composition. At times, this could include leftovers of both plant and animal origin. Many experiments considered food waste either in whole or in combination as a feeding substrate for BSF larvae. Food processing wastes comprise the low-value side streams from agroindustry. The most popular feed trials with food waste and agricultural side streams are brewer's spent grain, fruit pomace, seed husk, soybean side streams, spent coffee, pressed seed cakes, bread and cookie remains, fermented empty fruit bunches and corn straw, potato peels, cassava peels, etc.

On kitchen waste, the survival rate of BSF larvae was only 41% [39]. Although not very different, on canteen food waste the survival rate until the pupae stage was up to 80%. However, when larvae were fed purely on oil waste from the canteen, the whole population failed to reach the pupae stage. The inhibited mobility and respiration due to the viscous consistency of oil waste and lack of easily digestible nutrients is a plausible reason for the lower survival rate and biomass increase [89]. On agricultural side streams and side streams from the food industry, the larval survival rates were above 90% such as for apple pulp, brewer's spent grain, corn meal, chicory roots, and fruit puree. None of the larvae survived on tomato leaves [132]. Feeding rice straw at 12.5 mg FM/larva/d was found to result in only half of the larvae surviving. In contrast, up to 92% and 98% survived for the feeding rates of 100 mg FM/larva/d and 200 mg FM/larva/d, respectively [32]. That implies the possibility of overfeeding low-value substrates as the larvae are able to extract the required nutrients. The brewer's spent grain obtained from four different companies was formulated by either adding just water, brewer's yeast, or molasses. This resulted in a total of twelve different diet formulations. The diets significantly differed in their macronutrient contents but the survival rate was $\geq$85% for all treatments [105]. On spent coffee, sweet potato, and dough the survival rates were 98, 87, and 83%, respectively [133]. On cottonseed press cake, the survival rate was >99%. This suggests that the presence of anti-nutritional diet components like gossypol did not negatively affect the survival rate [75]. On fish-rendering products, the larval survival rate was just 1.5%. The possible heavy metal contents in the fish diet are considered to be detrimental to larval growth by the authors [39]. A survival rate of 89% was found on food waste with a fat content of 12% [132]. Similarly, the crude fat contents of approximately 9, 13, and 19% for vegetables, tofu side streams, and food waste diet had no hindrance on larval performance. For example, larvae weighed 179, 200, and 193 mg FM, respectively, when reared for 14 d on vegetables, tofu side streams, and food wastes [91]. Although larvae can survive on most diets, several factors and diet characteristics listed in this review affect the life history traits of larvae considerably. In addition, even if the larvae manage to stay alive the time taken for development is highly variable. On a rice straw diet at a 200 mg FM/larva/d feeding rate, the larvae reached the prepupal stage in 38 d, while lower feeding rates like 12.5 mg FM/larva/d extended the larval developmental time to 54 d [32]. At a 100 mg FM/larva/d feeding rate, the larval development time was ~25 d on diets consisting of vegetable wastes or tofu dreg [31]. The total developmental time to reach the pupal stage was 22 d on a food waste diet [89]. A developmental time of 31 d was taken on brewer's spent grain consisting of a sorghum–barley–water mix to reach the pupal stage. A development time of ~26 d was found in

three diet formulations composed of malt–barley–water, sorghum–barley–brewer's yeast, and barley–water diet mixes [105].

Larval weight is an important factor in choosing the diet mixture. The larval weights varied between 3 and 226 mg FM when fed on various food wastes and agricultural side streams. On kitchen waste and fruit and vegetable wastes, the larval weight was 173 mg and 123 mg FM, respectively [39]. Another study by Nguyen and colleagues (2015) obtained a larval weight of 226 mg FM on kitchen waste. The reason could be the difference in fat content [40]. In the former kitchen waste, the fat content of the diet was only approximately 5% and, in the latter, 20%. In addition, the fat:protein ratio, as well as the amino acid and fatty acid profile, might play a role [134]. The high protein and high-fat diet obtained by mixing agricultural side streams gave an 86% survival rate in comparison to a low-protein and high-fat diet (72%) until observing the first prepupa [41]. The prepupal weight on a rice straw diet at a 200 mg FM/larva/d feeding rate was only 13.6 mg DM in 38 d [32]. Another study on rice straw resulted in 100% inhibition of larval growth [61]. The addition of restaurant wastes to rice straw by an 80:20 ratio resulted in a larval weight of approximately 49 mg DM; harvested at the 50% prepupae stage. Based on additional optimization tests, restaurant solid waste and rice straw mix represented 70:30 and 0.35% of Rid-X, a commercial product with functional microbes and enzymes able to break down cellulose, lipids, and proteins, leading to a larval weight of approximately 61 mg DM [33]. The larvae reared on solid residual fraction, a residue produced after oil extraction from restaurant waste, weighed 65 mg FM [55]. Industrial food waste and household food waste yielded final larval weights of approximately 176 mg and 65 mg FM within 9 d, respectively [132]. The unexpected low larval weight of 65 mg FM on household waste cannot be explained by the nutritional quality of the diet. However, the possible presence of some harmful substances (pesticides, cleaning chemicals, etc.) might have inhibited larval growth [132]. Although the survival rate in apple pulp was up to 95.5%, the larval weight was just ~38 mg FM. It was postulated that the combination of a pH of 3.7, lower protein content (3.4%), high crude fiber (25.7%), and cellulose (21.5%) in apple pulp inhibits larval growth. Similarly, feeding fruit puree led to lower larval weights (80 mg FM) even with a 99% survival rate [132]. Fiber-rich palm oil side streams used by Klüber et al. (2022), yielded larvae of 187 mg FM in a *Bjerkandera adusta* fermented diet in ~30 d in comparison to ~149 mg FM in a non-fermented reference diet in ~41 d. The chicken feed diet in the same experiment yielded larvae of 303 mg FM in ~22 d [63]. Chia et al. (2018) prepared 12 different diets from brewer's spent grain (sorghum, barley), brewer's yeast, and cane molasses, and the best results were obtained from the following diet mixes. The larval weight in the sorghum–barley–water diet mix was ~150 mg FM, while ~200 mg FM was yielded from a barley–water diet mix. Larvae of similar weights (~150–200 mg FM) were harvested from barley–brewer's yeast, malt–barley–brewer's yeast–molasses, and sorghum–barley–brewer's yeast–molasses diet mixes. Larvae were collected as soon as they developed into pupae [105]. Composted cocoa pod husks in combination with food waste resulted in a larval weight of 112 mg FM within 18 d. The prepupal weight of 150 mg FM was obtained from larvae fed on tofu dreg [31]. The larval weight on the cottonseed press cake was approximately 160 mg FM [75]. The individual weight based on the biomass harvested was 165 mg FM in the apple and spent grain diet. A slightly lower weight of 145 mg FM was found on a diet consisting of spent grain and banana. The use of pure fruits drastically reduced the larval weight to 88–105 mg FM for banana, apple, and a mixture thereof, in comparison to spent grain alone with 136 mg FM [135]. Larval weights were 22 mg, 164 mg, and 171 mg FM on oil waste, food waste, and chicken feed, respectively [89]. On fish render, larvae gained 143 mg FM [39] and 167 mg FM [40]. The lower larval weight in fish renders in comparison to kitchen waste (173 mg FM) could be caused by the bioaccumulation of heavy metals.

The use of organic wastes as feed for larvae also aims at a reduction in substrate volume. Substrate reduction after the larval harvest has been measured in some experiments. A substrate reduction of up to 85% was observed for canteen food waste. The percent substrate reduction was 66 and 2.4% on chicken feed and oil waste, respectively [89].

Brewer's grain and corn meal had a similar waste reduction percentage of approximately 45%. Industrial food waste had a substrate degradation of 59%. Apple pulp and household food waste had approximately an 18% substrate reduction [132]. The substrate reduction was 79% for the fruits and vegetable diet, followed by 70% for bakery, 64% for cheese, 61% for sugar beet waste, and approximately 52% for brewer's grain and yeast [37]. The percentage of substrate reduction was 74% for the apple–spent grain substrate mix. A substrate degradation of 59% was found in an apple–banana diet mix [134].

It is evident that BSF larvae are capable of thriving on various organic substrates. However, the results of these feeding studies emphasize the complexity of the larval feeding regime, particularly the availability and ratio of required nutrients, the absence of contaminants, and the provision of appropriate amounts of feed. Moreover, differences in larvae performance could be attributed to dissimilarities in rearing conditions and substrate properties in terms of particle size, and substrate moisture among other factors. The distinguishable experimental approaches by the researchers have to be standardized or at least kept in mind to make an unbiased comparison of results.

5. Conclusions

For an optimized and sustainable production of BSF as a source of food and feed, ample research on available side streams and appropriate rearing conditions is crucial. Although the earlier studies conducted on BSF rearing provide good information on their performances, it is clear that the protocols and approaches in measuring the listed rearing variables differed tremendously. These differences make it challenging to compare the results between studies but even harder to translate knowledge to an industrial application. Here, a comprehensive overview of biotic and abiotic variables to be kept in mind is highlighted. These include substrate chosen as feed and its properties, environmental conditions, and container or cage type to facilitate optimum circumstances for BSF growth and development.

According to the studies analyzed, the mortality rate of larvae grown on agricultural side streams is relatively low while their performances differ between the diets, which is promising. Combining different side streams in a way that their nutritional profiles complement each other could compensate individual deficiencies. In consequence, low-value agricultural side streams can be adjusted to the nutritional requirements of BSF larvae, creating a sustainable feeding system that contributes to a circular economy. In addition, this review also guides define the range of variables to be tested in the future; ensuring an improvement first in research and long-term for BSF production.

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2.2 Peer reviewed article 2 – Bioconversion efficiency and chemical composition of *Hermetia illucens* larvae fed spent mushroom substrates.

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ORIGINAL ARTICLE Open Access

Bioconversion efficiency and chemical composition of *Hermetia illucens* larvae fed spent mushroom substrates

Anjani Nayak[1], Martin Rühl[1,2] and Patrick Klüber[2*]

Abstract
Spent mushroom substrate (SMS) is a by-product remaining after harvesting mushrooms. We evaluated the effect of substituting chicken feed with 0–100% of *Pleurotus eryngii* and *Lentinula edodes* SMS at different stocking densities (200–1000 larvae/box) on development, composition, and substrate reduction of black soldier fly (*Hermetia illucens*) larvae. Although the survival rate was not significantly different, feeding pure SMS led to a low growth rate. The substitution level of SMS negatively correlated with individual larval weight, total harvested biomass, larval growth rate (LGR), feed conversion ratio (FCR), substrate reduction, and waste reduction index (WRI) except for the 20% substitution. Feeding 40% SMS resulted in the highest number of prepupae. In the density experiment, the heaviest larvae (220–239 mg fresh weight) were obtained at 200 larvae/box in the 0% SMS group. The frass residue and FCR decreased with increased density. Remarkably, when feeding 20% SMS at 250 larvae/box, the harvested biomass, LGR, and FCR did not differ significantly from the 0% SMS control, whereas some of the higher densities led to a deterioration. In fact, the frass residue, substrate reduction, and WRI were even improved at 250 larvae/box in the 20% SMS group. The chemical analyses of larvae reared on 20% SMS at 250 larvae/box showed comparable ash and fat contents and a higher protein content compared to the 0% SMS group. Accordingly, up to 20% of a standard diet such as chicken feed can be replaced by low-cost SMS without disadvantages for breeding.

Keywords Bioconversion, Black soldier fly, Circular economy, Agricultural by-products, Insect rearing, Spent mushroom substrate

Introduction

The expected global increase in demand for animal protein is 14% per person in the next 30 years (Komarek et al. 2021). The livestock-production, processing, and transport do not only consume valuable resources but are responsible for up to 45% of the greenhouse gas emissions (Gerber et al. 2013). As a consequence of the growing demand for animal protein, the demand for feed is also steadily increasing. The quantity of cereals used in animal feed is estimated to reach over 1.1 billion tons by 2050 (Makkar 2018). It is also changing land use patterns, forcing the destruction of natural ecosystems such as

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(rain)forests across the world. Therefore, the exploration of sustainable alternatives, such as agricultural by-products, to commercially used feed is urgently needed. However, the digestion and absorption of certain by-products by conventional livestock may be limiting because of the presence of anti-nutritional factors (Samtiya et al. 2020) and contaminants (Jayathilake et al. 2022). Since insects have the capability to feed on various organic matter, they are considered promising bioagents that could be part of an effective waste management in the future, thus contributing to the circular economy (Boukid et al. 2021). Such insects, which are rich in their nutritional composition, can in turn be fed to animals. Thus, reducing the dependability on importing feed ingredients.

Larvae of the black soldier fly, *Hermetia illucens* (BSF; Diptera: Stratiomyidae), are well known for their remarkable capability in thriving on diverse biodegradable materials. It is hence identified as a potential candidate for converting agricultural by-products into nutrient-rich biomass (Siddiqui et al. 2022). This conversion process also produces frass, a mixture of insect exuviae, remaining feed and feces; which can be used as a soil supplement (Klammsteiner et al. 2020). The BSF is native to South America and requires warm and humid climatic conditions (Čičková et al. 2015). They are polyphagous insects with a voracious feeding appetite at their larval stage. The life cycle under optimum conditions is around 45 days (De Smet et al. 2018). The larval stage consisting of six instars covers 14–16 days in ideal conditions (Tomberlin et al. 2002, 2009). There are several studies on the ability of BSF larvae in bioconverting by-products including restaurant wastes (Zheng et al. 2012a, b; Leong and Kutty 2020; Nguyen et al. 2013), fish offal (St-Hilaire et al. 2007), beer production wastes (Chia et al. 2018), human and livestock feces (Sheppard et al. 1994; Newton et al. 2005; Myers et al. 2008; Rehman et al. 2017), and municipal organic wastes (Kalová and Borkovcová 2013) to nutrient-rich feed alternatives. Despite the promising and versatile application possibilities of BSF larvae in waste management, feeding of insects is regulated within the legislative framework of the European Union (EU). Since industrially reared insects are classified as "farmed animals", the same general rules and restrictions apply as for conventional livestock. Thus, farmed insects are subject to the feed ban rules laid down in Article 7 and Annex IV to Regulation (EC) No 999/2001 and, additionally, to animal feeding rules laid down in Regulation (EC) No 1069/2009. According to the abovementioned regulations, insects are not allowed to be fed with slaughterhouse or rendering-derived materials, feces, catering waste, and unsold supermarket or industrial products that are containing fish or meat (Veldkamp et al. 2022). On one hand, it constricts the available options to feed the insects sustainably without depending on importing feed grains. On the other hand, it provides a gateway to look at the other available plant-based by-products as feed for the larvae. Unfortunately, there are plenty of agricultural by-products ending up in landfills or are burned. Besides losing valuable resources there is also pollution caused by the way these wastes are currently handled (Nguyen et al. 2015). Therefore, identifying the by-products and utilizing them as an alternative feed source would solve multiple problems. This way the competition between the resources for food and feed production can also be minimized.

Among all the food products with emerging demand, mushrooms being healthy and rich in protein are particularly attractive. According to Market Data Forecast (2022), the European mushroom industry (consisting mainly of button, shiitake, and oyster mushrooms) had a market value of USD 13.7 billion in 2018 and is estimated to reach USD 21.7 billion by 2026. Mushroom cultivation includes a substrate on which the mycelium grows. The substrate consists of lignocellulose-rich wastes like wood logs, rice straw, horse and chicken manure, wheat straw, wood chips, sawdust, seed husks, coffee pulp, corn cobs, and bagasse among many others (Oei 2003). To ensure an optimal milieu for mycelial growth and fruiting body formation, substrate properties and processing steps must be adjusted to the requirements of the corresponding mushroom. Once the mushrooms have been harvested, the by-product remaining is the growing substrate, also known as spent mushroom substrate (SMS). Astonishingly, the production of 1 kg of mushrooms generates 3–5 kg of waste (Beckers et al. 2019; Leong et al. 2022). The amount of SMS waste generated in the EU alone is more than 3 billion kilograms per year (Ceurstemont 2020). This tremendous amount of waste has to be managed and mostly ends up in landfills, costing 10–50 € per ton (Beckers et al. 2019). SMS is already being examined for its suitability as fertilizer, alternative feed source, in pest management (Rinker 2017), for potential reuse in mushroom cultivation (Rinker 2017; Zied et al. 2020), and in ethanol production (Grover et al. 2015). Although there are several concepts to prevent SMS from being dumped in landfills, these solutions are not applied in practice. Another barely studied option is to utilize SMS as insect feed. In this way, the extensively produced and locally available by-product of the mushroom industry can be upcycled into high-value insect protein and fat within a short time.

The BSF larvae could become a biological tool in reducing the amount of SMS that is otherwise discarded. Encompassing all publications on feed trials with BSF larvae, there are only two studies so far highlighting the use of SMS (Li et al. 2021; Mao et al. 2023). In the former study, the SMS was mixed with canteen waste. However, the use of food waste from restaurants as larval feed is

not allowed under current EU regulations. Commercial BSF producers predominantly use chicken or pig feed as diet for larval fattening. The high costs of the feed and imported grains make it expensive and unsustainable, resulting in producers having difficulty competing with conventional animal and plant protein sources. The use of SMS as a complete substitute or, depending on the growth performance, as a feed supplement could therefore considerably reduce the costs of insect production.

The initial experiment focused on the ability of BSF larvae to survive on diets consisting of chicken feed replaced with different ratios of SMS. Subsequently, we aimed to find the ideal stocking density (200–1000 individuals) per 100 g feed to obtain the optimum survivability, growth performance, feed conversion efficiency, and waste reduction. Additionally, the larval nutritional composition was analysed to evaluate the potential of BSF larvae as novel feed.

Materials and methods

The experiments were conducted between July 2021 and June 2022 at the Fraunhofer Institute for Molecular Biology and Applied Ecology (Giessen, Germany).

Insects and feeding substrates

Eight-day-old BSF larvae were shipped vacuum-packed by Hermetia Baruth GmbH (Baruth/Mark, Germany) and used for the feeding trials. After receiving the larvae, they were unpacked and transferred into polypropylene boxes and kept for 1 h in a climate chamber at 27 ± 1 °C and 65 ± 5% relative humidity to recover natural behavior. To ensure uniform size and mass they were separated using a vibratory sieve shaker with a mesh size between 1.0 and 1.4 mm (AS 200, Retsch, Haan, Germany). The mean individual larval weight was determined by weighing five replications of 100 larvae with a precision balance (ALJ 160-4 A, Kern & Sohn, Balingen-Frommern, Germany). BSF larvae that were used for feeding trials had an average individual weight of 6.0 ± 1.2 mg.

The diets tested consisted of chicken feed (CF; GoldDott Eierglück, DERBY Spezialfutter, Muenster, Germany), SMS, or combinations thereof, with CF serving as a high-quality reference diet (Klüber et al. 2023). The SMS was obtained from an organic mushroom farm (Löckes Bio-Vertriebs GmbH, Büttelborn, Germany). For this, the SMS was collected as a by-product of king oyster (*Pleurotus eryngii*) and shiitake (*Lentinula edodes*) production on the same day of harvesting the fruiting bodies. First, remaining fruiting bodies attached to the surface of the SMS blocks were removed. Subsequently, the blocks were disintegrated by hand and dried immediately at 80 °C for 10 h in a laboratory kitchen oven (HB674GBS1, Siemens AG, Munich, Germany). The CF and dried SMS were prepared by grinding in a Mockmill 200 (Wolfgang Mock, Otzberg, Germany) and a Thermomix TM6 (Vorwerk, Wuppertal, Germany) to a particle size of 0.1–0.8 mm. The substrates were stored in airtight containers at room temperature until use. King oyster SMS and shiitake SMS were mixed in a 1:1 ratio for the feeding trials and are summarized hereafter as SMS.

Gradual substitution of standard feed with SMS

First, it was intended to clarify how the growth performance and survivability of larvae are affected if different proportions of CF are replaced by SMS. A total of six substitution levels were examined (0%, 20%, 40%, 60%, 80%, 100%), with 0% substitution representing the pure CF control diet.

We weighed 150 g (dry matter; DM) of each diet into four conical 12.5×12.5×11.5 cm (l × w × h) replicate boxes (BDPN24, MegaView Science, Taichung, Taiwan) and adjusted the moisture to 60% by adding warm tap water. Subsequently, 200 eight-day-old BSF larvae were transferred in each box by spreading them over the substrate surface. The donut-shaped lid was fitted with a circular 9 cm mesh insert for a proper air circulation. All boxes were incubated in a climate chamber under controlled conditions of 27 ± 1 °C and 65 ± 5% relative humidity in darkness. The boxes were rearranged randomly on every second day. No additional feed or water was added throughout the experiment. Based on the findings of preliminary experiments, larvae of all dietary groups were harvested ten days after the start of feeding (first prepupae observed). For this purpose, larvae were collected individually from the frass using spring steel tweezers, cleaned of coarse impurities, weighed, counted, and cold-inactivated at − 20 °C. The survival rate was determined as the percentage of larvae recorded during harvesting. The total harvested biomass represents the total amount of harvested insect biomass per box (larvae, prepupae, and pupae). On this basis, individual larval weight was calculated by dividing the total harvested biomass by the number of surviving larvae (given as fresh matter; FM). The frass residue is defined as the amount of digested substrate remaining after harvesting the larvae. The weight of the frass was obtained by differential weighing with the empty box. After mixing and homogenization, the moisture content was determined (DAB 100-3, Kern & Sohn, Balingen-Frommern, Germany) to reveal the dry weight of the frass residue. For the calculation of conversion efficiencies and substrate reductions it was assumed that the entire feed provided had been consumed by the BSF larvae (Oonincx et al. 2015). All data were recorded in four biological replicates. Besides larval growth rate (Eq. 1), the following parameters depicted in Eqs. (2–5) were calculated based on DM (Oonincx et al. 2015; Mohd-Noor et al. 2017; Jucker et al. 2020):

$$LGR\,(Larval\ growth\ rate\ (g/d)) = \frac{(Total\ biomass\ harvested\ (g) - Initial\ biomass\ inoculated\ (g))}{Number\ of\ rearing\ days\ (d)} \quad (1)$$

$$FCR\,(Feed\ conversion\ ratio) = \frac{Total\ feed\ provided\ (g)}{(Total\ biomass\ harvested\ (g) - Initial\ biomass\ inoculated\ (g))} \quad (2)$$

$$ECI\,(Efficiency\ of\ conversion\ of\ the\ ingested\ feed) = \frac{(Total\ biomass\ harvested\ (g) - Initial\ biomass\ inoculated\ (g))}{(Total\ feed\ provided\ (g) - Frass\ residue\ (g))} \quad (3)$$

$$SR(Substrate\ reduction\ (\%)) = \frac{(Total\ feed\ provided\ (g) - Frass\ residue\ (g)) \times 100}{Total\ feed\ provided\ (g)} \quad (4)$$

$$WRI\,(Waste\ reduction\ index) = \frac{(Total\ feed\ provided\ (g) - Frass\ residue\ (g))}{Number\ of\ rearing\ days\ (d)} \quad (5)$$

Optimization of stocking density

After demonstrating that BSF larvae were able to survive on substrate substituted with SMS, we aimed to identify the best combination of stocking density and SMS substitution level at which larvae grow best while exhibiting high conversion efficiency and substrate reduction. Since larval growth in the gradual substitution experiment was strongly inhibited when the SMS level was ≥60%, chicken feed was replaced by 50% or less to find density-related effects. Feed, frass and larval samples were stored at − 20 °C until further use.

A total of four different density populations (200, 250, 400, 1000 larvae/box) were combined with four SMS substitution levels (0%, 20%, 40%, 50%). The feeding trials were conducted under identical conditions as described in the gradual substitution experiment, with only 100 g of feed being provided. All data were recorded in four biological replicates, besides the 400 larvae/box approach which was replicated three times. The corresponding data collection and calculations were also performed according to the same methodology mentioned above.

Chemical analysis

The highest individual weight of larvae was found at a density of 250 larvae/box within the SMS containing groups. Moreover, when feeding 20% SMS at 250 larvae/box, the harvested biomass, LGR and FCR did not differ significantly from the 0% SMS control, whereas some of the higher densities led to a decrease. In fact, the frass residue, substrate reduction and WRI were even improved at 250 larvae/box in the 20% SMS group. Therefore, the feed, larvae, and frass were examined for their nutritional composition at a density of 250 larvae/box. Among the harvested biomass, prepupae and pupae were not included in the chemical analyses. All chemical analyses were conducted in triplicates except pH measurements which were done in duplicates.

All parameters studied refer to DM (except dry matter content). Feed (~ 150 g), L5 larvae (~ 100 g), and frass (~ 200 g) of the four replicates within a group were quantitatively transferred to a box and pooled. The samples were then ground with a mortar under liquid nitrogen and lyophilized at 0.8 mbar vacuum pressure and − 85 °C condenser temperature (Delta LSCplus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 72 h. Prior to lyophilization, the moisture content was determined thermogravimetrically (M35 Moisture Analyzer, Sartorius, Göttingen, Germany). For the analyses of crude ash, protein, and fat, 1 g of lyophilized feed and frass, as well as 0.5 g of lyophilized larvae were used. However, 3 g of the samples was used to determine crude fiber.

The initial pH value of the feed and the pH value of the frass after harvesting were measured (S47-K, Mettler-Toledo, Gießen, Germany). For this, 5 g of the sample was added to 40 mL distilled water, mixed thoroughly and centrifuged to measure the pH of the supernatant. The crude fiber content was determined according to the method of Scharrer-Kürschner, as described elsewhere (Matissek et al. 2018). The crude ash was measured by pre-ashing the samples over a Bunsen burner and incinerating them twice for 6 h at 550 °C in a muffle furnace (L 9/11, Nabertherm, Lilienthal, Germany). The content was then calculated by differential weighing. The determination of total nitrogen was conducted according to the method of Kjeldahl (Matissek et al. 2010), where the samples were subjected to digestion in concentrated sulfuric acid, followed by steam distillation (behrotest S5, behr Labor-Technik, Düsseldorf, Germany) and titration (TitroLine 5000, SI Analytics, Mainz, Germany). We used conversion factors based on the corresponding amino acid profile to calculate the crude protein content. Hydrolysis of samples (20–40 mg) for amino acid profiling was achieved using three different protocols. The total hydrolysis covers all amino acids except tryptophan, cysteine and methionine. Consequently, alkaline and oxidative digestion were performed for tryptophan and both sulfur-containing amino acids, respectively (Seidel et al. 2023). The released amino acids were then separated by a cation exchange column, derivatized with ninhydrin and detected at 570 nm–440 nm (proline) in the amino acid analyzer (S433, Sykam Chromatographie Vertriebs GmbH, Fürstenfeldbruck, Germany). To determine the crude fat content according to Weibull-Stoldt, the samples were disintegrated in boiling 4 mol·L^{-1} hydrochloric acid for 30 min. Thereafter, samples were filtered, washed neutrally with hot demineralized water, and dried to constant weight for ~ 1 h at 105 °C. The extraction of the fat was performed in a behr E6 system using petroleum ether

(behr Labor-Technik, Düsseldorf, Germany) and the content was determined gravimetrically. The extracted lipids were dissolved in 3 mL of iso-octane and stored at − 20 °C. According to the method of Hammer et al. (2021), fatty acids (FAs) bound in triglycerides were converted into fatty acid methyl esters (FAMEs) by means of alkali-catalyzed transmethylation. The generated FAMEs were separated by gas chromatography (7890B, Agilent, Waldbronn, Germany) using an Agilent VF-WAXms column (30 m × 0.25 mm, 0.25 µm film thickness) with 1.56 mL/min as carrier gas flow. After the column, the gas flow was split into two halves. One led to an Agilent 5977B MSD detector, while the other half led to an olfactory detection port that was not used for FA analysis. The temperature program and detector parameters have been described elsewhere (Hammer et al. 2021). The 37 Component FAME Mix (Supelco, Bellefonte, Pennsylvania, USA) was used to identify the FAMEs.

Data processing and statistics

Data curation and processing were carried out using Excel 2016 (Microsoft, Redmond, WA, USA). Statistical analysis and visualization were conducted in OriginPro 2022b (OriginLab, Northampton, MA, USA). The Shapiro–Wilk test was applied to verify whether the data of a population is normally distributed. The homogeneity of variance was calculated with Levene's test. Parameters recorded in the gradual substitution experiments and chemical analyses were subjected to a one-way analysis of variance (ANOVA) and means were separated using the Tukey's test (homogeneous variance). If the variance was inhomogeneous, a one-way Welch's ANOVA followed by Games–Howell post hoc test were conducted. The Pearson product–moment correlation was used for determining linear relationships between variables (Hilgers et al. 2019). Data obtained from the stocking density experiments were subjected to a two-way ANOVA and means were separated using the Tukey's test. An error level of $\alpha = 0.05$ for statistical significance was set for all analyses.

Results

Feeding gradual substitutions of SMS

First, the extent to which a standard feed can be substituted by SMS was examined in order to achieve identical or improved growth performance and biomass yield. All groups received exactly the same amount of 150 g DM of the corresponding diet, with high-quality CF being gradually replaced by 0–100%. The survival rate of pure CF and all substitution groups did not differ significantly, whereas the 40–80% SMS groups were found to have ≥3% higher survival rates compared to pure SMS ($F_{5,18} = 4.84$; $P \leq 0.04$; Table 1). There was no linear correlation between the survival rate and the SMS level ($r = -0.14$; $P = 0.51$). The highest individual larval weight (223–234 mg FM) was obtained in the 20% SMS group and was in fact 11.5% greater than the CF larvae ($F_{5,18} = 1012.06$; $P < 0.00009$). Contrastingly, individual larval weight was strongly negatively correlated with increasing SMS level ($r = -0.96$; $P < 0.00001$) and decreased drastically by up to 93.2% and 93.9% when compared to the CF and 20% SMS groups, respectively ($F_{5,18} = 1012.06$; $P < 0.00001$).

Ten days after starting the experiment, no prepupae or pupae were detected in populations provided pure CF and 80–100% SMS, while some larvae (≤6.5%) had already developed into prepupae in groups fed with 20% and 60% SMS. Here, the number of prepupae varied between 1–10/box. Feeding 40% SMS resulted in a significant acceleration of development, since 27% of the larvae (9–42 larvae/box) had already reached the prepupal stage on the day of harvest ($F_{5,18} = 8.78$; $P < 0.008$). The number of emerging prepupae was not correlated with the SMS

Table 1 Growth performance and bioconversion efficiency of BSF larvae fed different SMS substitution levels (0–100%)

Parameters	SMS substitution (%)					
	0	20	40	60	80	100
Total amount of feed (g DM)	150.00 ± 0.00^{a}	150.00 ± 0.00^{a}	150.00 ± 0.00^{a}	150.00 ± 0.00^{a}	150.00 ± 0.00^{a}	150.00 ± 0.00^{a}
Survival rate (%)	97.25 ± 0.75^{a}	98.75 ± 1.30^{a}	99.25 ± 1.03^{ab}	99.75 ± 0.43^{ab}	99.00 ± 1.17^{ab}	96.00 ± 1.62^{ac}
Individual larval weight (mg FM)	205.39 ± 2.22^{a}	229.08 ± 4.65^{b}	178.34 ± 2.35^{c}	115.43 ± 6.91^{d}	59.21 ± 7.02^{e}	14.06 ± 0.72^{f}
Number of prepupae and pupae (%)	0.00 ± 0.00^{a}	4.75 ± 2.86^{a}	27.00 ± 14.30^{b}	6.50 ± 3.57^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Total harvested biomass						
(g FM)	39.95 ± 0.47^{a}	45.25 ± 1.25^{b}	35.40 ± 0.79^{c}	23.03 ± 1.38^{d}	11.48 ± 1.13^{e}	2.70 ± 0.15^{f}
(g DM)	14.29 ± 0.22^{a}	17.59 ± 0.44^{b}	13.36 ± 0.58^{a}	8.14 ± 0.68^{c}	2.89 ± 0.40^{d}	0.66 ± 0.03^{e}
Frass residue (g DM)	122.68 ± 1.20^{a}	80.37 ± 2.22^{b}	83.78 ± 0.59^{c}	99.67 ± 0.67^{d}	128.91 ± 0.83^{e}	145.22 ± 1.00^{f}
Larval growth rate (LGR; g FM/d)	3.87 ± 0.05^{a}	4.41 ± 0.13^{b}	3.42 ± 0.08^{c}	2.18 ± 0.14^{d}	1.03 ± 0.11^{e}	0.15 ± 0.02^{f}
Feed conversion ratio (FCR)	3.76 ± 0.05^{a}	3.32 ± 0.09^{b}	4.24 ± 0.09^{c}	6.54 ± 0.41^{d}	13.21 ± 1.39^{e}	55.72 ± 3.00^{f}
Efficiency of conversion of the ingested feed (ECI)	0.52 ± 0.02^{a}	0.26 ± 0.01^{b}	0.20 ± 0.01^{c}	0.16 ± 0.01^{d}	0.14 ± 0.02^{d}	0.15 ± 0.04^{bcd}
Substrate reduction (%)	18.21 ± 0.80^{a}	46.42 ± 1.48^{b}	44.15 ± 0.39^{c}	33.56 ± 0.45^{d}	14.06 ± 0.56^{e}	3.18 ± 0.67^{f}
Waste reduction index (WRI; g DM/d)	2.73 ± 0.12^{a}	6.96 ± 0.22^{b}	6.62 ± 0.06^{c}	5.03 ± 0.07^{d}	2.11 ± 0.08^{e}	0.48 ± 0.10^{f}

Data represent means ± SD ($n = 200$). Different letters (a–f) within a row indicate statistically significant differences between groups ($P < 0.05$; one-way ANOVA or Welch's ANOVA)

level ($r=-0.15$; $P=0.49$). Both fresh matter and dry matter of the total harvested biomass were highest in the 20% SMS group at 43.3–46.6 g FM ($F_{5,18} = 907.75$; $P \leq 0.00003$) and 17.0–18.2 g DM ($F_{5,18} = 672.44$; $P<0.00001$), respectively, and exceeded the yield of the CF group by 13.3–23.1% (Table 1). The total harvested biomass was strongly negatively correlated with increasing SMS level and decreased by up to 94.0% FM ($r=-0.95$; $P<0.00001$) and 96.2% DM ($r=-0.93$; $P<0.00001$) when compared to the CF and 20% SMS groups, respectively. Interestingly, the frass residue in groups fed 20% and 40% SMS was substantially reduced by 34.5% and 31.7% respectively compared to the CF group ($F_{5,18} = 1396.15$; $P<0.00001$). The amount of frass residue was positively correlated with increasing SMS level ($r=0.97$; $P<0.00001$), whereby feeding 100% SMS resulted in a difference of only 4.8 g DM between the weighed frass residue and the feed initially supplied. With 4.2–4.6 g FM/d, the larval growth rate (LGR) was highest in the 20% SMS treatment and exceeded the CF control by 14% ($F_{5,18} = 890.35$; $P=0.00003$). In general, the LGR decreased sharply with an increase in SMS level ($r=-0.99$; $P<0.00001$). When feeding pure SMS, reductions of 96.1% and 96.6% were observed compared to CF and 20% SMS, respectively ($F_{5,18} = 890.35$; $P<0.00001$).

Besides the biomass yield and larval development, parameters such as utilization efficiency and waste reduction also contribute to circular economy. The 20% SMS substitution improved the feed conversion ratio (FCR) by 11.7% (3.2–3.5) in comparison to pure CF ($F_{5,7.8} = 197.13$; $P=0.009$). FCR correlated positively with increasing SMS level ($r=0.81$; $P=0.00002$) and reached values up to 14.8- and 16.8-fold higher than in the CF and 20% SMS groups, respectively, reflecting the low growth rates and high frass residues reported above. Larvae of all substitution groups were found to have a significantly ≥ 50% lower efficiency of conversion of the ingested feed (ECI) than those provided pure CF ($F_{5,8.0} = 167.67$; $P \leq 0.0003$). Here, ECI was negatively correlated with increasing SMS level ($r=-0.83$; $P=0.00001$). The 20–60% SMS groups showed 1.8–2.5-fold higher substrate reductions compared to their CF counterparts ($F_{5,18} = 1398.30$; $P<0.00001$), wherein feeding 20% SMS led to the greatest reduction (Table 1). The same was also noted for the waste reduction index (WRI). In fact, the 20–60% SMS groups achieved 1.8–2.5-fold higher daily reduction values than the CF group ($F_{5,18} = 1403.29$; $P<0.00001$), with 20% SMS resulting in the greatest WRI. Both substrate reduction and WRI were negatively correlated with increasing SMS level ($r=-0.97$; $P<0.00001$).

Optimization of stocking density

While the majority of parameters recorded were significantly improved by substituting 20% SMS, it became apparent that the values decreased markedly in the 40–60% SMS groups (Table 1). Therefore, it was decided to consider a maximum replacement of 50% for further trials. As several diets were not fully digested by the larvae at a density of 200 individuals/box, the amount of feed supplied was reduced from 150 to 100 g DM and variations in stocking density (200–1000 larvae/box) were tested.

All groups received exactly the same amount of 100 g DM of the corresponding diet. In general, survival rate of all groups was high with 92–100%. We found statistically significant differences in the average survival rate for the density ($F_3=31.38$; $P<0.00001$), but not for the SMS level ($F_3=1.29$; $P=0.29$). No significant interaction between these terms was calculated ($F_9=1.17$; $P=0.34$). A Tukey post-hoc test revealed that the highest density reduced the survival rate significantly compared to lower density groups when fed with 0% ($P \leq 0.0003$) or 40% ($P \leq 0.00003$) SMS diets (Fig. 1A). Individual weight differed significantly for the density ($F_3=527.78$; $P<0.00001$) and the SMS level ($F_3=347.00$; $P<0.00001$), wherein interactions of both variables were verified ($F_9=10.81$; $P<0.00001$). The highest individual larval weight (220–239 mg FM) was obtained at 200 larvae/box in the 0% SMS group, which was ≥ 16.8% greater than in all SMS containing diets. In general, the weight of the larvae decreased successively with increasing SMS level and density. Feeding 50% SMS at 1000 larvae/box resulted in a weight reduction of 50.3% and 77.1% ($P<0.00001$) compared to the highest and lowest density within the CF control, respectively (Fig. 1B). Besides the 0% SMS group, individual weight was highest at 200–250 larvae/box in all diets containing SMS ($P \leq 0.0002$).

The number of larvae that developed into prepupae or pupae differed significantly for the density ($F_3=855.72$; $P<0.00001$) and the SMS level ($F_3=101.40$; $P<0.00001$), and both variables interacted with each other ($F_9=54.02$; $P<0.00001$). When comparing different densities within the same SMS level, the number of prepupae or pupae counted was significantly higher at 1000 larvae/box than in all other groups ($P<0.00001$). At 200–400 larvae/box, SMS substitution had a minor effect on the appearance of prepupae and pupae, whereas their quantity increased with rising SMS level at 1000 larvae/box, reaching a maximum of 496 at 40% SMS (Fig. 1C). The same was observed for the relative proportion of prepupae or pupae, which was significantly affected by the density ($F_3=285.59$; $P<0.00001$), the SMS level ($F_3=124.27$; $P<0.00001$), and their interactions ($F_3=19.76$; $P<0.00001$). In general, highest proportions for all densities were found at 40% SMS. In the 200–400 larvae/box densities, prepupae or pupae formation varied between the SMS levels in a non-linear pattern. Feeding 40% SMS led to a significant increase of ≥ 66.2% at 250 larvae/box

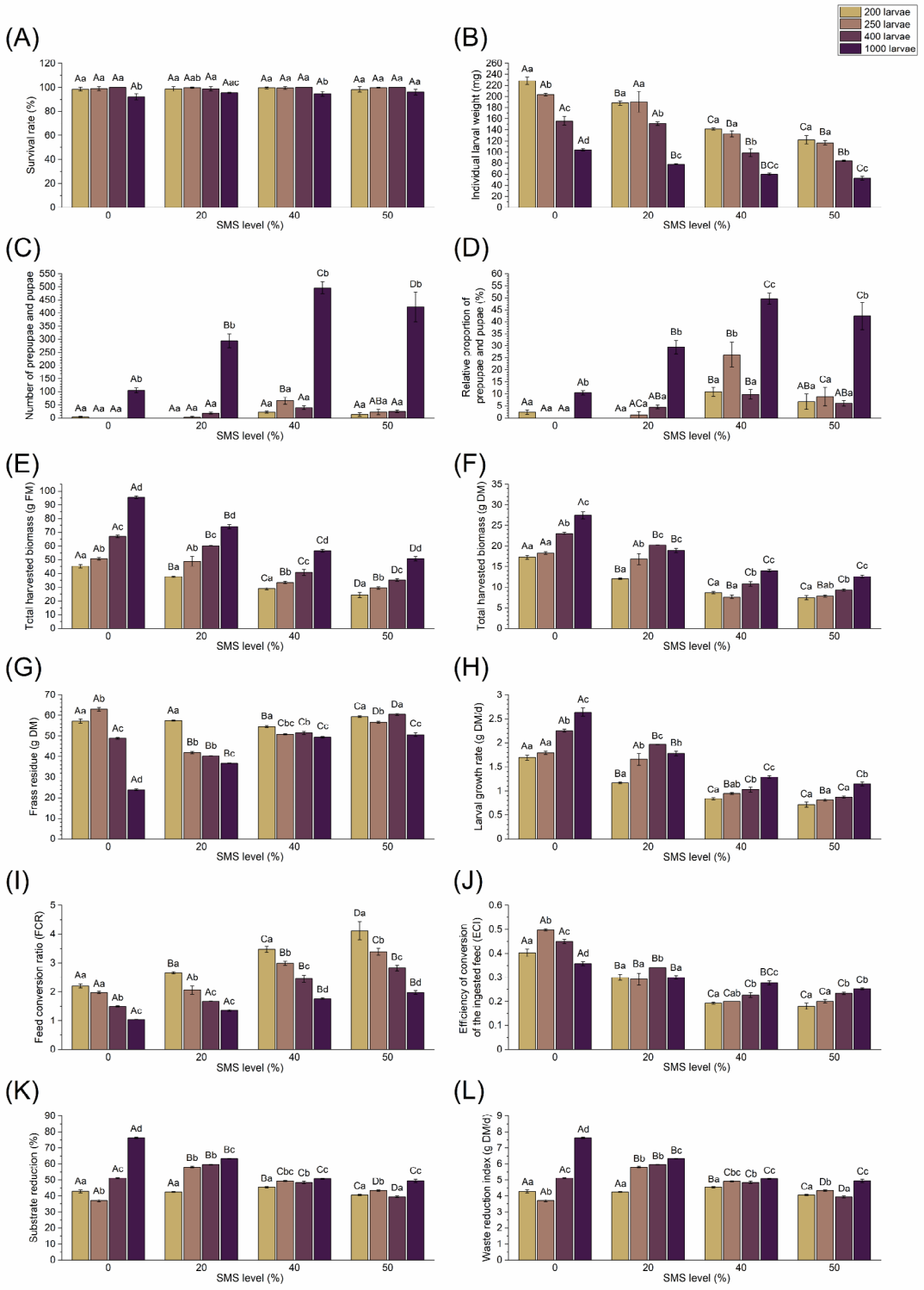

Fig. 1 Performance and bioconversion of BSF larvae under various densities (200–1000 larvae/box) and SMS substitutions (0–50%). Data represent means ± SD. (**A**) survival rate, (**B**) individual larval weight, number (**C**) and relative proportion (**D**) of prepupae and pupae, total harvested biomass in FM (**E**) and DM (**F**), frass residue (**G**), larval growth rate (**H**), feed conversion ratio (**I**) and efficiency of conversion (**J**), substrate reduction (**K**) and waste reduction index (**L**). Different letters indicate statistically significant differences, with lowercase letters (a–d) referring to data within an SMS group and uppercase letters (A–D) referring to data within a density group ($P < 0.05$; two-way ANOVA)

compared to the other SMS levels ($P<0.00001$). The proportion of prepupae or pupae was highest at 1000 larvae/box compared to all other densities independent of the SMS level ($P\leq0.04$; Fig. 1D).

Fresh and dry matter of the total harvested biomass were highest in the 0% SMS diet, followed by 20% SMS substitution with a mean reduction of 14.7% FM and 20.9% DM, respectively. Both variables were significantly affected by the density ($F_3=1321.00$; $P<0.00001$ and $F_3=388.37$; $P<0.00001$), the SMS level ($F_3=968.80$; $P<0.00001$ and $F_3=1258.58$; $P<0.00001$), and interactions thereof ($F_9=41.33$; $P<0.00001$ and $F_9=31.00$; $P<0.00001$). Here, the harvested biomass in all groups grew between 96.4 and 111.0% FM ($P<0.03$) and 57.2–68.2% DM with increasing density, whereas higher SMS levels led to a reduction of biomass yield (Fig. 1E, F). As a consequence, biomass decreased by up to 47.4% FM and 59.8% DM when CF was replaced by 50%. However, biomass yield DM did not differ between the highest SMS levels of 40–50% within the same density ($P\geq0.13$). We found statistically significant differences in the average frass residue for the density ($F_3=2042.79$; $P<0.00001$) and the SMS level ($F_3=1024.58$; $P<0.00001$), wherein interactions of both variables were verified ($F_9=625.53$; $P<0.00001$). In general, the frass residue decreased while the density increased throughout all diets, particularly in groups fed with 0–20% SMS level. When comparing the lowest and highest densities tested, the amount of frass residue was reduced by 58.4% ($P<0.00001$) and 36.0% ($P<0.00001$) for 0% SMS or 20% SMS, respectively (Fig. 1G). With 27.0% difference, the reduction of frass residue at 20% SMS and 250 larvae/box was remarkably higher compared to the lowest density ($P<0.00001$) and all other diet groups ($P<0.00001$). An SMS level of ≥40% led to higher amounts of frass residue across all densities.

LGR was significantly affected by the density ($F_3=317.93$; $P<0.00001$) and the SMS level ($F_3=1326.80$; $P<0.00001$), wherein interactions of both variables were calculated ($F_9=33.73$; $P<0.00001$). Here, the LGR increased with higher densities and peaked at 1000 larvae/box, whereby the 0% SMS group reached a≥32.4% higher rate than their counterparts ($P<0.00001$). However, LGR of the 20% SMS group did not differ significantly between 250 and 1000 larvae/box ($P=0.22$), while 400 larvae/box peaked and exceeded both by 16.0% and 9.7%, respectively ($P\leq0.01$). We observed that the LGR decreased with increasing SMS level, although it did not differ between the highest SMS levels of 40–50% within the same densities ($P\geq0.09$; Fig. 1H). SMS level ($F_3=368.10$; $P<0.00001$), density ($F_3=434.06$; $P<0.00001$), as well as their interactions ($F_9=9.01$; $P<0.00001$) significantly affected the FCR. The FCR improved with increasing density independent of the diet. For this, the best ratios were obtained in the 0–20% SMS groups at 1000 larvae/box ($P\leq0.004$). Importantly, the conversion ratio of 0% and 20% SMS at 250–1000 larvae/box did not differ significantly ($P\geq0.10$). In contrast, the FCR of the 40–50% SMS diets increased significantly ($P\leq0.006$; Fig. 1I). The ECI was affected by the density ($F_3=35.17$; $P<0.00001$) and SMS level ($F_3=1027.76$; $P<0.00001$), whereby interactions of both variables were verified ($F_9=51.06$; $P<0.00001$). Generally, ECI was highest in the 0% SMS group at 250 larvae/box ($P\leq0.0004$). Besides 400 larvae/box ($P\leq0.005$), no differences were determined between the densities within the 20% SMS group ($P>0.99$). No linear effect of the density was determined throughout the diets. Feeding higher SMS substitutions led to a successive decline in ECI, although no further decrease was found for larvae provided with 50% SMS instead of 40% SMS ($P\geq0.21$; Fig. 1J).

The proportion of substrate reduced differed significantly for the density ($F_3=2050.27$; $P<0.00001$) and the SMS level ($F_3=1028.75$; $P<0.00001$), and both variables interacted with each other ($F_9=631.30$; $P<0.00001$). Besides the 1000 larvae/box density, no linear effect of substrate reduction with SMS level was observed. As density increased, the substrate reduction improved independently of the diet. The substrate reduction was highest at 1000 larvae/box ($P\leq0.003$), with 0% SMS exceeding the other diets by ≥17.1% ($P<0.00001$). It was found that feeding 20% SMS at 250–400 larvae/box resulted in a significantly≥13.4% higher substrate reduction in comparison to all other diets within the same densities ($P<0.00001$; Fig. 1K). The same pattern was noticed for the WRI. The WRI differed significantly for the density ($F_3=2089.72$; $P<0.00001$) and the SMS level ($F_3=1051.88$; $P<0.00001$), and both variables interacted with each other ($F_9=640.85$; $P<0.00001$). As density increased, WRI improved regardless of the diet, with 1,00 larvae/box being the highest ($P\leq0.004$). In general, the WRI was highest for the 0% SMS diet, followed by 20% SMS substitution, where the indices did not differ significantly at 200 larvae/box ($P>0.99$; Fig. 1L). Feeding 20% SMS at 250–400 larvae/box resulted in a significantly higher WRI than all other diets when comparing the same densities ($P<0.00001$).

Nutritional analysis

The highest individual weight of larvae was found at a density of 250 larvae/box within the SMS containing groups. Furthermore, when feeding 20% SMS at 250 larvae/box, the harvested biomass, LGR and FCR did not differ significantly from the 0% SMS control, whereas some of the higher densities led to a decrease. In fact, the frass residue, substrate reduction and WRI were even improved at 250 larvae/box in the 20% SMS group. Therefore, the nutritional composition of the feed, larvae, and frass at a density of 250 larvae/box were examined.

Table 2 Chemical composition of the diets substituted with 0–50% SMS

Parameters	SMS substitution (%)			
	0	20	40	50
Moisture (%)	13.5 ± 0.5[a]	16.1 ± 0.0[a]	13.3 ± 0.3[a]	13.2 ± 0.4[a]
Crude ash (%DM)	13.1 ± 0.1[a]	11.1 ± 0.1[b]	10.1 ± 0.3[c]	8.8 ± 0.3[d]
Crude fiber (%DM)	2.4 ± 0.4[a]	9.3 ± 0.3[b]	15.9 ± 1.2[c]	18.7 ± 0.3[c]
Crude fat (%DM)	2.5 ± 0.2[a]	1.9 ± 0.0[ab]	1.2 ± 0.4[ab]	1.2 ± 0.2[b]
Total nitrogen (%DM)	2.7 ± 0.0[a]	2.3 ± 0.0[b]	2.1 ± 0.1[c]	1.9 ± 0.1[c]
Kjeldahl factor	6.51	6.52	6.52	6.43
Crude protein (%DM)	17.3 ± 0.1[a]	15.0 ± 0.3[b]	13.4 ± 0.4[c]	12.0 ± 0.7[d]

Besides the moisture content, analyzed parameters are given as a percentage of DM (%DM). Data represent means ± SD ($n = 3$). Different letters (a–d) within a row indicate statistically significant differences between groups ($P < 0.05$; one-way ANOVA or Welch's ANOVA)

Table 3 Chemical composition of BSF larvae reared on diets substituted with 0–50% SMS

Parameters	SMS substitution (%)			
	0	20	40	50
Moisture (%)[†]	6.4 ± 0.3[a]	6.6 ± 0.8[a]	7.1 ± 0.1[a]	7.8 ± 0.0[b]
Crude ash (%DM)	15.7 ± 0.1[a]	14.9 ± 0.2[a]	17.5 ± 0.4[b]	17.6 ± 0.5[b]
Crude fiber (%DM)	3.4 ± 0.2[a]	4.6 ± 0.2[b]	5.7 ± 0.3[c]	5.8 ± 0.3[c]
Crude fat (%DM)	21.0 ± 0.5[a]	20.8 ± 1.0[a]	14.2 ± 0.9[b]	11.2 ± 0.3[c]
Total nitrogen (%DM)	5.8 ± 0.1[a]	6.3 ± 0.1[b]	7.2 ± 0.1[c]	7.2 ± 0.1[c]
Kjeldahl factor	5.84	5.85	5.86	5.84
Crude protein (%DM)	34.0 ± 0.8[a]	36.9 ± 0.8[b]	42.2 ± 0.5[c]	41.9 ± 0.8[c]

Besides the moisture content, analyzed parameters are given as a percentage of DM (%DM). Data represent means ± SD ($n = 3$). Different letters (a–c) within a row indicate statistically significant differences between groups ($P < 0.05$; one-way ANOVA or Welch's ANOVA)

[†]After lyophilization

Table 4 Chemical composition of BSF frass obtained from diets substituted with 0–50% SMS

Parameters	SMS substitution (%)			
	0	20	40	50
Moisture (%)[†]	13.9 ± 1.3[a]	13.3 ± 0.2[ab]	15.6 ± 0.3[a]	15.7 ± 0.1[ac]
Crude ash (%DM)	21.6 ± 0.0[a]	25.7 ± 1.0[ab]	19.7 ± 0.6[ac]	16.2 ± 0.5[d]
Crude fiber (%DM)	3.2 ± 0.4[a]	21.5 ± 0.8[b]	29.9 ± 0.7[c]	30.0 ± 0.7[c]
Crude fat (%DM)	0.8 ± 0.0[a]	0.8 ± 0.1[a]	1.0 ± 0.3[a]	0.9 ± 0.1[a]
Total nitrogen (%DM)	2.5 ± 0.1[a]	2.0 ± 0.0[b]	1.7 ± 0.0[c]	1.7 ± 0.0[c]
Kjeldahl factor	5.64	5.82	5.91	5.97
Crude protein (%DM)	14.0 ± 0.4[a]	11.9 ± 0.2[b]	9.8 ± 0.2[c]	10.3 ± 0.3[c]

Besides the moisture content, analyzed parameters are given as a percentage of DM (%DM). Data represent means ± SD ($n = 3$). Different letters (a–d) within a row indicate statistically significant differences between groups ($P < 0.05$; one-way ANOVA or Welch's ANOVA)

[†]After lyophilization

The diets used in this study had a comparably low moisture content of <20% and varied in their chemical composition (Table 2). Here, the highest crude ash content was found in the 0% SMS diet ($F_{3,8}$ = 139.28; $P \leq 0.00007$). As the SMS proportion increased, the crude fiber content also increased by up to 7.8-fold ($F_{3,4.3}$ = 579.74; $P = 0.00001$), whereas the crude fat content decreased by half at 50% SMS compared to pure CF ($F_{3,3.3}$ = 11.99; $P = 0.01$). The same applied for the total nitrogen and crude protein contents, which were reduced by ~30% when CF was replaced by 50% SMS ($F_{3,8}$ = 55.79; $P < 0.00001$ and $F_{3,8} = 59.70$; $P < 0.00001$).

The different diets fed to the BSF larvae led to significant differences in their chemical composition. Lyophilization reduced the larval moisture content from 67.7 to <7.8% (Table 3). The crude ash content of larvae provided 50% SMS was 11.1 to 15.5% higher than that of larvae fed pure CF or 20% SMS ($F_{3,8}$ = 27.56; $P < 0.003$). The same was observed for the crude fiber content, which increased by 42.0% when CF was replaced by 50% SMS ($F_{3,8} = 41.15$; $P = 0.00005$). The crude fat revealed an opposite pattern, with the lowest content (46.7% reduction) found in the larvae fed the 50% SMS diet ($F_{3,8}$ = 91.34; $P < 0.00001$). All SMS containing diets improved the total nitrogen ($F_{3,8}$ = 57.21; $P \leq 0.02$) and, thus, crude protein content ($F_{3,8}$ = 58.55; $P \leq 0.02$) in BSF larvae significantly compared to pure CF, with 40–50% SMS showed the highest values (Table 3).

After harvesting the larvae, the frass of the different groups had an average moisture content of 63.2%, which was reduced to <15.7% by lyophilization (Table 4). The crude ash content of frass obtained from pure CF did not differ significantly from 20 to 40% SMS, while a 50% SMS replacement led to a 24.9% lower content ($F_{3,3.3}$ = 66.77; $P = 0.01$). Frass from all SMS containing diets had ≥6.6-fold higher crude fiber contents compared to pure CF ($F_{3,8}$ = 708.33; $P < 0.00001$). There was no significant difference in the crude fat content of the frass among the groups ($F_{3,3.9}$ = 1.72; $P = 0.30$). All SMS containing frass showed significantly lower total nitrogen ($F_{3,8}$ = 138.94; $P \leq 0.00004$) and crude protein contents ($F_{3,8}$ = 105.06; $P \leq 0.0002$) compared to pure CF. There were no significant differences in crude fiber, protein, and total nitrogen contents between the 40–50% SMS frass (Table 4).

The amino acid content decreased with increasing SMS substitution in the feed, although there was no major difference between 40 and 50% SMS substitution (Fig. 2A). Glutamine was the predominant amino acid in all diets, whereas methionine, cysteine and tryptophan were limiting. In general, the amino acid content of the larvae was markedly higher than that of the feed or the frass and contained great amounts of aspartic acid, glutamine and histidine (Fig. 2A–C). Larvae fed 40–50% SMS achieved higher amino acid contents than their counterparts fed lower SMS levels. The amino acid content of the frass was considerably reduced, and tryptophan was almost completely removed (Fig. 2C).

The dominant FA in the diets was C18:2 (38.6–46.2%), followed by C18:1 and C16:0 (Table 5). Besides the FAs found in the diets, additional ones were detected in the larvae, namely C12:0 (58.3–73.9%), C14:0 (9.7–11.6%),

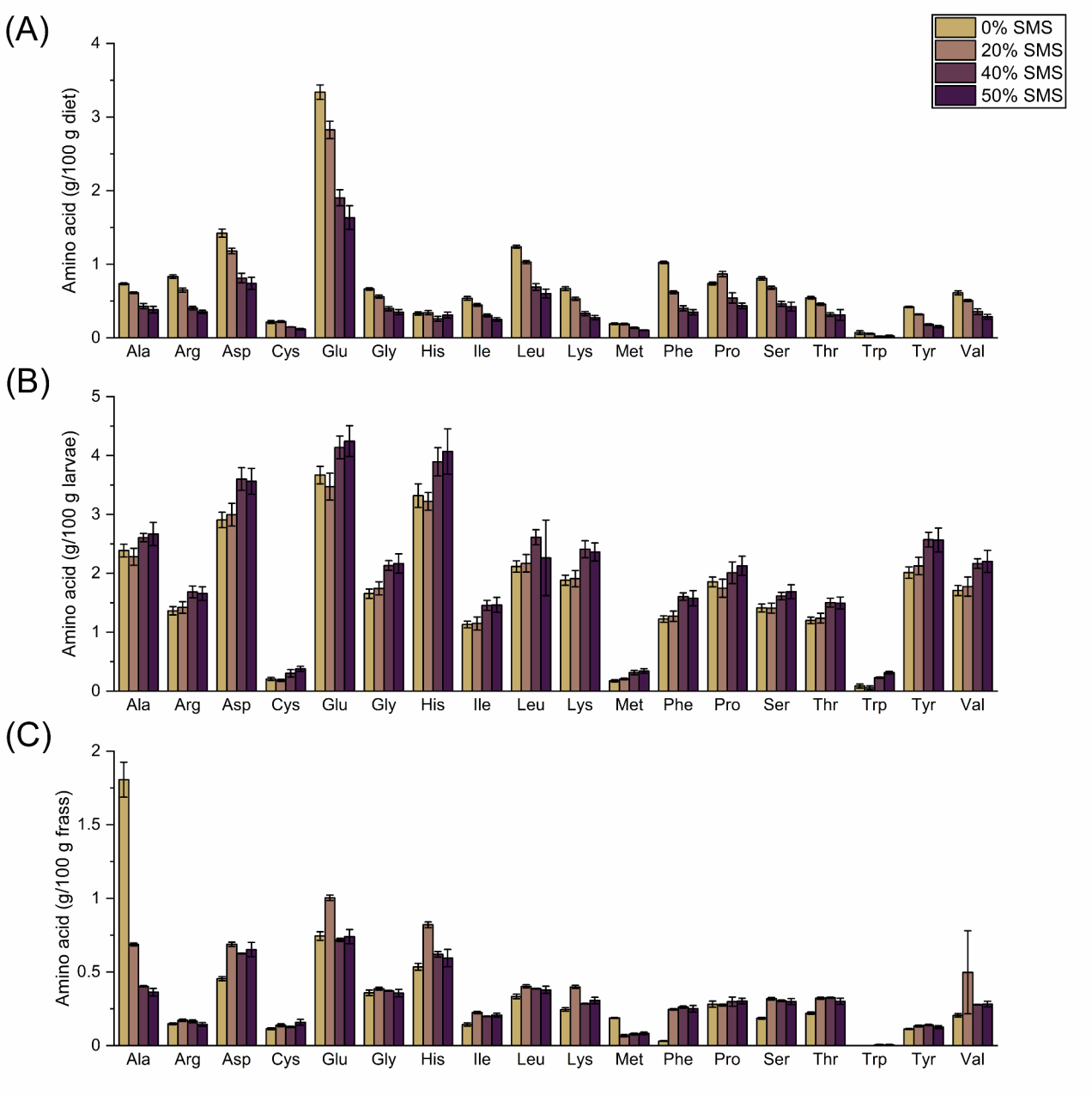

Fig. 2 Amino acid composition of (**A**) diets substituted with 0–50% SMS, (**B**) BSF larvae reared on them, and (**C**) the corresponding frass. Data represent means ± SD ($n = 3$) and are given as g/100 g sample

and C16:1 (1.8–3.2%). The frass had the most diverse FA profile, especially in the 40–50% SMS groups. C16:0 (22.6–35.4%) and C18:1 (23.0–34.6%) were the most abundant FAs in all frass groups, followed by C18:2 (10.4–34.7%). Here, we also detected methyl-branched FAs, which are rare to find in nature (Table 5).

Discussion

Spent mushroom substrate, also known as spent mushroom compost, mushroom bran, mushroom residue, or mushroom soil (Oei et al. 2007) contains around 80% of unused nutrients (Moon et al. 2012), making them a promising feed ingredient (Bapista et al. 2023; Foluke et al. 2014). However, there has been limited research into the use of SMS as feed for insects (Kim et al. 2014; Li et al. 2020, 2021; Mao et al. 2023). This study evaluated the potential of using *Pleurotus eryngii* and *Lentinula edodes*

Table 5 Fatty acid profile of diets substituted with 0–50% SMS, BSF larvae, and the frass

Fatty acids	Substrate				Larvae				Frass			
SMS level (%)	0	20	40	50	0	20	40	50	0	20	40	50
C12:0	–	–	–	–	63.5 ± 1.1	73.9 ± 1.0	59.9 ± 2.3	58.3 ± 1.1	–	–	–	–
C14:0	–	–	–	–	11.6 ± 0.2	9.8 ± 0.2	10.8 ± 0.6	9.7 ± 0.2	–	–	3.5 ± 0.5	2.7 ± 0.6
C14:0*	–	–	–	–	–	–	–	–	–	3.5 ± 0.0	13.5 ± 0.7	6.8 ± 0.2
C15:0*	–	–	–	–	–	–	–	–	–	–	4.0 ± 0.2	2.7 ± 0.3
C16:0*	–	–	–	–	–	–	–	–	–	–	4.7 ± 0.2	2.6 ± 0.4
C16:0	23.6 ± 0.4	24.4 ± 0.8	25.8 ± 0.3	25.7 ± 0.2	12.5 ± 0.4	9.5 ± 0.3	15.4 ± 1.2	15.6 ± 0.4	34.4 ± 0.6	35.4 ± 0.1	28.4 ± 0.2	22.6 ± 0.4
C18:0	2.5 ± 0.1	3.2 ± 0.2	3.3 ± 0.0	3.0 ± 0.0	1.2 ± 0.1	–	1.2 ± 0.1	1.5 ± 0.0	6.2 ± 0.1	7.4 ± 0.0	4.9 ± 0.5	3.4 ± 0.3
Σ saturated fatty acids	26%	28%	29%	29%	89%	93%	87%	85%	41%	46%	59%	41%
C16:1	–	–	–	–	3.2 ± 0.1	1.9 ± 0.1	2.2 ± 0.0	1.8 ± 0.1	–	–	2.1 ± 0.1	3.6 ± 0.1
C18:1	27.7 ± 0.1	29.9 ± 0.4	31.5 ± 0.3	32.7 ± 0.4	6.9 ± 0.4	4.9 ± 0.4	8.0 ± 0.3	9.5 ± 0.4	23.0 ± 0.6	33.6 ± 0.0	28.5 ± 0.4	34.6 ± 0.9
C19:1	–	–	–	–	–	–	–	–	–	1.7 ± 0.0	–	–
Σ monounsaturated fatty acids	28%	30%	32%	33%	10%	7%	10%	11%	23%	35%	31%	38%
C18:2	46.2 ± 0.5	42.5 ± 0.4	39.4 ± 0.6	38.6 ± 0.6	1.1 ± 0.1	–	2.5 ± 0.1	3.6 ± 0.2	34.7 ± 0.1	17.1 ± 0.0	10.4 ± 0.2	21.0 ± 0.4
C18:3	–	–	–	–	–	–	–	–	1.7 ± 0.1	1.3 ± 0.0	–	–
Σ polyunsaturated fatty acids	46%	42%	39%	38%	1%	0%	3%	4%	36%	19%	10%	21%

Data represent means ± SD ($n = 3$) and are given as a percentage of total fatty acids (*methyl branched fatty acids; – not detected or sum of less than 1%)

SMS as one of the rearing substrates for BSF larvae. Both species belong to the highest-produced mushrooms in terms of yield (Zhang et al. 2015) and industrialization (Li 2018), indicating large quantities of corresponding SMS. Other SMS that fall into these categories originate from *Agaricus bisporus* and *A. arvensis* (Bapista et al. 2023; Girotto and Piazza 2022), *Auricularia cornea*, *A. auricularia*, and *A. heimuer* (Du et al. 2022; Wei et al. 2020; Li et al. 2021), *Pleurotus citrinopileatus* and *P. ostreatus* (Li et al. 2020, 2021), and *Flammulina velutipes* (Kim et al. 2014) production. Although the composition of SMS varies, they mainly contain around 40% cellulose, 28% hemicellulose, and 32% lignin. Thus, like our study, others usually tested a gradual substitution of standard feed with SMS. In this work, we gradually replaced chicken feed with an SMS mixture. Similarly, gradual substitutions of wheat and rice bran with 0–70% *L. edodes* SMS (Li et al. 2020) and wheat bran with *Pleurotus eryngii* and *F. velutipes* SMS (Kim et al. 2014) were carried out for feeding *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. Li et al. (2021) conducted feeding experiments for BSF larvae with *L. edodes* SMS and food waste. It is to be noted that food waste is not allowed as feed in the EU (Regulation (EC) No 999/2001). Another study fed SMS from not specified mushrooms and distiller's grain to BSF larvae (Mao et al. 2023).

The survival rates of BSF larvae in the current substitution experiment were between 96 and 99.8%. Similarly, the survival of BSF larvae was not significantly different when fed with Gainesville diet (control consisting of alfalfa, wheat bran, and corn meal) or SMS of *A. heimuer*, *L. edodes*, *P. eryngii*, or *P. citrinopileatus* (Li et al. 2021). However, Li et al. (2020), postulated that all *T. molitor* larvae died when fed with 100% of *A. auricularia*, *P. ostreatus*, or *P. citrinopileatus* SMS, whereas survival for *L. edodes* and *P. eryngii* SMS were 37% and 1.3%, respectively. The substitution of wheat bran and rice bran with *L. edodes* SMS resulted in > 90% survival, with the lowest rate (~ 92%) observed at 70% substitution of wheat bran. These results highlight the potential of rearing insects, particularly BSF larvae, on SMS. Some mushrooms may produce secondary metabolites that might affect the insect's performance or, in some cases, lead to a lower survival. This is not the factor that influenced our study, as the survival rate was high even at 100% SMS level (Fig. 1A).

However, we found differences between the individual larval weights when chicken feed was substituted with SMS. The highest individual larval weight (223–234 mg FM) was not in the control diet but with 20% SMS substitution. As the SMS level increased, the individual larval weight decreased, which might be related to a reduction of nutritional value (Table 2). In the stocking density trials, the individual weight was significantly different both

in terms of SMS level and larval density. The reduction in individual weight with increasing density might be because of the growing competition between the larvae. Similarly, the individual weight of *T. molitor* larvae reduced as the proportion of *P. eryngii* SMS increased in the diet (Kim et al. 2014). After 30 d, the weight of *T. molitor* larvae on pure *L. edodes* (0.5 mg) and *P. eryngii* (negligible to be measured) SMS were comparatively lower than those reared on the control diets (3.6–6.8 mg). *T. molitor* larvae weighed 4.8 mg on 60:40 SMS: wheat bran and 4.6 mg on 60:40 SMS: rice bran diet. The authors hypothesize that pure SMS could not meet the nutritional requirements of the larvae, which might be due to its physical, chemical, and biological properties (Li et al. 2020). Feeding mixtures therefore appear to compensate for such deficiencies. Cai et al. (2017) prepared a mixture of wheat bran or kitchen waste and root waste from *F. velutipes* as feed for BSF larvae. In our study, the mushroom stems attached to the SMS blocks were removed to maintain uniformity. However, using the SMS blocks with mushroom stems would provide additional nutrients. The larval weight on 50:50 *L. edodes*: *P. eryngii* SMS in the current study was 14 mg (Table 1). According to Li et al. (2021), BSF larvae weighed 69 mg and 38 mg on pure *L. edodes* or *P. eryngii* SMS, respectively. The substitution of food waste with *L. edodes* SMS resulted in larval weights between 160 and 242 mg (Li et al. 2021). When substituting similar levels of CF, we yielded larval weights ranging between 59 and 229 mg. The reason for this difference in range could be the feeding rate. In this work, 150 g DM feed/200 larvae were used, while Li et al. (2021) provided three times more with 90 g DM feed/40 larvae. Interestingly, the highest weights in both studies were not in the control diet. In fact, the highest larval weights were obtained at 60% SMS with 260 mg (Li et al. 2021) and at 20% SMS with 229 mg, exceeding the corresponding controls by ~ 11%.

The number of prepupae and pupae formed was highest at 40% SMS, while none were found in the 100% SMS diet (Table 1; Fig. 1D). Similarly, no prepupae were detected when pure *L. edodes* and *P. eryngii* SMS were fed (Li et al. 2021). Here, a density of 1000 larvae accelerated the prepupae or pupae formation compared to lower densities. However, the prepupae and pupae were of smaller size. Presumably, the lower nutrient availability in SMS caused them to develop faster, although individuals were smaller, to reach the adult stage despite unfavourable conditions. Another possible reason could be higher temperatures in the rearing boxes due to increased larval interactions at 1000 larvae density, leading to faster development. For *T. molitor*, a diet of 40:60 wheat bran: *L. edodes* SMS also resulted in smaller pupae compared to those reared on pure wheat bran or a lower substitution of SMS (Li et al. 2020). According to Li et al. (2021), 32–55% of BSF developed to prepupae on food waste replaced with 20–40% of *L. edodes* SMS. In the substitution experiment, BSF prepupae proportion was 4.8 and 27.0% on CF replaced with 20 or 40% SMS, respectively. Astonishingly, the food waste diet promoted a faster development than the CF diet.

The total harvested biomass in the substitution experiment was 2.7 g FM at 100% SMS and 40.0 g FM for pure CF. Mao et al. (2023) inoculated 0.2 g of BSF larvae on wet distiller's grain and a not specified SMS-based diet. Here, total harvested larvae biomass was ~ 2.4 and 22.8 g FM for 100% SMS and pure wet distiller's grain, respectively. Since neither the number of inoculated larvae nor their density is given, a comparison with our results is difficult. All other publications that used SMS to feed insects have not addressed the total harvested biomass, giving no path for comparison.

Frass residue in the substitution experiment was highest when larvae were fed with 100% SMS. Mao et al. (2023) obtained similar results, indicating that the highest frass residues occur with a 100% SMS diet compared to standard diets or mixtures thereof. The authors conclude that the lower nutritional content in SMS is the cause for the lower utilization by the larvae. Furthermore, indigestible components such as fibers, particularly lignocelluloses present in plant-based substrates, are also likely to limit utilization by the larvae (Klüber et al. 2022; Tables 2 and 4).

In the current study, the LGR was highest (4.2–4.6 g FM/d) in the 20% SMS substitutions, while pure SMS resulted in an LGR of 0.2 g FM/d. Similarly, feeding fiber-rich diets such as olive pomace and silage grass, led to a comparable low LGR of 0.2 and 0.7 g FM/d (Veldkamp et al. 2021). Like LGR, FCR was best (3.3) in the 20% SMS diet, whereas higher SMS substitutions caused higher FCRs. According to Oonincx et al. (2015) the FCR was best (~ 1.4) for a high protein-high fat diet and lowest for a low protein-low fat diet (~ 2.6). With 26% crude fiber content, apple pulp also had a higher FCR (8.9) than the CF control (2.1) and a 74.2% lower individual larval weight (Broeckx et al. 2021). This justifies, that such by-products cannot fully meet the requirements for larval growth without mixing or pretreating them (Klüber et al. 2022). Substituting CF with SMS resulted in lower ECI (Fig. 1J; Table 1). Mao et al. (2023) also found an ECI of 24.7% for the 75:25 wheat distiller's grain: SMS diet and of 2.2% for pure SMS diet. Similar to our diets containing high levels of SMS, ECI was 0.26 for olive pomace and 0.14 for silage grass (Veldkamp et al. 2021), indicating the difficulty of the conversion of fiber-rich feed into biomass by BSF larvae (Li et al. 2015).

The substrate reduction was highest (46.4%) on the 20% SMS diet and lowest (3.2%) on the 100% SMS diet. Substrate reduction of 64.3% and 33.11% was obtained for

pure distiller's grain and pure SMS, respectively (Mao et al. 2023). The higher substrate moisture (10%) and longer feeding period (5 d longer) in the latter study might have stimulated a better decomposition of the feed. Cai et al. (2017) postulated a substrate reduction of 42.3% when mushroom root waste was fed to BSF larvae. In accordance with our data, substrate reduction has been shown to reduce gradually as wheat bran was replaced by olive pomace (Ramzy et al. 2022).

The waste reduction index was highest (7 g DM/d) at 20% SMS substitution, while pure CF and pure SMS had 2.7 and 0.5 g DM/d, respectively. Higher SMS substitutions generally resulted in lower WRI. Cai et al. (2017) observed a similar tendency with a WRI of 2.4 g DM/d for Gainesville diet (control) and 1.6 g DM/d for mushroom root waste. The difference between the WRI of pure SMS and mushroom root waste may be due to the fact that the latter one has a total amino acid content of 6.1 g/100 g DM, which is about twice as high as SMS with 3.1 g/100 g DM (data not shown). In contrast, olive pomace had an identical WRI as pure SMS (Veldkamp et al. 2021).

We observed that the substrates were not fully utilized by the larvae at a density of 200 larvae/box. Hence, higher densities were considered to optimize the substrate utilization (200–1000 larvae per 100 g DM feed). Several densities have already been tested for various substrates, as discussed by Nayak et al. (2024). Although higher larval densities showcase intraspecific competition for resources, there could be beneficial effects due to higher bacterial accumulation and hence associated availability of nutrients (Barragan-Fonseca et al. 2018). Similar to our data, the individual larval weight was shown to decrease as the stocking density increase (Barragan-Fonseca et al. 2018). However, Lopes et al. (2023) postulate that increasing larval density up to 6.3 larvae/cm^2, increased bioconversion efficiency and larval yield. We also observed an increase of larval yield when the density increased, whereas the efficiency of conversion increased, remained the same or decreased depending on the SMS level (Fig. 1F, J). Yakti et al. (2022) also highlight the differences in larval growth and chemical composition due to changes in larval density.

Besides the larval performance, it is of great interest to perform chemical analyses in order to identify suitable by-products, develop new feeding concepts, evaluate the quality of the products, and suggest appropriate uses for them. With 13.1% DM, the crude ash content of pure CF was highest among all diets tested and reduced gradually as the SMS level increased. Other studies report ash contents between 4 and 6% DM for vegetable, grain, and press cake diets fed to BSF (Bonelli et al. 2020; Galassi et al. 2021; Schreven et al. 2020; Broeckx et al. 2021). Lower ash contents of 0.4–0.9% DM were found for sweet potato, spent coffee, and dough-based diets (Romano et al. 2022). It is not yet clear to what extent ash content influences the larval development. As discussed above, fiber-rich by-products have a high proportion of indigestible components for BSF larvae. In combination with low protein and fat content, this can severely restrict larval growth. These by-products can be improved through a sensible combination with other substrates or a targeted fermentative pretreatment, as shown by Klüber et al. (2022). Li et al. (2021) did not provide the feed composition but the larval crude ash content in the 70:30 food waste: *L. edodes* SMS diet was similar to the 0% SMS diet of the current study. The larval crude fiber content is not addressed in the majority of publications. With 17.8% DM, Li et al. (2021) determined a crude fiber content that was >3-fold higher than in our larvae. This is probably due to feed residues that were present in the larval gut at the time of harvest. The larval crude protein content increased from 34% DM at 0% SMS to 42% DM at 50% SMS substitution, which is similar to or slightly lower than the literature data. Here, the crude protein content of the diets seems to have a considerable influence. For example, Oonincx et al. 2015 postulated comparable larval crude protein contents of 38–46% DM when feeding diets with 13–23% DM protein (see Table 2), while larvae reared on 30–39% DM protein diets had around 50% DM crude protein (Galassi et al. 2021). In general, however, it should be mentioned that the overwhelming majority of studies state a crude protein content that is too high, as they assume a conversion factor of 6.25. We, on the other hand, worked with a corrected Kjeldahl factor, which was calculated based on the amino acid profile and is 5.85 on average. The amino acid profile of the 50% SMS diet in the current study is similar to that of mushroom root waste, but no information on the larval composition is provided (Cai et al. 2017). Histidine, glutamic acid, cysteine, aspartic acid, alanine, and tyrosine contents of our BSF larvae were comparable or slightly higher than larvae reared on sweet potato and spent coffee (Romano et al. 2022).

The crude fat content of the larvae reared on chicken feed was 26% DM (Galassi et al. 2021). In comparison, Li et al. (2021) found a relatively lower crude fat content of 17.8% DM in larvae fed a 70:30 food waste: *L. edodes* SMS diet. According to Barrgan-Fonseca et al. (2018), the larval fat content varies greater than their protein content depending on the diet. FAs identified from the feed used in the current study had a chain length of C16–C18, including monounsaturated and polyunsaturated ones (Table 5). The frass was found to have a higher variety of FAs, some of them were also methyl-branched, which are extremely rare in nature (Hochmuth and Piel 2009). The substrate's composition, along with the presence of distinct microbial communities within the larval gut that

aid in FA metabolism, or the potential interplay between these factors, may contribute to the observed higher variety of FAs, including methyl-branched ones, in the frass. In particular in larvae, C12:0 and C14:0 proportions were higher than those reported in other studies (St-Hilaire et al. 2007; Romano et al. 2022; Galassi et al. 2021).

The chemical composition of the frass shows the overall utilization of the nutrients from the feed. Here, crude fiber content increased in the frass, while crude protein and fat contents decreased. As suggested by Cai et al. (2017), BSF frass from mushroom by-products could be a promising organic fertilizer.

Commercial BSF producers predominantly use chicken or pig feed as diet for larval fattening, which is neither sustainable nor cost-efficient. *L. edodes* and *P. eryngii* SMS are generated in large quantities during the production of mushrooms and can help to reduce the amount of such feedstuffs by partially replacing them. At the same time, large quantities of these organic by-products could be returned to the food and feed chain and thus contribute to circular bioeconomy efforts. Further research should be conducted on the use of SMS from other mushrooms, combinations thereof, and mixtures with different by-products as feed for insect rearing. Besides, feeding fresh SMS (including mushroom stems) immediately after harvesting could be promising.

Abbreviations

ANOVA	Analysis of variance
BSF	Black soldier fly
CF	Chicken feed
DM	Dry matter
ECI	Efficiency of conversion of ingested feed
EU	European Union
FA	Fatty acid
FAME	Fatty acid methyl esters
FCR	Feed conversion ratio
FM	Fresh matter
LGR	Larval growth rate
SMS	Spent mushroom substrates
SR	Substrate reduction
WRI	Waste reduction index

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Author contributions

Conceptualization, A.N., P.K. and M.R.; methodology, A.N. and P.K.; software, A.N. and P.K.; validation, A.N., P.K. and M.R.; investigation, A.N. and P.K.; resources, M.R.; data curation, A.N. and P.K.; writing—original draft preparation, A.N.; writing—review and editing, A.N., P.K. and M.R.; visualization, A. N., and P.K.; supervision, P.K. and M.R.; project administration, M.R. and P.K.; funding acquisition, M.R. All authors have agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are included in the manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

The hidden drivers: Unraveling the impact of density, moisture, and scale on *Hermetia illucens* rearing

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Abstract

The black soldier fly (*Hermetia illucens*) is a saprophagous insect known for bioconverting organic waste, potentially offering environmental benefits, such as contributing to waste reduction and nutrient cycling. The performance of larvae varies significantly with factors substrate moisture, larval density, and scale of production. Three experiments were conducted using a mix of spent mushroom substrate (SMS) and chicken feed (CF). In the first experiment, 250 larvae were reared on 100 g dry matter (DM) feed at moisture levels of 65–75%. Results showed that the average individual larval weight, total biomass, and feed conversion ratio (FCR) improved with increased moisture. In the second experiment, 300 and 350 larvae/box were tested at 70% and 75% moisture. The highest average individual larval fresh weight (158.6 mg) was observed at 70% moisture with 250 larvae, while the highest biomass was achieved at 75% moisture with 300 larvae. Finally, different scales (10–2,500 g feed with 25–6,500 larvae) were tested with a similar feeding rate. The highest individual larval weight was recorded at the 100 g scale, with no clear correlation between weight and scale. However, the 50 g scale achieved the highest substrate reduction (33.2%). Overall, this study underscores the need to adjust moisture, density, and scale to nutrient conversion efficiency when using SMS, CF or other diets. The optimal results for the SMS feed mix were observed at 75% substrate moisture, 250 larvae per 100 g DM, and at approximately 2 larvae per cm^2.

Introduction

The popularity of *Hermetia illucens*, the black soldier fly (BSF; Diptera: Stratiomyidae), has surged significantly in both academic and commercial sectors over the years [1]. The initial studies were mainly based on the larval bioconversion ability [2] and larvae as an alternative feed ingredient for various animals [3]. Recently, the trend has expanded to explore other potential applications, such as using BSF larvae for cosmetics, biodiesel, and various biotechnological products [4–9]. This underscores the importance of sustainable BSF larvae production to meet the growing demand driven by its multisectoral use and popularity [10–13].

Competing interests: The authors have declared that no competing interests exist.

Despite this, the knowledge on sustainably optimized feed and rearing methods is limited. The possible reasons include the vast array of plant and animal-based feed options [14, 15], differences in their nutritional composition [16] and physical properties [17], as well as variations in experimental methodologies. The side streams such as fruit and vegetable wastes [18, 19], or other organic wastes [15, 20] are well investigated. However, side streams from the edible mushroom production, although promising, are less explored as feed for BSF larvae.

The production of edible mushrooms increased more than 30 times in less than 50 years globally [21]. China is the world's largest mushroom producer, responsible for approximately 80% of global output [21]. Additionally, over 100 other countries are involved in mushroom cultivation [22]. This is unsurprising, as materials needed for mushroom cultivation are widely available around the world. Mushrooms are grown on organic substrates like crop residues, wood chips, sawdust, and husks, among others. After mushroom harvesting, organic materials containing residual fungal mycelium, referred to as spent mushroom substrates (SMS), are often discarded [23]. The increasing demand for mushrooms has led to a significant production of SMS [24, 25]. The composition of SMS varies depending on the initial materials used in cultivation. Typically, it consists of cellulose, hemicellulose, lignin, residual fungal mycelium, carbohydrates, proteins, and minerals [26]. The abundant availability of SMS and its potential use as feed for BSF larvae make it an ideal resource for further investigation. Two mushroom species, namely king oyster (*Pleurotus eryngii*) and shiitake (*Lentinula edodes*), have been used as feed for BSF larvae by Li et al. (2021) [27]. However, the number of studies on the use of SMS and its influence on larval performance is limited. Moreover, the influence of factors such as moisture, larval density, and scale are rarely explored.

BSF larvae are known to exhibit varying effects on their survival, growth, feed conversion ratio, and substrate reduction with different substrate moisture levels [17, 28–30]. For example, among the moisture contents of 70–80%, larval growth showed a positive correlation with increasing moisture [31]. A similar result was reported for larval yield by Palma et al. (2018) within a moisture content range of 48–68% wet basis [30]. According to Fatchurochim et al. (1989), BSF larvae can grow and develop into pupae in substrates with moisture content between 30% and 70% by weight [32]. However, the physicochemical characteristics of the substrate determine whether it is too dry, too wet, or optimal for larval growth at a given moisture range. Hence, it is necessary to examine whether the moisture content has to be specifically adapted depending on the diet and its physicochemical properties [33]. The simplest approach is to experiment with different moisture levels for the same substrate and observe the larval performance.

Density plays a crucial role in BSF feeding trial, as it can promote intraspecific competition [34], due to larval aggregation [35] or increase microbial load, leading to better nutrient uptake [36, 37]. Various ranges of BSF larval density have been considered in experiments: Parra-Paz et al. (2015) assessed densities of 1–6 larvae/cm^2 [5], Opare et al. (2022) tested 1–10 larvae/cm^2 [38], while Barragan-Fonseca et al. (2018) examined lower densities of 0.3–2.5 larvae/cm^2 [36]. Given the importance of larval biomass yield and developmental time in industrial-scale productions, further research on density optimization using sustainable feeds is essential.

Various studies have been conducted at different scales. The scale of an experiment refers to the size of the study in terms of the amount of feed and the number of larvae used. In BSF research, the impact of scale is not extensively studied [39]. Most BSF feed trials are conducted on a lab or bench-top scale [40, 41], which is advantageous for fast and resource-efficient testing of larval performance [42]. However, understanding how factors change during scaling up is essential for expanding BSF production using by-products. Additionally, some studies have conducted just the large-scale feed trials without comparing them to smaller-scale processes [39, 41, 43]. A large-scale experiment was carried out using up to 1400 kg FM feed with a larval density of 10 larvae/cm^2, however no comparisons were conducted based on scale [43]. Yakti

et al. (2022) used four box sizes (scales) with low or high densities of larvae each [42]. The number of larvae in the smallest box with low density included 814 larvae (4.2 larvae/cm^2) and the largest box with high density consisted of 13,000 larvae (6.3 larvae/cm^2). Here, the authors declared that the scale of the experiment is known to alter the larval composition [42]. There has been no scale experiment using SMS as feed for BSF larvae so far. If the scale experiments in laboratory setup yields different results, further optimization and tailoring will likely be necessary before establishing an optimized production methodology for industrial replication [40, 41].

Taking all these factors into account, three research questions were formulated:

1. How does the moisture content influence larval performance on an SMS-based diet?
2. Does increasing larval density (larvae/cm^2) improve larval growth performance and feed conversion at substrate moisture levels above 60%?

1. What is the impact of varying experimental scales on the growth performance and survival rates of larvae in a laboratory setup?

Materials and methods

The experiments were carried out at the Fraunhofer Institute for Molecular Biology and Applied Ecology (Giessen, Germany) between July 2022 and August 2023.

Insects and feeding substrates

Eight-day-old BSF larvae, vacuum-packed and shipped by Hermetia Baruth GmbH (Baruth/Mark, Germany), were used for the feeding trials. Upon arrival, the larvae were unpacked, transferred into polypropylene boxes, and placed in a climate chamber at 27 ± 1˚C and 65 ± 5% relative humidity for 1 h to allow them to recover their natural behavior. For an assured uniform size and mass, the larvae were separated using a vibratory sieve shaker with a mesh size of 1.0–1.4 mm (AS 200, Retsch, Haan, Germany). The average individual larval weight was then determined by weighing five replicates of 100 larvae each using a precision balance (ALJ 160-4A, Kern & Sohn, Balingen-Frommern, Germany). For experiments with less than100 larvae, they were counted individually. BSF larvae that were used for feeding trials had an average individual weight of 7.0 ± 0.0 mg.

The diet consisted of equal parts chicken feed (CF; GoldDott Eierglück, DERBY Spezialfutter, Muenster, Germany) and SMS. The SMS was sourced from an organic mushroom farm (Löckes Bio-Vertriebs GmbH, Büttelborn, Germany). It was collected as a by-product from the production of king oyster (*Pleurotus eryngii*) and shiitake (*Lentinula edodes*) mushrooms on the same day the fruiting bodies were harvested. Initially, any remaining fruiting bodies on the surface of the SMS blocks were removed. The blocks were then manually disintegrated and immediately dried at 80˚C for 10 h using a laboratory kitchen oven (HB674GBS1, Siemens AG, Munich, Germany). The CF and dried SMS were processed to a particle size of 0.1–0.8 mm using a Mockmill 200 (Wolfgang Mock, Otzberg, Germany) and a Thermomix TM6 (Vorwerk, Wuppertal, Germany). The prepared substrates were stored in airtight containers at room temperature until further use. For the feeding trials, king oyster SMS and shiitake SMS were combined in a 1:1 ratio and are hereafter referred to as SMS.

Optimization of substrate moisture

The dry matter (DM) weight of each diet, amounting to 100 g, was distributed into three replicate cylindrical boxes measuring 12.5 cm diameter and 11.4 cm height each (BDPN24,

MegaView Science, Taichung, Taiwan). The dry matter content of the substrates was determined thermogravimetrically using a moisture balance (DAB 100–3, Kern & Sohn, Balingen-Frommern, Germany). Moisture levels of 65%, 70%, and 75% were achieved by adding warm tap water. Following this, 250 eight-day-old BSF larvae were evenly distributed over the substrate surface in each box (2.04 larvae/cm^2). A circular 9 cm mesh insert was placed in the donut-shaped lid to ensure adequate air circulation. All boxes were then placed in a climate chamber under controlled conditions of 27 ± 1˚C and 65 ± 5% relative humidity in darkness. The boxes were randomly repositioned every second day. No additional feed or water was added throughout the experiment. Following the outcomes of preliminary experiments, larvae from all moisture groups were harvested ten days after the feeding commenced. Each larva was individually retrieved from the frass using spring steel tweezers, cleaned to remove coarse impurities, weighed, counted, and then cold-inactivated at -20˚C. The survival rate was calculated as the percentage of larvae, prepupae, and/or pupae recorded during the harvesting process in comparison to the initially inserted larval numbers. The total harvested biomass denotes the total amount of insect biomass collected per box, comprising larvae, prepupae, and pupae, minus the initially inserted larval weight. Based on this, the average individual larval weight was determined by dividing the total harvested biomass by the number of surviving larvae, expressed as fresh matter (FM). The feed conversion ratio **(Eq 1)** was calculated based on DM [44]:

$$FCR\ (Feed\ conversion\ ratio) = \frac{Total\ feed\ provided\ (g)}{(Total\ biomass\ harvested\ (g) - Initial\ biomass\ inoculated\ (g))} \quad (1)$$

Density optimization for selected moisture contents

Higher moisture contents (70% and 75%) showed improved larval growth, prompting a retest with increased larval densities (300 and 350 larvae per box i.e., 2.45 and 2.85 larvae/cm^2, respectively) to evaluate survival and growth performance. This experiment was carried out under the same conditions as for the moisture optimization. The objective was to investigate whether and how the variables moisture and density interact. Feed and larval samples were stored at -20˚C for subsequent analysis. Data collection and calculations followed the previously mentioned methodology.

Investigation of scale effects on growth performance and bioconversion

After optimizing density and substrate moisture, the focus was on determining the extent to which the scale of the feeding trials has an influence on larval growth and bioconversion efficiency. Therefore, at first, it was intended to clarify how the survivability and the growth performance of larvae are affected if the same experiments are done in different scales. A total of five scales were examined (10 g feed + 25 larvae, 50 g feed + 125 larvae, 100 g feed + 250 larvae, 1,000 g feed + 2,500 larvae, 2,500 g feed + 6,500 larvae), with 75% substrate moisture. The diet used was 50% CF and 50% SMS. The feeding rate was the same in all the setups i.e., 0.4 g DM/larva.

For the smaller scale setups (10 g, 50 g, 100 g DM feed), the corresponding amount of each diet was weighed (ALJ 160-4A, Kern & Sohn, Balingen-Frommern, Germany) into three cylindrical boxes with 12.5 cm diameter and 11.4 cm height replicate boxes (BDPN24, MegaView Science, Taichung, Taiwan), as described in the previous chapters. A circular 9 cm mesh insert was placed in the donut-shaped lid to ensure adequate air circulation in the smaller scale boxes. The substrate moisture was adjusted to 75% by adding warm tap water. Subsequently,

25 and 125 eight-day-old BSF larvae were counted individually and transferred in each box by spreading them over the substrate surface. For other scales, the average weight of individual larvae was calculated by weighing five replicates of 100 larvae each using a precision balance (ALJ 160-4A, Kern & Sohn, Balingen-Frommern, Germany). The substrate was weighed using the same balance for the 10 g, 50 g, and 100 g DM feed approaches. Because of the larger amount of feed, the 1,000 g DM (Kern 572–39, Kern & Sohn, Balingen-Frommern, Germany) and 2,500 g DM (ACS-Z, CELMI, Buccinasco, Italy) feed were weighed using different balances and transferred into three cuboidal replicate boxes of 17.0 × 25.5 × 38.0 cm (Santos Box XS, Keter Italia S.p.A., Roncadelle, Italy) and 39.4 × 29.4 × 24.7 cm (AUER packaging, Amerang, Germany), respectively. A 1,000 mesh/6.5 cm^2 cloth (A113a, Bioform, Nuremberg, Germany) was used for a proper air circulation and to prevent larval escape for 1,000 g and 2,500 g feed boxes. The number of larvae per cm^2 were 0.20, 1.02, 2.03, 5.77, and 5.61 for the feed 10–2,500 g, respectively. The variations in the density per cm^2 is due to the different box sizes. All boxes were incubated in a climate chamber under identical conditions as in previous experiments. The boxes were rearranged randomly on every second day. No additional feed or water was added throughout the experiment and boxes were harvested 10 d after the trial has started. The harvesting procedure and storage protocols mirrored those applied in the previous experiments. Likewise, survival rate, total biomass, average individual larval weight, and FCR were calculated following the same methods. The FCR measures the relationship between the quantity of feed provided and the weight gained by the larvae. In addition, frass was collected, weighed and the DM content determined. The efficiency of conversion of ingested feed (ECI), substrate reduction (SR), and waste reduction index (WRI) were calculated following Eqs (2–4) [44, 45]. The equations ECI and FCR assume that larvae consume all the provided feed, unlike conventional livestock, where feed intake can be measured accurately. The factors total harvested biomass (DM and FM) and frass weight were normalized based on the number of larvae to allow comparability between the different scales.

$$ECI\ (Efficiency\ of\ conversion\ of\ the\ ingested\ feed) = \frac{(Total\ biomass\ harvested\ (g) - Initial\ biomass\ inoculated\ (g))}{(Total\ feed\ provided\ (g) - Frass\ residue\ (g))} \quad (2)$$

$$SR\ (Substrate\ reduction\ (\%)) = \frac{(Total\ feed\ provided\ (g) - Frass\ residue\ (g)) \times 100}{Total\ feed\ provided\ (g)} \quad (3)$$

$$WRI\ (Waste\ reduction\ index) = \frac{(Total\ feed\ provided\ (g) - Frass\ residue\ (g))}{Number\ of\ rearing\ days\ (d)} \quad (4)$$

Data processing and statistics

Data curation and processing were carried out using Excel 2016 (Microsoft, Redmond, WA, USA). Statistical analysis and visualization were conducted in OriginPro 2024b (OriginLab, Northampton, MA, USA). The Shapiro–Wilk test was applied to verify whether the data of a population is normally distributed. The homogeneity of variance was calculated with Levene's test. Variables recorded in the moisture and scale experiments were subjected to a one-way ANOVA and means were separated using the Tukey's test (homogeneous variance). If the variance was inhomogeneous, a one-way Welch's ANOVA followed by Games–Howell post hoc test was conducted. The Pearson product–moment correlation was used for determining linear relationships between variables [46]. Data obtained from the density experiment were

Table 1. Growth performance and feed conversion ratio of BSF larvae reared at different moisture levels (65–75%).

	Substrate moisture (%)		
Variables	**65**	**70**	**75**
Total amount of feed (g DM)	100.0 ± 0.0 [a]	100.0 ± 0.0 [a]	100.0 ± 0.0 [a]
Survival rate (%)	100.0 ± 0.1 [a]	97.9 ± 3.0 [a]	93.2 ± 3.0 [a]
Average Individual larval weight (mg FM)	105.9 ± 1.7 [a]	158.6 ± 9.0 [b]	233.3 ± 8.2 [c]
Total harvested biomass (g FM)	26.5 ± 0.7 [a]	39.3 ± 0.5 [b]	53.1 ± 1.3 [c]
Total harvested biomass (g DM)	7.9 ± 0.2 [a]	13.6 ± 0.2 [b]	18.4 ± 0.4 [c]
Feed conversion ratio (FCR)	3.8 ± 0.1 [a]	2.5 ± 0.0 [b]	1.8 ± 0.0 [c]

Data represent means ± SD ($n = 3$). Different letters (a–c) within a row indicate statistically significant differences between groups ($P < 0.05$; one-way ANOVA or Welch's ANOVA). DM = dry matter, FM = fresh matter, FCR = feed conversion ratio

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subjected to a two-way ANOVA and means were separated using the Tukey's test. An error level of $\alpha = 0.05$ for statistical significance was set for all analyses. For the scale experiment, total harvested biomass, frass weight and WRI were normalized based on the corresponding number of larvae.

Results

Optimization of substrate moisture

The first trial examined how varying substrate moisture content affects larval growth and development (Table 1). The goal was to determine the optimal moisture level among 65, 70, and 75% for a substrate composed of 100 g DM feed, with an equal mix of CF and SMS. The survival rate of larvae was $\geq$ 93% and did not differ significantly between the moisture groups ($P = 0.12$; Table 1). However, a negative linear correlation between the survival rate and the moisture level was calculated ($r = -0.74$; $P = 0.02$). The average individual larval weight (233.3 mg FM) was highest in the 75% moisture level group and increased with rising moisture levels ($r = 0.99$; $P < 0.01$). Here, larvae grew significantly better and reached an average individual weight that was 49.8% and 120.3% higher when compared to the 70% and 65% moisture groups, respectively ($P < 0.01$). Both fresh matter and dry matter of the total harvested biomass were highest in the 75% moisture group, outperforming the other groups by 35.0–133.7% ($P < 0.01$; Table 1). The total harvested biomass was strongly positively correlated with increasing moisture level ($r = 0.99$; $P < 0.01$). Providing the larvae feed with 75% moisture improved the feed conversion ratio (FCR) by 50.3% in comparison to the 65% moisture group ($P < 0.01$; Table 1). Here, FCR correlated negatively linearly with increasing moisture level ($r = -0.98$; $P < 0.01$).

Density optimization for the selected moisture contents

In general, larval performance improved with higher substrate moisture content. Consequently, substrate moisture levels of 70% and 75% were selected for further investigation. Building on the previous experiment, where larval density was set at 250 larvae per 100 g DM, this study explored variations in stocking density ranging from 250 to 350 larvae per box (S2 Table).

All groups received the same amount of 100 g DM feed consisting of CF and SMS (1:1 *w/w*). The survival rate of all groups ranged between 83.7–100.0% with statistically significant differences between the moisture groups ($P < 0.01$). A significant interaction between the variables moisture and density was calculated ($P < 0.01$). Individual average weight differed significantly

Table 2. Growth performance and feed conversion ratio of BSF larvae reared at different moisture levels (70–75%) and varying larval densities (250–350).

	Substrate moisture (%)					
	Larval density					
Variables	**70%**	**70%**	**70%**	**75%**	**75%**	**75%**
	250 L	**300 L**	**350 L**	**250 L**	**300 L**	**350 L**
Survival rate (%)	97.9 ±3.0 ^{aA}	98.0 ±2.4 ^{aA}	100.0 ± 0.0 ^{aA}	93.2 ± 3.0 ^{aA}	96.4 ± 2.0 ^{aA}	83.7 ± 4.0 ^{bB}
Average Individual larval weight (mg FM)	158.6 ±9.0 ^{aA}	105.3 ± 1.6 ^{bA}	97.3 ± 4.7 ^{bA}	233.3 ± 8.2 ^{aB}	212.3 ± 4.2 ^{aB}	178.3 ±8.1 ^{bB}
Total harvested biomass (g FM)	39.3 ±0.5 ^{aA}	28.8 ±0.4 ^{bA}	32.3 ± 1.9 ^{abA}	53.1 ± 1.3 ^{aB}	60.0 ± 2.3 ^{abB}	50.7 ± 4.7 ^{acB}
Total harvested biomass (g DM)	13.6 ±0.2 ^{aA}	8.5 ±0.1 ^{bA}	9.3 ± 0.5 ^{bA}	18.4 ± 0.4 ^{aB}	19.8 ± 0.8 ^{abB}	16.3 ± 1.5 ^{acB}
Feed conversion ratio (FCR)	2.6 ± 0.0 ^{aA}	3.5 ± 0.1 ^{bA}	3.1 ± 0.2 ^{bA}	1.9 ± 0.0 ^{aB}	1.7 ± 0.1 ^{aB}	1.6 ± 0.2 ^{aB}

Data represent means ± SD (n = 3). Different letters within a row indicate statistically significant differences, with lowercase letters (a–c) referring to data within the moisture group and uppercase letters (A–B) referring to data within a density group ($P < 0.05$; two-way ANOVA). DM = dry matter, FM = fresh matter, FCR = feed conversion ratio

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for the moisture ($P < 0.01$) and the density level ($P < 0.01$), wherein interactions of both variables were verified ($P = 0.01$). The highest average individual larval weight (233.3 mg FM) was obtained at 250 larvae/box in the 75% moisture group, which was ≥ 9.9% greater than in all other groups. In general, the weight of the larvae decreased successively with increasing density ($r = -0.47$; $P = 0.05$) and was positively correlated with increasing moisture level ($r = 0.86$; $P < 0.01$; Table 2). There was an 83.2% reduction in weight at 350 larvae/box reared at 70% moisture level in comparison to 75% moisture ($P < 0.01$). There was a significant difference (101.6%) within the 300 larvae/box density at different moisture levels. Within the 70% moisture treatments, the average individual larval weight was highest at a larval density of 250. Fresh and dry matter of the total harvested biomass was highest (60.0 g FM and 19.8 g DM) in the 75% moisture group at 300 larvae/box, followed by the 250 larvae/box group within the same moisture level with a mean reduction of 13.0% FM ($P = 0.10$) and 7.6% DM ($P = 0.42$), respectively. Both variables were significantly affected by the moisture ($P < 0.01$ and $P < 0.01$) and the density ($P < 0.01$), and interactions thereof were calculated ($P < 0.01$). Here, the harvested biomass improved between 35.1–108.3% FM and 35.3–132.9% DM with increasing moisture. In general, the lowest biomass was harvested at 300 larvae/box and a moisture level of 70% (Table 2). No linear relationship was determined for the density and total biomass (FM: $r = -0.17$; $P \geq 0.51$).

Moisture level ($P < 0.01$), density ($P < 0.01$), as well as their interactions ($P < 0.01$) significantly affected the feed conversion ratio (FCR). Regardless of the larval density, FCR improved with increasing moisture level ($P < 0.01$). The best feed conversions were obtained within the 75% moisture group (1.6–1.9), whereby no density effect was recorded ($P \geq 0.13$). FCR was highest at 250 larvae/box and differed significantly from the 300 and 350 larvae/box groups ($P < 0.01$ and $P < 0.01$) at 70% moisture level. FCR was not found to have a linear relationship for the density ($r = 0.20$; $P = 0.42$). Given that 250 larvae/box achieved optimum results and required 16.6% less individuals, this density was considered most promising.

Investigation of scale effects on growth performance and bioconversion

Based on the previous experiments, the optimal moisture level for a feed mixture of 50% CF and 50% SMS was determined to be 75%, yielding the highest average individual larval weight, harvested biomass and FCR. Among the tested larval densities, 250 and 300 larvae/box produced the best outcomes in terms of average individual larval weight, biomass, and FCR. The results indicated almost significant difference between the densities of 250 and 300

Table 3. Growth performance and bioconversion efficiency of BSF larvae reared at different scales (10–2,500 g DM feed provided to 25–6,500 larvae) using similar feeding rates of 0.4 g DM/larva.

	Feed provided (g DM)				
	10	**50**	**100**	**1,000**	**2,500**
Variables	**25 Larvae**	**125 Larvae**	**250 Larvae**	**2,500 Larvae**	**6,500 Larvae**
Survival rate (%)	100.0 ± 0.0 [a]	98.7 ± 0.4 [a]	92.0 ± 2.9 [ab]	98.8 ± 0.5 [a]	72.3 ± 4.8 [b]
Average Individual larval weight (mg FM)	122.7 ± 3.5 [a]	155.9 ± 2.3 [b]	166.5 ± 2.6 [bc]	151.7 ± 2.4 [bd]	142.7 ± 11.4 [ab]
Total harvested biomass (g FM)	2.9 ± 0.1	18.3 ± 0.3	36.4 ± 0.7	354.1 ±3.8	619.0 ±27.3
Total harvested biomass * (g FM)	*0.11 ± 0.00 [b]	*0.15 ± 0.00 [a]	*0.15 ± 0.00 [a]	*0.14 ± 0.00 [a]	*0.10 ± 0.00 [c]
Total harvested biomass (g DM)	0.9 ± 0.0	6.4 ± 0.1	11.5 ±0.5	107.6 ± 7.1	159.9 ±7.9
Total harvested biomass * (g DM)	*0.04 ±0.00 [c]	*0.05 ± 0.00 [a]	*0.05 ± 0.00 [ab]	*0.04 ± 0.00 [b]	*0.02 ± 0.00 [d]
Feed conversion ratio (FCR)	3.5 ± 0.1 [b]	2.7 ± 0.0 [c]	2.8 ± 0.1 [c]	2.8 ± 0.0 [c]	4.1 ± 0.2 [a]
Efficiency of conversion of the ingested feed (ECI)	0.3 ± 0.0 [ab]	0.4 ± 0.0 [ac]	0.5 ± 0.0 [a]	0.5 ± 0.0 [ad]	0.5 ± 0.3 [a]
Frass weight (g DM)	6.8 ± 0.2	33.4 ± 0.4	76.0 ±2.6	790.7 ± 12.8	2095.8 ±159.3
Frass weight * (g DM)	*0.27 ± 0.01[a]	*0.27 ± 0.00 [a]	*0.30 ± 0.01 [ab]	*0.32 ± 0.01 [b]	*0.32 ± 0.02 [ab]
Substrate reduction (%)	31.9 ± 1.9 [a]	33.2 ± 0.7 [a]	24.0 ± 2.6 [ab]	20.9 ± 1.3 [b]	16.2 ±6.4 [ab]
Waste reduction index (WRI; g DM/d)	0.3 ± 0.0	1.7 ± 0.0	2.4 ± 0.3	20.9 ± 1.3	40.4 ± 15.9
Waste reduction index * (WRI; g DM/d)	*0.013 ± 0.00 [a]	*0.013 ± 0.00 [a]	*0.010 ± 0.00 [ab]	*0.008 ± 0.00 [b]	*0.006 ± 0.00 [ab]

Data represent means ± SD ($n = 3$). Different letters (a–d) within a row indicate statistically significant differences between groups ($P < 0.05$; one-way ANOVA or Welch's ANOVA). * = normalized data. DM = dry matter, FM = fresh matter, FCR = feed conversion ratio, ECI = efficiency of conversion of the ingested feed, WRI = waste reduction index

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larvae/box at 75% moisture content (Table 2). Given that 250 larvae per box maintained optimal results despite being fewer than 300, this density was considered to be better and chosen as benchmark for the investigation of scale effects at 75% substrate moisture. Each group was provided with the same (0.4 g DM/larva) feeding rate, which was adapted to different scales between 10–2,500 g feed and 25–6,500 larvae, respectively.

The survival rate differed significantly among the scale groups ($P < 0.01$), with the 10–1,000 g feed groups achieved $\geq$ 92%. In the 2,500 g scale, a survival rate of 72.3% was recorded, which was 21.5–38.3% lower compared to the smaller scales (Table 3). A negative correlation between the survival rate and the scale was determined ($r = -0.85$; $P < 0.01$).

The highest average individual larval weight (166.5 mg FM) was obtained in the 100 g scale group and was 35.7% and 9.8% higher than in the 10 g ($P < 0.01$) and 1,000 g scales ($P < 0.02$), respectively. Contrastingly, the 2,500 g scale did not differ significantly from the 100 g scale ($P = 0.27$). The 10 g scale produced the larvae with the lowest (122.7 mg FM) average individual larval weight. No linear relationship between the average individual larval weight and scale could be calculated ($r = -0.09$; $P = 0.73$). Both fresh matter and dry matter of the total harvested biomass were significantly different among the scales ($P < 0.01$ and $P < 0.01$). Based on the normalized data, both variables were highest in the 50 g and 100 g scales with a weight of 0.15 g FM and 0.05 g DM, respectively (Table 3). The lowest normalized total biomass (0.10 g FM and 0.02 g DM) was obtained from the 2,500 g scale and was 50% ($P < 0.01$) and 150% ($P < 0.01$) lower compared to the 50–100 g scale groups, respectively. The total harvested biomass was negatively correlated with increasing scale (FM: $r = -0.70$; $P < 0.01$; DM: $r = -0.78$; $P < 0.01$). The FCR was significantly different across the treatments ($P < 0.01$). For the FCR, the smallest and largest scales had the worst FCR, while the scales between 50–1,000 had the best values ranging between 2.7–2.8. FCR correlated positively with increasing scale ($r = 0.71$; $P < 0.01$). The efficiency of conversion of the ingested feed (ECI) was lowest (0.28) in the 10 g scale and increased by 89.3% in the 1,000 g scale ($P = 0.01$).

Contrastingly, the ECI was not significantly different between the 10 g and the 2,500 g scales ($P = 0.81$). Although the ECI increased successively with increasing scale, it is not linearly related to the scale. ($P = 0.21$). The normalized frass weight revealed significant differences across the scales ($P < 0.01$) and was highest (0.32 g DM) at 1,000 g and 2,500 g scales (Table 3). Interestingly, the 1,000 g scale differed significantly from the 10 g ($P < 0.02$) and 50 g scales ($P < 0.01$), whereas the 2,500 g scale did not ($P = 0.28$ and $P = 0.24$). The frass weight was found to increase with scale ($r = 0.67$; $P < 0.01$). With 33.2%, the 50 g scale group achieved the highest substrate reduction, representing a 2.1-fold higher reduction compared to the 2,500 g scale ($P < 0.01$). The substrate reduction was negatively correlated to the scale ($r = -0.77$; $P < 0.01$). The waste reduction index (WRI), larval ability in reducing the given feed, differed significantly between the treatments ($P < 0.01$). Here, the 10 g and 50 g scale groups yielded the highest WRI (0.013 g DM/d), reflecting 2.2-fold higher daily reduction values than the 2,500 g group ($P < 0.01$and $P < 0.01$). The WRI was negatively correlated with increasing scales ($r = -0.79$; $P < 0.01$).

Discussion

Optimization of substrate moisture

Substrate moisture is crucial in rearing BSF larvae [29, 32, 47, 48] because it interacts with substrate texture, microbial activity, and larval movements impacting larval feed consumption, in turn larval survival, development time, and prepupae size [30, 49]. Besides, moisture influences effective separation of larvae from the frass while harvesting [31]. In the current study, the survival rate showed no significant difference for the moisture levels 65, 70, and 75%. The lowest survival (~93%) was however found at 75% moisture level (S1 Table). It could be because a thin layer of water formed in this study in boxes with 75% moisture content, similar to the studies of Bekker et al. (2021) [29]. Comparably high survival rates (> 95%) were found at moisture levels of 60, 70, and 80% [28] and at 70, 75, and 80% [31]. The water holding capacity can vary for different substrates at the same moisture level. This change in water retention or release can impact larval survival and growth rate due to variations in substrate texture and the amount of free water present [33]. Our study found that SMS can be used as feed for BSF, as feeding 100% SMS or mixing it with CF poses no threat to larval survival [50]. Additionally, we observed that a 75% moisture is the upper limit; exceeding this value causes a layer of water to form on the substrate, leading to larval drowning and escape. However, larvae fed with fresh SMS could perform differently. We concur that drying and grinding the substrate is necessary to ensure a well-distributed and homogenized feed for the larvae. A significant difference in larval average individual weight was observed across moisture levels when rearing on equal amounts of CF and SMS, with weights increasing as moisture levels rose, reaching up to 243 mg FM at 75% moisture (S1 Table). A similar outcome was reported in another study using three feed types and five different moisture levels (55–75%), where the highest weight (237 mg FM) larvae were obtained at 75% on a crumbled pellet diet [33]. The larval weights observed in the current study underscore the potential of substituting CF with SMS. The lower average larval weight (115–178 mg FM) with 40–60% of SMS in Nayak et al. (2024) is due to the lower substrate moisture [50]. Thus, indicating the importance of moisture adjustment. The harvested biomass depends directly on the average individual weight of the larvae and their survival. Similar to average individual weight, the total biomass harvested from 250 larvae/box was highest at 75% moisture, increasing with higher substrate moisture (Table 1). The average individual larval weight and total biomass achieved in this study demonstrate the potential of replacing CF with SMS, with minimal impact on yield. In the current study, substrate moisture levels exceeding 75% were not tested, as it could prolong the presence of a

water layer, restricting the oxygen supply required by the larvae. Consequently, it is assumed that larvae would not perform better in a 50% CF and SMS mixture with moisture levels above 75%. However, alternative feed substitutes to SMS might perform better at substrate moisture levels exceeding 75%. In this study, feed conversion ratio (FCR) was also affected by moisture, with an improved FCR of 1.9 at 75% compared to 3.8 at 65%. The higher moisture levels may have restricted O_2 penetration into the substrate and thus increased anaerobic microbial activity, which could have led to an improvement in feed conversion. While the majority of studies did not specify the FCR, a ratio of 1.9 is among the best for various diets [19]. Substrate moisture significantly impacts BSF larvae performance, and a fixed moisture level seems to be not ideal due to variables like water-holding capacity [51]. Instead, the moisture levels should be adjusted for each substrate based on its physical properties [17]. Moisture control can also be achieved using water-absorbing ingredients like rice bran, rice husk, and coconut coir powder [52], or through aeration [31]. Active aeration was not employed in this study. However, it could be both a viable option and a necessity for larger-scale larval production in a bioreactor. With proper ventilation, BSF larvae can even thrive on substrates with up to 90% moisture content [51].

Density optimization for the selected moisture contents

Larval density, typically measured in larvae per cm^2 [36], is crucial when using BSF for bioconversion [38, 53]. In this study, densities of 300 and 350 larvae per 720 ml container were tested, alongside a 250 larval density for comparison, resulting in densities of 2.45, 2.85, and 2.04 larvae/cm^2, respectively. Other studies have used densities ranging from 0.31 larvae/cm^2 [36] to 10 larvae/cm^2 [38, 43, 54]. These variations in larval densities complicate direct comparisons of larval performance [55]. Nevertheless, a comparison of studies is made from the available data for larval performance.

In this study, the highest density (350 larvae/box) at 75% moisture resulted in a significantly lower survival rate of 83.7% in comparison to other treatments (S2 Table). Dzepe et al. (2020) found a decreasing survival rate with increasing density for larval numbers of 1, 2, 4, 6, 8, and 10 larvae/cm^2 [47]. It was found that average individual larval weight decreased as density increased, regardless of moisture level. However, larval weights were consistently higher at 75% moisture across all densities. The highest average individual larval weight in this study (233.3 mg FM) occurred at 75% moisture with a density of 250 larvae, while the lowest weight (97.3 mg FM) was at 70% moisture with a density of 350 larvae (Table 2). This trend has been observed in other studies as well [47, 53]. In general, differences in average individual larval weight are not only because of the density and moisture but other factors such as feed type and nutritional properties [53]. Schreven et al. (2022) concluded the same as the larval average individual weights were significantly different between the feed types (CF, CF and cameline seed press cake mix, chicken manure) [37]. Here, the larval weights decreased tremendously from 70.4 mg FM to 24.5 mg FM in chicken manure for densities of 50 and 200 larvae/container, respectively. For CF, the weight difference between the same densities was just 26.4 mg FM. Total harvested biomass in this study refers to the biomass at the end of the experiment minus the inoculated biomass. There was no linear relationship between density and total biomass (FM: $r = -0.16$, $P = 0.51$ and DM: $r = -0.30$, $P = 0.22$). The highest biomass (60.0 g FM and 19.8 g DM) was obtained for the density of 300 larvae at 75% moisture. The current results indicate that a substrate moisture level of 75% is more favorable than 70%. This adjustment provides a simple and cost-effective method to boost larval output. The increased moisture might have enhanced nutrient absorption by the larvae, contributing to the improved results. Variations in larval numbers and survival rates make it challenging to compare total harvested

biomass across studies, and few have reported this metric. Gligorescu et al. (2022), for example, reported harvesting 950 and 2,000 g FM larvae per box (40 × 60 × 20 cm) at larval densities of 7 and 10 larvae/cm^2, respectively [43]. Their study, however, involved a much larger scale, with 14,000 and 20,000 larvae per box. FCR was lowest (1.58) at 350 larvae/box at 75% substrate moisture, while it was highest (3.5) in the 300 larvae/box group at 70% moisture. Our results indicate that FCR does not have a linear correlation with density ($r = 0.20$, $P = 0.42$). However, Yakti et al. (2022) declared that the FCR values significantly differed between the densities (4.2–6.3 larvae/cm^2) [42]. The FCR of 1.4–2.6 were measured for high protein-high fat and low protein-low fat diets, respectively [44]. The FCR values from other studies range between 2.6–4.6 [43], 3.1–4.2 [42], and 13.4 [2]. It is hence clear that the FCR is also diet dependent.

Our findings indicate that larvae fed a CF and SMS mixed diet show optimal growth at 75% substrate moisture and a density of 250 among the tested variables (S2 Table). This observation aligns with other studies; for instance, both extremely high and low densities [38, 47] and extreme moisture levels can be detrimental [34]. Higher densities increase competition but are also linked to elevated phenoloxidase levels, enhancing insect immunity [54]. Parra Paz et al. (2015) recommend larval densities between 1.2 and 5 larvae/cm^2 [5]. The optimal combination of moisture and density depend on the substrate and should be determined through pilot tests, though lab results may not directly translate to larger scales. Considering all these, it can be summarized that the stocking density, substrate moisture, and their interactions are key factors influencing BSF larvae performance and are critical for BSF production.

Investigation of scale effects on growth performance and bioconversion

Several authors have advocated for large-scale experiments, as results from small-scale studies cannot be directly applied to industrial settings [42]. Key reasons include shifts in environmental conditions, resource management, and operational costs [56]. The scale experiment is crucial in overcoming these challenges, as it determines the feasible number of treatments and replicates based on available time and manpower. Tasks like counting larvae, preparing feed, and harvesting are time-intensive. However, large-scale studies are essential as BSF gains popularity as an alternative animal feed. In our study, we compared scales using the same feeding rate (0.4 g DM/ larva) and climatic conditions. The smallest scale (10 g DM feed, 25 larvae) was 25 times smaller than the largest one (2,500 g DM feed, 6,500 larvae). Although the largest scale is still smaller than industrial bioreactors, the aim was to observe fundamental changes. Yang and Tomberlin (2020) compared small- and large-scale setups by rearing BSF larvae in 1.0 L and 29.5 L containers, using 307 g of feed and 614 larvae in the small scale, and 5 kg of feed with 10,000 larvae in the large scale [57]. Scaling up might alter various factors, especially in industrial settings with different rearing systems, locations, harvesting or ventilation systems [40]. Large-scale studies typically use 7 kg [40, 41] to 10 kg [42] of feed and 10,000–13,000 larvae. It is important to note that the largest scale in our study is 3–5 times smaller, yet notable differences in larval performance were observed. This is also because we have covered a broader spectrum of scales. We found significant differences in the survival rate. The lowest survival was obtained in the 2,500 g scale group (S3 Table). This is not surprising as it has been postulated that survival rate and larval growth might vary from laboratory to industrial scale [31, 42]. The survival rate was found to be 28.2% greater on the industrial scale than on a bench-top scale [57]. Yakti et al. (2022) also found that the larval mortality rate was significantly higher in small scale [42]. The contradictory result in our study could be the effect of layer of water that reduced the substrate aeration in the larger scale. The smallest scale of 10 g feed we tested was not ideal as the BSF larvae tend to live in aggregation [58]. This was not

possible because of lower amount of substrate (and its depth). Miranda et al. (2020) reared 10,000 larvae in 7 kg FM feed (70% moisture) [41]. There, the average individual larval weight of larvae on the day of harvest (9 d) was between 152–170 mg FM for the four diets tested (swine, dairy and poultry manure, Gainesville diet). We observed the highest average individual weight (166.5 mg FM) in the100 g DM feed approach. In contrast, higher density (1,000 and 2,500 g DM feed) groups had a lower larval weight probably because of the less aeration within the substrate as there was a thin layer of water in the largest scale for up to three days. Moreover, we observed higher temperatures at higher densities which could be due to increased movement and interaction with conspecifics, microbial activity, varied air movements among others, which may have resulted in higher energy consumption and reduced weight gain. Similarly, the bench-top scale resulted in an average individual larval weight of 174.4 mg FM and was 24.7% higher than that of the industrial scale [57]. The total biomass obtained per larva (normalized data) decreased with upscaling (Table 3). However, the lowest scale (10 g) also had a lower total harvested biomass indicating the lower scale is not ideal even in lab-scale approaches. The FCR values in our study ranged between 2.7–4.1 (S3 Table). We found no significant difference in the FCR values for the scales 50–1,000 g feed. Gligorescu et al. (2022) state FCR values of 2.3–5.5, indicating successful and efficient production in a semi-industrial setting [43]. A lower FCR indicates better feed efficiency, meaning less feed is needed to produce a given amount of body mass. However, an FCR of 4.1 in our study is considered decent even in comparison to other small-scale studies which had much higher FCR values, namely 5.8 for municipal organic waste [20] or 10.3 for poultry and dairy manure [59]. The use of catering and household waste as BSF feed resulted in an FCR of 1.7–3.6 [60]. This highlights that the FCR, like other variables, depends on various factors such as diet composition, particle size, pH levels, moisture content, larval density, and temperature in BSF production. The efficiency of conversion of ingested feed (ECI) articulates the amount of the ingested feed converted into larval biomass. In general, the ECI increased with upscaling. The ECI parameter is not yet discussed in terms of scale in any studies. In the current study, the substrate reduction decreased with upscaling, resulting in more frass at higher scales that needs to be managed. The substrate reduction was only 16.2% in the 2,500 g scale, while it was 31.9% in the lowest scale group. Such lower substrate reduction at a larger scale can pose a challenge, particularly in industrial settings, unless the resulting frass is repurposed as a soil supplement. The waste reduction of 59.4–74.0% was measured for 10,000 larvae fed with 8 kg of a diet consisting of apple, banana, and spent grains [40]. These general differences between the studies may be because of varying fiber contents and nutrient qualities.

The scale of BSF production has recently gained attention as industrial setups expand globally [40, 43]. Our scale comparison highlights its importance for optimizing production during scaling. While bench-top experiments remain crucial for fast and economically assessing various substrates [40], results may not directly translate across different scales. This can also be seen from our results where the five different scales used led to different larval performance for the variables analyzed. The variation in larval performance may also be attributed to the vacuum packaging used during their early stages for transportation to the laboratory. While the effects of vacuum packaging on young larvae are not well understood, it could potentially have an impact. However, vacuum packaging is a standard method with which young BSF larvae are delivered from the BSF companies. Future research should integrate both small- and large-scale experiments for more comprehensive validation of results. As private companies investing in large-scale BSF production often keep their findings confidential due to competitive pressures [39], studies like ours are valuable for exploring new possibilities in mass production of BSF larvae.

In this study, we primarily conducted experiments using a 100 g DM feed mix. Returning to the initial research questions, we conclude that increasing the moisture level to 75%

enhanced larval performance, as evidenced by improvements in larval weight, total biomass, and FCR. Moreover, it was observed that densities exceeding 250 larvae/box did not improve larval performance, even with substrate moisture levels above 60%. Through optimizing moisture level and larval density, we determined that a CF and SMS mix at 75% moisture and a density of 250 larvae/box yielded the best results. In the scale experiment, the highest individual larval weight (169 mg FM) and optimal FCR (2.7) were obtained with 100 g DM feed. The five laboratory scales also revealed that the scale of the experiment, even within similar feed and climate settings, resulted in differences in larval performance in terms of survival, individual larval weight, total biomass, and FCR. These results suggest that for large-scale applications, variables must be carefully controlled and minimally adjusted to replicate similar outcomes.

Supporting information

S1 Table. Optimization of substrate moisture. A comparison of three treatments with three replicates each. In the treatment column % indicates the percentage of substrate moisture and L indicates number of larvae used per treatment. Survival % = percentage of survived larvae or pupae, FM = fresh matter, DM = dry matter, FCR = feed conversion ratio.
(XLSX)

S2 Table. Density optimization for selected moisture contents. A comparison of six treatments with three replicates each. In the treatment column % indicates the percentage of substrate moisture and L indicates number of larvae used per treatment. Survival % = percentage of survived larvae or pupae, FM = fresh matter, DM = dry matter, FCR = feed conversion ratio.
(XLSX)

S3 Table. Investigation of scale effects on growth performance and bioconversion. A comparison of five treatments with three replicates each. In the treatment column g indicates the amount of substrate on dry matter basis and L indicates number of larvae used per treatment. Survival % = percentage of survived larvae or pupae, FM = fresh matter, DM = dry matter, FCR = feed conversion ratio, ECI = efficiency of conversion of ingested feed, WRI = waste reduction index.
(XLSX)

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