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Pretreatments to improve black soldier fly larvae performance on fibrous biowastes and safeguarding insect-based food and feed

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“And when you can’t go back,

you have to worry only about the best way of moving forward.”

- Paulo Coelho

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Summary

To ensure food security and meet the rising demands of the growing population, the global food production will need to upscale. However, the current food system's resource-intensive practices, land conversion and greenhouse gas emissions are pushing several planetary boundaries, leading to challenges such as biodiversity loss and climate change. Among the factors contributing to these environmental impacts is the production of animal sourced protein, driven by the use of several unsustainable feed ingredients (e.g., soybean and fish meal). The exploration for a more sustainable protein source is needed.

A promising alternative involves nutrient upcycling from biowastes to produce protein-rich feeds suitable for aquaculture and livestock. Larvae of the black soldier fly, *Hermetia illucens* L. (BSFL) can efficiently convert various biowastes into a high-protein, high-fat insect biomass (partially) substituting feeds for pig, poultry, fish and pet nutrition. The incorporation of BSFL-based feed not only reduces land utilization and greenhouse gas emissions but also enables the valorization of biowastes and byproducts. The thesis was dedicated to examining two aspects of the insect production chain, pre-processing of biowastes and post-processing of insect products to support the viability and safety of an insect-based system.

The first part of the thesis addressed BSFL's challenges with low-value biowastes high in lignocellulosic fibers, resulting in poor development, marked by reduced bioconversion efficiency and longer developmental times. Lignocellulosic fibers, composed of lignin, cellulose, and hemicellulose, hinder microbial and larval degradation. Therefore, this thesis investigated biowaste pre-treatments to increase degradability by larvae and/or microorganisms in the biowaste or larval digestive tract. Given the limited research on biowaste pre-treatments within the BSFL context, this work evaluated the potential applicability of pretreatment methods used in other bioprocessing technologies, including physical (e.g., mechanical and thermal), chemical (e.g., alkaline and acids) and biological (e.g., bacteria and fungi) methods.

This thesis focused on evaluating the identified potential pretreatments aimed at improving BSFL performance. We investigated the use of ammonia pretreatment for lignocellulose degradation and its effect on BSFL performance. An optimal ammonia dose with 5 % and pretreatment time of three days was identified for effective fiber degradation and assessed for enhancing larval rearing. However, ammonia pretreatment for all substates decreased BSFL rearing performance metrics by more than half compared to the untreated control. Further analysis revealed that ammonia pretreatment exhibited dose-dependent toxicity towards BSFL. Therefore, ammonia pretreatment was deemed not suitable for BSFL.

Following chemical pretreatment, this thesis focused on thermal and mechanical methods, to investigate their impact on BSFL processing of fibrous biowastes. The applied thermal pretreatment resulted in either no significant improvement or decreased larval performance on all substrates, regardless of treatment duration. However, mechanical pretreatment showed promising results, demonstrating higher larvae performance. This work highlights the need to assess numerous additional pretreatments across a variety of biowastes.

Although improving BSFL development on low-value high fibrous biowastes is necessary, ensuring that the end-product is safe is equally important. Therefore, this thesis aimed to address the potential presence of pathogens in BSFL reared on various biowastes by exploring the use of an effective decontamination technology. The focus was on a non-thermal treatment technology, low-energy electron beam (LEEB) for post-processing of dried insect products. LEEB has gained interest due to its ability to reduce microbial concentrations in low moisture goods with minimal product deterioration, while potentially extending shelf-life. Given the diverse microbial communities in edible insects and insect-derived products post processing treatments are crucial for product safety. This thesis explored the application of LEEB treatment (250 keV and 12 kGy) on dried BSFL and yellow mealworm. Inoculated *Escherichia coli* K-12 was effectively reduced by a 4-$\log_{10}$ on dried BSFL. A subsequent six-month shelf-life study on naturally contaminated dried BSFL and yellow mealworm demonstrated that microbial counts in both LEEB-treated BSFL and mealworm remained lower than the control throughout the shelf-life. LEEB treatment had no impact on the peroxide value for both insects and drying treatments.

To conclude, BSFL has the potential as a viable alternative protein source, addressing the growing global demand for more sustainable animal feed solutions. The challenges posed by low-value biowastes high in lignocellulosic fibers were addressed through the investigation of biowaste pretreatments in this work. Moreover, this thesis emphasized the significance of effective decontamination technologies, with LEEB treatment proving to be a gentle and effective approach to support the safety of dried insect products. As BSFL production holds the potential for efficient waste valorization and protein-rich feed production, continued research in optimizing their rearing processes and addressing safety concerns remains imperative.

Zusammenfassung

Um die Ernährungssicherheit zu gewährleisten und die steigende Nachfrage der wachsenden Bevölkerung zu befriedigen, muss die weltweite Nahrungsmittelproduktion ausgeweitet werden. Die ressourcenintensiven Praktiken des derzeitigen Lebensmittelsystems, die Landumwandlung und die Treibhausgasemissionen stoßen jedoch an die Grenzen des Planeten und führen zu signifikanten Problemen, wie dem Verlust der biologischen Vielfalt und dem Klimawandel. Zu den Faktoren, die zu diesen Umweltauswirkungen beitragen, gehört die Produktion von tierischem Eiweiß, die durch die Verwendung verschiedener nicht nachhaltiger Futtermittelzutaten (z. B. Sojabohnen und Fischmehl) vorangetrieben wird. Daher ist die Suche nach einer alternativen Proteinquelle notwendig.

Eine vielversprechende Alternative ist das Upcycling von Nährstoffen aus Bioabfällen, um proteinreiche Futtermittel für Aquakulturen und Viehzucht herzustellen. Die Larven der schwarzen Waffenfliege, *Hermetia illucens* L. (BSFL), können verschiedene Bioabfälle effizient in protein- und fettreiche Insektenbiomasse umwandeln und damit Futtermittel für Schweine, Geflügel, Fische und Haustiere (teilweise) ersetzen. Die Verwendung von Futtermitteln auf BSFL-Basis reduziert nicht nur den Flächenverbrauch und die Treibhausgasemissionen, sondern ermöglicht auch die Verwertung von Bioabfällen und Nebenprodukten. In dieser Arbeit wurden zwei Aspekte der Insektenproduktionskette untersucht: die Vorverarbeitung von Bioabfällen und die Nachverarbeitung von Insektenprodukten, um die Marktfähigkeit und Sicherheit von Prozessen auf Insektenbasis zu unterstützen.

Der erste Teil der Arbeit befasste sich mit den Problemen von BSFL bei Nutzung von minderwertigen Bioabfällen, die einen hohen Anteil an Lignozellulosefasern aufweisen. Dies führt zu einer schlechten Larvenentwicklung, die durch eine geringere Biokonversionseffizienz und längere Entwicklungszeiten gekennzeichnet ist. Lignozellulosefasern, die aus Lignin, Zellulose und Hemizellulose bestehen, behindern den mikrobiellen und larvalen Abbau. Daher wurde in dieser Arbeit untersucht, wie Bioabfälle vorbehandelt werden können, um die Abbaubarkeit durch Larven und/oder Mikroorganismen im Bioabfall- oder Larvenverdauungstrakt zu erhöhen. In Anbetracht der begrenzten Forschung über die Vorbehandlung von Bioabfällen im Rahmen der BSFL Produktion wurde in dieser Arbeit die potenzielle Anwendbarkeit von Vorbehandlungsmethoden bewertet, die in anderen Bioverarbeitungsprozessen eingesetzt werden, einschließlich physikalischer (z. B. mechanischer und thermischer), chemischer (z. B. alkalischer und saurer) und biologischer (z. B. Bakterien und Pilze) Verfahren.

Diese Arbeit konzentrierte sich auf die Bewertung der identifizierten potenziellen Vorbehandlungsverfahren zur Verbesserung der BSFL Produktion. Wir untersuchten die Verwendung der Ammoniak-Vorbehandlung für den Lignozelluloseabbau und ihre Auswirkungen auf die BSFL Performance. Eine optimale Ammoniakdosis von 5 % und eine Vorbehandlungszeit von drei Tagen wurden für einen effektiven Faserabbau ermittelt und im Hinblick auf die Verbesserung der Larvenaufzucht bewertet. Allerdings verringerte die Ammoniak-Vorbehandlung bei allen Substraten die Leistungsdaten der BSFL-Aufzucht um mehr als die Hälfte im Vergleich zur unbehandelten Kontrolle. Eine weitere Analyse ergab, dass die Ammoniak-Vorbehandlung eine dosisabhängige Toxizität für BSFL aufwies. Daher wurde die Ammoniak-Vorbehandlung für die BSFL als ungeeignet erachtet.

Nach der chemischen Vorbehandlung konzentrierte sich diese Arbeit auf die thermische und mechanische Vorbehandlung, um deren Auswirkungen auf die BSFL Verarbeitung von faserigen Bioabfällen zu untersuchen. Die genutzte thermische Vorbehandlung führte bei allen Substraten, unabhängig von der Behandlungsdauer, entweder zu keiner signifikanten Verbesserung oder zu einer verminderten Leistung der Larven. Die mechanische Vorbehandlung zeigte jedoch vielversprechende Ergebnisse und führte zu einer höheren Performance der Larven. Diese Arbeit unterstreicht die Notwendigkeit, zahlreiche zusätzliche Vorbehandlungen für eine Vielzahl von Bioabfällen zu prüfen.

Obwohl es notwendig ist, die Entwicklung von BSFL aus geringwertigen, faserreichen Bioabfällen zu verbessern, ist es ebenso wichtig, die Sicherheit des Endprodukts zu gewährleisten. Ziel dieser Arbeit war es daher, das potenzielle Vorhandensein von Krankheitserregern in BSFL, die auf verschiedenen Bioabfällen gezüchtet wurden, durch den Einsatz einer wirksamen Dekontaminierungstechnologie zu untersuchen. Der Schwerpunkt lag dabei auf einer nicht-thermischen niedrig Elektronenstrahl basierten Technologie (LEEB) für die Nachbehandlung von getrockneten Insektenprodukten. LEEB hat aufgrund seiner Fähigkeit, die mikrobiellen Konzentrationen auf trockenen Produkten bei minimaler Qualitätsverschlechterung zu reduzieren und gleichzeitig die Haltbarkeit zu verlängern, an Interesse gewonnen. Angesichts der vielfältigen mikrobiellen Gemeinschaften in essbaren Insekten und aus Insekten gewonnenen Produkten sind Nachbehandlungen für die Produktsicherheit entscheidend. In dieser Arbeit wurde die Anwendung der LEEB-Behandlung (250 keV und 12 kGy) auf getrockneten BSFL und gelben Mehlwürmer untersucht. Inokulierte *Escherichia coli* K-12 wurden auf getrockneten BSFL effektiv um 4 $\log_{10}$ reduziert. Eine anschließende sechsmonatige Haltbarkeitsstudie von natürlich kontaminierten getrockneten BSFL und gelben Mehlwürmern zeigte, dass die Keimzahlen sowohl in den LEEB-behandelten BSFL als auch in den Mehlwürmern während der gesamten Haltbarkeitsdauer niedriger waren als in der Kontrolle. Die LEEB-Behandlung hatte weder bei den genutzten Insekten noch bei den unterschiedlich angewandten Trocknungsverfahren einen Einfluss auf den Peroxidwert.

Zusammenfassend lässt sich sagen, dass BSFL das Potenzial hat, eine alternative Proteinquelle zu sein, die der wachsenden weltweiten Nachfrage nach nachhaltigen Futtermitteln gerecht wird. Die Herausforderungen, die sich aus geringwertigen Bioabfällen mit einem hohen Anteil an Lignozellulosefasern ergeben, wurden in dieser Arbeit durch die Untersuchung von Vorbehandlungsverfahren für Bioabfälle angegangen. Darüber hinaus wurde in dieser Arbeit die Bedeutung effektiver Dekontaminierungstechnologien hervorgehoben, wobei sich die LEEB Behandlung als schonender und effektiver Ansatz zur Förderung der Sicherheit von getrockneten Insektenprodukten erwies. Da die BSFL Produktion das Potenzial für eine effiziente Abfallverwertung und eine proteinreiche Futtermittelproduktion birgt, sind weitere Forschungen zur Optimierung der Aufzuchtverfahren und Sicherheit unerlässlich.

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Introduction

Rapid urbanization as a result of increased population growth, expecting to exceed 9 billion by 2050, intensifies the challenges in waste management. As cities expand and economies thrive, the availability of products and services increases, leading to a rise in waste generation. By 2050 waste production is expected to reach 3.4 billion tons, with food and green waste currently constituting 44% of global waste (Kaza et al., 2018). To address these growing challenges, the shift from a linear economy to a more circular economy has emerged as a solution to foster sustainable development. In low- and middle-income countries where waste management remains a challenge, the utilization of the black soldier fly larvae (BSFL), *Hermetia illucens* L., technology offers a promising opportunity for waste valorization. BSFL can reduce waste by 12–68 % dry mass (DM), transforming it into a protein- and fat-rich insect biomass suitable as feed for pets, pigs, poultry and fish (Gold et al., 2018; Gold et al., 2020).

Additionally, as food production will need to increase by 70% to meet the global demand, BSFL offer an alternative to traditional protein rich feeds such as soybean meal and fishmeal, known for their negative environmental impacts (Aiking, 2011). The production of marketable products from BSFL reared on biowaste offers opportunities for revenue generation and entrepreneurship, potentially stimulating economic growth in the global south.

One of the main advantages of BSFL is their ability to be reared on a wide range of biowaste streams including, but not limited to: animal manure (Craig Sheppard et al., 1994; Newton et al., 2005; Nguyen et al., 2015; Yu et al., 2011), food waste (Bosch et al., 2019; Nguyen et al., 2015; Nyakeri et al., 2017a), fruit waste (Mohd-Noor et al., 2017), brewery side streams (Meneguz et al., 2018b), fecal sludge (Lalander et al., 2013; Peguero et al., 2021) and human feces (Banks, Gibson, and Cameron 2014). However, larvae development can vary depending on the biowaste type. For example, the bioconversion rate, which indicates the amount of dry larval biomass produced per unit of waste, can range from 1.9 – 6% for animal manures (Gold et al., 2018; Liu et al., 2018) and 13 – 21% for food waste (Lalander et al., 2019; Nyakeri et al., 2017b). When attempting to create a profitable business from the upscaling of waste, understanding critical factors that influence BSFL development is essential.

Factors such as diet, larval density, feeding regime and temperature during the larval stage can significantly impact their development (Barragan-Fonseca et al., 2017; Miranda et al., 2019; Yakti et al., 2022). Studies have highlighted that protein and non-fibrous carbohydrates can increase larval development while

lignocellulosic fibers (i.e., lignin, cellulose hemicellulose) have demonstrated negative impact on larval growth (Barragan-Fonseca et al., 2019; Gold et al., 2020b; Liu et al., 2018). These indigestible carbohydrates present a challenge for both BSFL and biowaste microorganisms in the degradation of high fiber biowastes. The use of pretreatment, applied to other bioprocessing technologies, to assist in fiber degradation could also be implemented within BSFL production.

Although BSFL's ability to consume a wide variety of biowastes is a main advantage, the presence of potential pathogens, such as foodborne bacteria, in the feeding substrate raises concerns about pathogen accumulation in the larval digestive tract. For example, Lalander et al. (2013) observed helminth eggs, *Ascaris suum* ova, in the larval gut when rearing BSFL on contaminated fecal sludge. To reduce the microbial load of the larvae, common thermal treatments such as blanching, boiling and drying are used (Saucier et al., 2022; Vandeweyer et al., 2017). While these treatments effectively inactivate most microorganisms, heat resistant pathogens such as *Salmonella* spp. and bacterial spores can remain in heat treated products (Lang et al., 2016; Zhang et al., 2018). For instance, pathogens such as *Salmonella* spp., *E. coli*, and *B. cereus* have been detected in dried BSFL (Grabowski and Klein, 2017; Kashiri et al., 2018; Wynants et al., 2019). Identifying suitable post processing treatments to ensure safety of dried insect products while maintaining product quality is essential.

Therefore, this thesis focuses on two key aspects of the BSFL production chain: the pre-processing of the biowastes and post-processing of dried insect products. To gain a comprehensive understanding of the factors that contribute to the development of BSFL, and biological contaminants associated with insect rearing, **Chapter 1**, provides an in-depth literature-based summary.

Following chapter 1, the first part of the thesis delves into the use of pretreatments to improve larval performance on high fiber biowastes. **Chapter 2** discusses the different pretreatment strategies that could potentially be used within BSFL production. The utilization of pretreatments in the context of BSFL has remained largely unexplored, prompting the need for investigation. This chapter examines pretreatment methods applied in other bioprocessing technologies, such as anaerobic digestion and composting, to enhance methane production or improve composting efficiency.

Based on the review, alkaline pretreatments, particularly ammonia pretreatment, has been widely used for improving methane production. Therefore, **Chapter 3** investigates the use of ammonia pretreatment for fiber degradation on spent grain, cow manure, grass clippings and oat by-product. To test a different category of pretreatment, **Chapter 4** evaluates on the use of physical pretreatments, specifically mechanical and thermal on spent grain, cow manure, and grass clippings.

While the first aspect of the thesis assessed how to improve larval development on fibrous biowastes another aspect was ensuring product safety. **Chapter 5** focuses on investigating the use of a non-thermal treatment technology, low energy electron beam (LEEB) to support the safety of dried insect products for food and feed production. This chapter investigates LEEB's application for both dried BSFL and dried yellow mealworm.

Chapter 6, offers a comprehensive summary of the main conclusions of this thesis. Additionally, this chapter also highlights further research perspectives in relation to the findings of this this research.

1. Chapter: Background

1.1. Food insecurity

Food security is a critical aspect of the United Nations' 2030 Agenda for Sustainable Development Goals (SDG), with SDG 2 aiming to achieve zero hunger by ensuring access to safe and nutritious food (United Nations, 2023). Prior to the Covid-19 pandemic more than 820 million people still suffered from food insecurity (United Nations, 2020). Unfortunately, the pandemic has only further worsened this issue, placing millions more are at risk of hunger due to job losses, economic downturns, and disruptions in the global food supply chain (United Nations, 2020). Addressing food security becomes even more crucial in light of the increasing food demand expected with the projected global human population surpassing 9 billion by 2050.

Food systems play a significant role in environmental challenges, contributing to climate change as almost a third of all greenhouse gas emissions comes from the food system (Foley et al., 2005; Vermeulen et al., 2012). Among the pressing concerns in the food system is the increasing demand for animal-sourced protein (i.e., meat, milk, eggs, and fish), with the demand for meat expected to be 58% higher in 2050 than in 2010 (Makkar et al., 2014). However, this comes with significant ecological consequences, as livestock production currently accounts for 75% of all agricultural land use, and contributes to deforestation, freshwater degradation and biodiversity loss (Foley et al., 2011). The pursuit of SDG 2 underscores the urgency of finding sustainable and alternative proteins such as, cultured meat (Bhat et al., 2015), single-cell proteins (Anupama and Ravindra, 2000), plant-based protein (Day et al., 2022) and insects (Van Huis, 2013) as solutions that tackle food security while addressing environmental challenges.

1.2. Biowaste management

In addition to food security, waste management is an equally pressing concern. Waste generation is a byproduct of urbanization and economic growth (Hoornweg and Bhada-Tata, 2012). As global income level and living standards rise, so does the increase in consumption, consequently increasing biowaste materials typically generated from agricultural industries and municipal solid wastes (Hoornweg and Bhada-Tata, 2012). In 2016, the amount of global waste generated was estimated to be around 2.1 billion tons, with a substantial 44% attributed to food and green waste (Kaza et al., 2018). The global waste is expected to increase to 3.4 billion tones by 2050 (Kaza et al., 2018).

The escalating waste production poses significant environmental consequences, including heightened pollution levels, depletion of natural resources, and strain on ecosystems. Insufficient waste management practices can result in the release of harmful pollutants and greenhouse gas emissions. Conventional waste treatment options typically consist of incineration or landfill (Hoornweg and Bhada-Tata, 2012), with the latter typically being the preferred choice (Figure 1.1a) (Nanda and Berruti, 2021). However, both these options are considered major sources of pollution because of their negative impact on the environment and public health (Hoornweg and Bhada-Tata, 2012).

In low- and middle-income countries, biowaste management remains a challenge, particularly in the context of household waste among urban and peri-urban areas, as it often remains uncollected. Biowaste comprises of a wide variety of organic material, influenced by several local factors including waste collection system, and socioeconomic factors (Puig-Ventosa et al., 2013). Despite constituting around 80% of the solid municipal waste, the organic fraction, is typically left unaccounted for (Diener et al., 2011). Consequently, the need for biowaste treatment technologies that can unlock the value within biowaste is necessary.

(a) (b)

Figure 1.1 (a) Landfill filled with mixture of plastic, biowaste, textiles and other miscellaneous items; (b) Biowaste when separated can be used for BSFL rearing (D.A. Peguero, 2023).

1.3. Black soldier fly larvae as a biowaste treatment technology

An insect that has gained particular interest is the larvae of the black soldier fly *Hermetia illucens* L., (BSFL). BSFL are known as voracious feeders because of their ability to reduce substantial volumes of biowaste and side streams by 26–68 % dry mass (DM) (Gold et al., 2018). BSFL can consume a wide variety of biowaste including animal manure (Peguero et al., 2023), food waste (Gligorescu et al., 2022), fruit waste

as shown in Figure 1.1b (Meneguz et al., 2018b), brewery side streams (Peguero et al., 2023) and human feces (Banks et al., 2014). Through their effective breakdown and conversion of organic matter into a nutrient-rich insect biomass, BSFL play a crucial role in preventing the loss of nutrients that would otherwise contribute to pollution and resource depletion. Notably, BSFL upcycle these otherwise lost nutrients, producing a biomass high in protein (32–58 % DM) and fat (15–39%) (Gold et al., 2018).

Given their high protein and lipids, BSFL can be used to (partially)-substitute, traditional protein livestock feed ingredients such as fishmeal and soybean meal. Further processing, involving defatting and protein solubilization, separate these fractions to obtain a protein-rich larval meal and oil from the lipids suitable for pigs, poultry, fish and pets (Figure 1.2). Beyond their role in nutrient recycling for protein and lipid production, BSFL leave behind a compost-like residue beneficial for plant growth (Figure 1.2).

In comparison to other bioprocessing technologies such as composting, BSFL bioconversion has the potential to produces fewer greenhouse gases (Mertenat et al., 2019). The feeding process is characterized by continuous larval movement throughout a high moisture content substrate, which favors aeration and oxygen supply for the aerobe microorganism, thus reducing methane emissions, which are produced only under anaerobic conditions (Chen et al., 2019). Depending on the scale, insect rearing can be implemented with simple equipment and minimal land area, rendering its breeding economically feasible and environmentally appealing for contributing to environment preservation (Grau et al., 2023; Spykman et al., 2021).

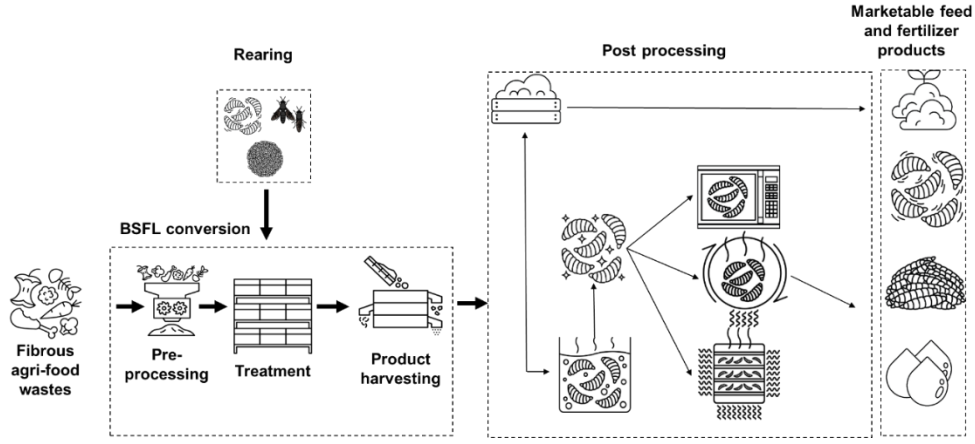

Figure 1.2. Schematic of BSFL production system (Eawag/SANDEC).

1.3.1. *Hermetia illucens*, Black soldier fly

Hermetia illucens, commonly known as black soldier fly (BSF), belongs to the order Diptera and family Stratiomyidae (Table 1.1). BSF originated in the Americas and was initially widespread from Argentina to central USA, and abundantly found in both tropical and warmer temperate regions (Craig Sheppard et al., 1994). However, with the advent of globalization, BSF has been introduced to numerous regions across the globe including Africa, Asia, Australia, and Europe (Gujarathi and Pejaver, 2013; Maquart et al., 2020; Martínez-Sánchez et al., 2011). In recent decades, BSF has been globally distributed for usage within large-scale industries, as evidenced by the identification of domesticated strains from individual samples across 57 countries (Kaya et al., 2021).

The adult BSF has a slender black body featuring two transparent windows situated on the first abdominal segment and range in length of 15–20 mm (Oliveira et al., 2015). Additionally, the adult possesses a single pair of iridescent flight-wings and antennae extending from its head (Gujarathi and Pejaver, 2013; Lemke et al., 2022; Rehman et al., 2023). Despite the common belief that adults do not consume food, recent research contradicts this notion, revealing the presence of a fully functional alimentary canal capable of food transit and digestion (Bruno et al., 2019a). As such, the practice of providing sugar-water to adults has been applied in mating cages (Oonincx et al., 2016). The provision of sugar-water or protein feeding substrate has proven effective in extending the lifespan of both male and female adults (Bruno et al., 2019a; Nakamura et al., 2016), enhancing oviposition and improving egg hatchability (Bertinetti et al., 2019). Adults feed through extra-oral digestion due to the transformation of their digestive system during pupation (Bruno et al., 2019a). The non-piercing mouthpart restricts the amount of regurgitated saliva and the potential pathogen transmission by adults, resulting in negligible zoonotic risk (Lemke et al., 2022). However, this does not exclude the possibility of them being a mechanical vector for pathogens or being susceptible to them (Lemke et al., 2022; Tettamanti et al., 2022).

Table 1.1. Taxonomical classification of black soldier fly (image: K. Mikka, 2023) (Singh and Kumari, 2019).

Taxonomical classification		
	**Kingdom**	Animalia
	Phylum	Arthropoda
	Class	Insecta
	Order	Diptera
	Family	Stratiomyidae
	Genus	Hermetia
	Species	*H. illucens*

The life cycle of BSF consists of six life stages: eggs, neonates, larvae, prepupae, pupae and adult fly, as shown in (Figure 1.3) (De Smet et al., 2018). The cycle begins when adult flies emerge from the pupa stage,

with the average adult's lifespan lasting on average one to two weeks (with or without water), potentially extending up to 70 days, until their energy reserves are depleted (Nakamura et al., 2016). The mating behaver of male and female flies is influenced by light, showing increased success under direct sunlight (Heussler et al., 2018; Tomberlin and Sheppard, 2002). Mating occurs typically after two days of emergence, with females engaging in only one mating event in their lifetime (Singh and Kumari, 2019).

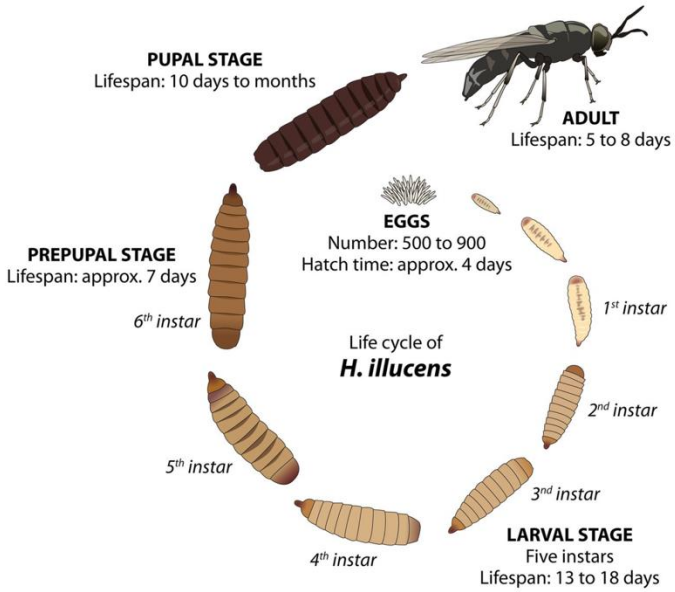

Figure 1.3. Schematic of BSFL lifecycle (De Smet et al., 2018).

Following mating, the female adult fly engages in oviposition, seeking small, dry cracks and crevices near moist decomposing organic matter, serving as a food source for the developing neonates (Figure 1.4) (Raksasat et al., 2020). The average female fly can lay between 500–900 eggs (De Smet et al., 2018). After around four days, the laid eggs hatch into cream-color neonate larvae, which migrate to the available food source and begin feeding. The duration of the larval stage can vary, potentially lasting up to 4 weeks, depending on the quality and availability of the substrate (Rehman et al., 2023). As the larvae reach their final larval stage, they can individually weigh up to 220 mg wet/larva and reach up to 27 mm in length and 6 mm in width (Lalander et al., 2019; Salomone et al., 2016). Upon reaching the fifth instar stage, the larvae transform into prepupae, acquiring a darker brown-gray appearance, and cease the feeding stage (De Smet et al., 2018). Afterwards, the prepupae seek a dry, shaded and protected environment for pupation. Finally, the adult fly emerges from the pupa shell to find mates, copulate, and commence the life cycle again.

Figure 1.4. Black soldier fly on eggie ball (Dortmans et al., 2017).

1.4. BSFL digestive system

The digestive tract of any organisms serves as the essential site for metabolic processes associated with food intake, digestion and absorption (Rajagopal, 2009). Environmental factors and the microbial enzyme production could impact these metabolic processes, potentially resulting in efficient waste degradation, and fostering growth improvements (De Smet et al., 2018). BSFL feed through their mouthparts situated at the anterior end of their elongate-oval shape to feed themselves, and are able to ingest liquids and solids (Gold et al., 2018). A recent study found larvae were able to ingest solids between 22–100 μm, depending on the larval age and weight (Lievens et al., 2023).

The alimentary canal of BSFL, commonly referred to as the digestive tract, is a tubular organ that extends along the length of the body from the anterior oral opening (mouthpart) to the posterior area near the anus (Linser and Dinglasan, 2014). The larval digestive tract can be divided into three main regions: the foregut, midgut, and hindgut (Bruno et al., 2019b). The foregut primarily functions to transport, store, filter and grind ingested food before being delivered to the midgut, where food digestion and nutrient absorption occurs (Chapman, 2013). The final region of the larval digestive tract is the hindgut, responsible for reabsorption of water or useful substances, such as amino acids, from the food content before excretion (Bonelli et al., 2019; Dow, 1987; Seyedalmoosavi et al., 2022).

The midgut is the longest part of the larval digestive tract composed of three regions (anterior, middle and posterior), each with different pH and microbiota (Bruno et al., 2019b; Chapman, 2013; Gold et al., 2018). Bonelli et al. (2019) found that the anterior midgut is characterized by an acidic pH 6, followed by the middle midgut featuring a strongly acidic pH 2 and the posterior midgut with an alkaline pH 8.5. Within the

midgut, cells are actively involved in the production and secretion of digestive enzymes, which work to break down the ingested diet into smaller molecules that can be readily absorbed for nutrient uptake. Gold et al. (2020) found that the overall midgut residence time ranged from 154–200 mins but was highly dependent on the protein and non-fibrous carbohydrate content of the substrate.

1.4.1. Microorganisms in larval digestive tract

The digestive tract of insects harbor a variety of microorganisms such as protists, fungi, archaea, and bacteria (Engel and Moran, 2013). For BSFL, the midgut appears to be the main site for digestion, unlike insects that primarily use extraoral digestion methods (Bruno et al., 2019b). Kim et al. (2011) found the BSFL digestive tract demonstrated high activity of amylase, lipase, and protease activity, while extracts from their salivary glands exhibited less than 10% of the total enzyme activity. BSFL were found to possess a broader range of digestive enzymes in the gut ,with higher levels of activity, compared to those present in housefly. This distinction explains why BSFL are classified as the most efficient scavengers among all fly species (Kim et al., 2011).

Further investigation revealed that different bacterial concentrations characterized different regions of the midgut, with the posterior midgut showing a higher bacterial load compared to the anterior midgut (Bruno et al., 2019b). Bonelli et al. (2019) found that the proteolytic activity was significantly higher in the posterior midgut region, exceeding levels in the anterior and middle midgut regions by more than forty- and twenty-fold, respectively, indicating its major role in protein digestion. In contrast, α-amylase activity was highest in the anterior midgut, although starch was also digested to a significant degree in the posterior (Bonelli et al., 2019). In contrast to the anterior and posterior midgut, the middle midgut region appeared to have no direct association with the digestive processes (Bonelli et al., 2019). Instead it demonstrated a connection to pathogen inactivation due to the high lysozyme activity and strong acidic pH (Bonelli et al., 2019).

Given the beneficial impact the gut microbial community has on digestion, it has been suggested that certain BSFL bacterial isolates from the gut could enhance growth, in terms of polysaccharide degradation (Callegari et al., 2020). A study identified 15 bacterial isolates capable of cellulose degradation (Callegari et al., 2020). However, quantitative verification of metabolic activity should be performed under different pH levels, considering the variation in pH levels within the BSFL midgut (Callegari et al., 2020).

Overall, understanding how substrate-characteristics such as nutrient content, substrate pH and temperature have in shaping or optimizing the larval gut microbiota, can enable us to improve feed efficiency and subsequently improve larval mass gain (De Smet et al., 2018).

1.5. Substrate characteristics influencing BSFL

Multiple substrate characteristics influence the efficiency of BSFL biowaste treatment. The nutrient composition in the substrate plays an important role in larval development, impacting larval growth, development time, survival rates and chemical composition quality (Barragan-Fonseca et al., 2017; Lalander et al., 2019). Various factors contribute to the nutrient composition including aspects such as, seasonality, waste collection methods and income, leading to challenges in commercializing a product that develops differently depending on the substrate characteristics. Moreover, recent studies are investigating the influence of physical properties, such as moisture content and bulk density on larval development, aiming for a more comprehensive understanding on the substrate characteristics (Bekker et al., 2021; Yakti et al., 2023). Properly optimizing these parameters could significantly enhance bioconversion efficiency across different biowastes, ensuring consistent and reliable biomass production.

1.5.1. Moisture content

Moisture content with the substrate plays a significant role on larval development. This factor not only influences substrate texture but also affects larval movement, feeding efficiency, nutrient absorption, growth and survival (Makkar et al., 2014; Palma et al., 2018). One previous study suggested optimal moisture contents were between 40–70% (Fatchuochim et al., 1988). However, recent studies suggest that optimal substrate moisture contents are in the range of 65–80% for improved larval performance (Bekker et al., 2021; Cammack and Tomberlin, 2017; Cheng et al., 2017; Palma et al., 2018). While lower substrate moisture contents with 44–55% have led to lower survival rates of 30% (Chen et al., 2019), BSFL have been grown on higher substrate moisture contents of 90–92%, with survival rates ranging between 76–96% (Lalander et al., 2019; Peguero et al., 2023). This indicates that survival outcomes also depend on the interaction of other substrate properties.

Moisture content plays a role with substrate microbial activity dynamics, which can affect BSFL development (Chen et al., 2019). Substrates with lower moisture content may foster higher microbial activities due to increased porosity and improved oxygen transfer rates within the substrate, with the microorganisms serving as competitors for resources also used by the larvae (Palma et al., 2018). For example, Bekker et al. (2021) found substrate moisture content ranging from 44–55% promoted microbial substrate degradation to carbon dioxide (CO_2), converting 12–22% of substrate carbon into CO_2. Consequently, lower substrate moisture contents led to a reduction in larval wet mass by 18–25% compared to those reared on substrate moisture contents ranging from 65–75%.

The relationship between substrate moisture content, microbial dynamics and larval development highlights the role moisture content plays in optimizing the rearing process of BSFL. By altering the substrate moisture content, greenhouse gas emissions can be reduced, thereby addressing environmental impacts (Chen et al., 2019).

1.5.2. pH

The acidity or alkalinity of the substrate, expressed as potential of hydrogen (pH), is an important parameter that influences the availability and effectiveness of BSFL and substrate microorganisms. These microorganisms are essential for breaking down complex organic matter into simpler nutrients to be assimilated by the larvae. pH levels can vary among different types of biowastes. For example, animal manure tends to be initially more alkaline, whereas food waste leans towards acidity (Ma et al., 2018). The pH variability can have important implications for the overall efficiency of the process. However, few studies have investigated pH, yielding conflicting findings. For example, Pang et al. (2020) found that a pH of 11 in food waste yielded the highest individual larval weight of 95 mg (wet mass), while pH 3 inhibited growth (Pang et al., 2020). Conversely, Meneguz et al. (2018a) observed no significant difference in final larval weights on Gainesville diet for pH values of 4, 6, 7.5, and 9.5. However, Ma et al. (2018) found that larval weights on Gainesville diet increased within pH 6–10, with lowest larval weight at pH 2 (160 mg) and the highest at pH 6 (210 mg). A consistent trend among these studies indicates a pH <3 results in the lowest performance. However, it appears difficult to conclude the optimal pH. Interestingly, Bonelli et al. (2019) found that when the substrate pH was 8, the secretion of protease (assisting in protein digestion) from the larval gut was significantly higher than on lower or higher pH levels. Additionally, all studies observed that the final pH values of the residue tended to shift more alkaline when the initial pH was ≥5 (Ma et al., 2018; Meneguz et al., 2018a; Pang et al., 2020). Overall, a need for additional studies exists in order to reach a definitive conclusion regarding the impact of pH on BSFL and substrate microorganisms.

Additionally, pH could be used as a way to mitigate CO_2 emissions. For example, Pang et al. (2020) found cumulative CO_2 emissions were lower for pH 11 on harvest day (88 g/kg DM) compared to the highest CO_2 emissions observed at pH 5 (161 g/kg DM). The author attributes this to CO_2 potentially converting into carbonate at the substrate's higher pH level. Another contributing factor could be the potential inhibition of microorganisms within the substrate due to the extreme pH condition, leading to reduced CO_2 emissions. For example, Mendonca et al. (1994) observed that high pH values (12.0) negatively affected Gram-negative foodborne pathogens, such as *E. coli* 0157:H7, and *Salmonella enteritidis* by disrupting the microbial cell membrane. The high pH resulted in reduction of 8-$\log_{10}$ colony forming units (cfu)/ml. From a product safety perspective, pH appears to impact the pathogen proliferation, which could serve as a safety strategy.

1.5.3. Protein and carbohydrate content

The composition of nutrients in the substrate plays a crucial role in the development of BSFL affecting factors such as development time, survivability, and bioconversion efficiency (Nguyen et al., 2013). Essential nutrients consist of organic matter, protein, non-fiber carbohydrates (NFC), fiber and lipids (Cohen, 2005; Gold et al., 2020b). Insects utilize dietary carbohydrates as an energy source, although the effectiveness of specific carbohydrates varies among different insect species (Cohen, 2005). Protein is broken down into amino acids, where the amino acids are subsequently absorbed and transported to the cells (Cohen, 2005). They are then resynthesized into the proteins that form integral components of the insect's body, such as muscle tissues, cell membranes and enzymes (Cohen, 2005). Similar to humans, insects require eight to ten essential amino acids (Cohen, 2005). As carbohydrates and protein are two essential nutrients for a suitable substrate, several studies have investigated the optimization of larval performance by formulating feeds according to specific protein-carbohydrate ratios (e.g., 1:1, 1:2, 1:3) (Barragán-Fonseca et al., 2018; Barragan-Fonseca et al., 2019; Cammack and Tomberlin, 2017; Gold et al., 2020b). Gold et al. (2020b) found formulating different biowastes (e.g., cow manure, slaughterhouse waste, human feces and more) using a protein-carbohydrate ratio of 1:1 led to improved larval performance compared to when the larvae were reared on the individual biowaste. Additionally, the formulations resulted in a median bioconversion rate that was 8.6% higher when compared to bioconversion rate of chicken feed (Gold et al., 2020b). However, Barragan-Fonseca et al. (2019) highlighted that the overall protein and carbohydrate content might be more crucial than the ratio. When considering the influence of substrate nutrient composition on larval nutrient content, it has also been observed that the larval fat and ash content appear to be dependent on the rearing substrate (Spranghers et al., 2016). For example, dietary protein has been found to increase larval fat content (Barragan-Fonseca et al., 2018; Beniers and Graham, 2019). Moreover, substrates with lower organic matter contents (<90 % DM) have been associated with higher larval ash contents (14–23% DM) compared to substrates with higher organic matter contents.

1.5.4. Lignocellulosic fibers

Biowastes containing high lignocellulosic fibers (>45%) influence larval performance leading to low bioconversion rates. For example, animal manure which can have a high total fiber content >50% DM has led to low bioconversion rates (2–6% DM) compared to bioconversion rates (>15% DM) on highly nutritious feeds such as food waste or chicken feed with lower fiber contents. Lignocellulosic fibers are composed of cellulose and hemicellulose (both consist of carbohydrates) and lignin (composed of aromatic units), organized into bundles that are tightly interconnected (Figure 1.5). Cellulose, accounting for 30–50% of the lignocellulosic biomass, is made up of D-glucose linked by β-1,4 glycosidic bonds, which can be further broken down into glucose (Hall et al., 2010; Liu et al., 2008a). Cellulose consists of both crystalline

and amorphous structures, with the amorphous portion being more readily digestible (Laureano-perez et al., 2005). The cellulose strains are organized into bundles known as cellulose fibrils (Hendriks and Zeeman, 2009). Hemicellulose, making up 20–35% of lignocellulosic biomass, serves as a connection between cellulose and lignin, adding rigidity to the complex molecule (Laureano-perez et al., 2005; Liu et al., 2008). Hemicellulose is a polymer composed of pentoses, hexoses and sugar acids (Saha, 2003), with the xylan and the glucomannan fractions showing effective extraction in an alkaline environment (Hendriks and Zeeman, 2009). Lignin, representing 10–25% of lignocellulosic biomass, is the largest non-carbohydrate fraction, consisting of different phenolic alcohols (Liu et al., 2008a). The recalcitrance of lignin makes its degradation more difficult compared to cellulose and hemicellulose.

The BSFL digestive system is inefficient in breaking down lignin, as indicated by a negative correlation between larval weight and lignin content (Liu et al., 2018; Zheng et al., 2012). In one study, the inclusion of hemicellulose fractions galactose, xylan and arabinose as additives in chicken feed resulted in significantly lower bioconversion rates compared to chicken feed with no additives (Cohn et al., 2022), further highlighting the impact fibers have on larval performance. However, other studies have reported that BSFL reduced lignin in dairy manure and rice straw by 31% and 50%, respectively, (Liu et al., 2021; Rehman et al., 2017b). Biowaste pretreatments are essential for breaking down the indigestible fiber fractions into more easily degradable components.

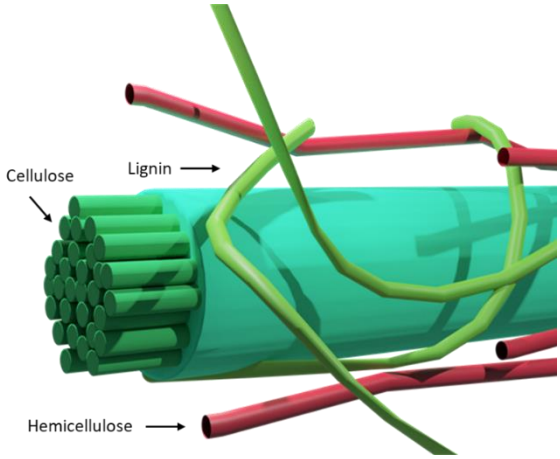

Figure 1.5. Schematic of the lignocellulosic structure (R. Axelrod/ETH Zurich/SFP).

1.5.5. Larval density, temperature and humidity

Apart from substrate properties, there are several other factors that influence larval performance, such as ambient temperature, relative humidity, and larval density (Barragan-Fonseca et al., 2017). BSFL are known for their tendency to aggregate during feeding, crawling towards the food from below, and then congregating on the upper layer, forming a fountain-like structure of larvae (Shishkov et al., 2019). Larval aggregation typically leads to higher substrate temperatures which may enhance feed assimilation, particularly with larger populations (Green et al., 2003; Li et al., 2023). Higher larval densities are linked to increased bacterial concentrations, potentially allowing larvae improved access to nutrients recycled by bacteria (Barragan-Fonseca et al., 2018). Therefore, optimizing larval density (i.e., number of larvae per square area) could contribute to either improving or inhibiting larval performance (Barragan-Fonseca et al., 2018). Various studies have examined different larval densities between 1–10 larva/cm^2 (Barragan-Fonseca et al., 2018; Dzepe et al., 2020; Parra Paz et al., 2015; Yakti et al., 2022).

The results among the studies tend to indicate that individual larva obtained more weight with lower larval densities <4 larvae/cm^2 (Barragan-Fonseca et al., 2018; Dzepe et al., 2020; Parra Paz et al., 2015; Yakti et al., 2022). For example, Dzepe et al. (2020) found the highest larval weight (i.e., 150 mg wet mass) at density 1 larva/cm^2, with the shortest development time of 12 days compared to higher densities. Barragan-Fonseca et al. (2018) found that lower densities (0.3–1 larvae/cm^2) and higher nutrient content accelerated larval development (13 days) compared to larvae grown on the higher density (4 larvae/cm^2). Similarly, Green et al. (2003) also observed that higher densities led to extended food ingestion periods and increased developmental time for the larvae of *Phormia regina,* driven by the need to acquire sufficient nutrition. Although higher larval densities (6 larvae/cm^2) have reached higher substrate temperatures (35–39°C) this did not lead to accelerated growth or increased weight, but rather reduced larval weight at harvest, likely due to the earlier depletion of nutrients in the feed (Yakti et al., 2022). However, all these studies used chicken feed as the substrate. In contrast when using former foodstuff as the substrate, Gligorescu et al. (2022) found a high larval density of 10 larvae/cm^2 compared to 7 larvae/cm^2 resulted in improved larval performance, and increased larval mass by around 33%. This indicates the need for further larval density studies using a wider range of biowastes.

Ambient temperature and relative humidity are also factors that have been widely studied with observations indicating temperatures around 25–32°C (Harnden and Tomberlin, 2016; Sheppard et al., 2002; Shumo et al., 2019a; Tomberlin et al., 2009) and humidity ≥50% (Holmes and Vanlaerhoven, 2012) are ideal for promoting larval development. Given the tendency of larvae to aggregate and generate heat, recent studies have begun to monitor substrate temperature. Recording substrate temperature alongside larval density is necessary as one study found that at a larval density of 4 larvae/cm^2, substrate temperature reached 43°C

even when ambient temperature was as low as 20°C (Li et al., 2023). This could be beneficial as protease activity has been observed to peak when substrate temperature reached 45°C (Kim et al., 2011).

1.6. BSFL end-products

1.6.1. Animal feed

BSFL are known for producing an insect biomass rich in both fat and protein insect biomass, making them suitable for livestock feed, such as pigs and poultry. In poultry nutrition, soybean meal and oil are commonly used as protein and fat sources (De Marco et al., 2015; Heuel et al., 2021). Recently, there has been a growing interest in integrating BSFL into poultry diets (Abd El-Hack et al., 2020). When considering poultry feed quality, investigations center around amino acid profiles and digestibility of the amino acids (Abd El-Hack et al., 2020). Certain essential amino acids such as lysine, methionine, and threonine are particularly important for pigs and poultry, with BSFL having high levels of these essential amino acids compared with soybean meal (Lu et al., 2022). Research has investigated different uses of incorporating BSFL into poultry animal feeds with full-fat larval meal or with fully defatted BSFL meal (Lu et al., 2022). For example, one study found that there was no significant loss in broilers' performance when exchanging soybean-based feeds with BSFL meal (Heuel et al., 2022) .

1.6.2. Aquaculture

Various studies conducted on freshwater species have demonstrated the potential of incorporating BSFL-based feeds into fish diets, highlighting the potential of insects as a partial or complete replacements for fish and soybean meals (Gasco et al., 2023). BSFL are rich in fat, which also serve as an energy source to fulfill fish metabolic needs (Gasco et al., 2023). The fatty acid concentration of BSFL, mainly consisting of saturated fatty acids, has been shown to be primarily influenced by nutritional content of the substrate they are reared on (Ewald et al., 2020).

The inclusion of BSF meal as a replacement for fish or soybean meal has shown positive impact on improving health conditions in several fish species (Mohan et al., 2022). For example, partially replacing 25% of fish meal and fish oil with BSF prepupae resulted in comparable feed conversion ratio and weight gain for rainbow trout (St-Hilaire et al., 2007).

1.6.3. Frass

Apart from the potential for sustainable protein and lipid yield from BSFL, the residual byproduct, commonly referred to as frass, produced during insect rearing, has the potential to contribute to the agri-food system (Barragán-Fonseca et al., 2022). Comprised mainly of insect feces, remnants of shed

exoskeletons, and undigested feed, frass contains valuable nutrients, bioactive molecules, and beneficial microorganisms that can enrich the soil–plant environment (Fuhrmann et al., 2022; Poveda, 2021). The chitin and its derivatives, resulting from the shedding of BSFL exoskeletons, contribute to positive plant performance by inhibiting pathogens and supporting beneficial soil microorganisms. Chitin-containing soil amendments have been demonstrated to enhance plant growth (Sharp, 2013) and modify plant physiology, attracting mutualist insects, such as pollinators and suppressing pests (Barragán-Fonseca et al., 2022).

1.7. Addressing safety concerns in BSFL production

1.7.1. Microbiological safety

Due to BSFL's ability to consume a wide variety of biowastes, addressing microbiological safety for insect-based feeds is critical to avoid transmission of disease. The hygiene concerns associated with edible insects or insect-based feeds stem from both the substrate, and the potential presence of pathogenic microorganisms on the external surface of the insect and/or within the larval digestive tract. For example, when inoculating fecal sludge with helminth eggs, *Ascaris suum* ova (320 ova/g wet mass fecal sludge), a concentration of 10 ova/larva were found within the larval gut (Lalander et al., 2014). Helminths, parasitic worms, are more prevalent in tropical and sub-tropical countries, infecting more than 2 billion people globally (Riaz et al., 2020). Another identified concern are pathogenic spore-forming bacteria, such as *Clostridium* spp and pathogenic species of *Bacillus cereus*, known to cause foodborne diseases (Delbrück et al., 2021; Vandeweyer et al., 2021) These spore-forming bacteria are heat-resistant and have been identified in BSFL among other pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Listeria* spp. and mycotoxin-producing fungi (Kashiri et al., 2018; Raimondi et al., 2020; Vandeweyer et al., 2021, 2020; Wynants et al., 2019). Although thermal treatments are effective in microbial inactivation, specific pathogens such as *Salmonella* spp. and bacterial spores are heat resistant and can remain in heat treated products (Lang et al., 2016; Zhang et al., 2018).

1.7.2. Treatment technologies in post-processing of insect products

Post-processing treatment technologies are critical to inactivate potential pathogens that may persist on the surface or within the larval digestive tract. Common thermal treatments are applied such as blanching, boiling and drying which can reduce the microbial load of insects (Saucier et al., 2022; Vandeweyer et al., 2017). Drying methods include various approaches such as roasting, frying, sun-drying, freeze-drying and microwave-assisted drying (Melgar-Lalanne et al., 2019). During the drying process, as water evaporates and cells undergo osmotic stress, the cells are exposed to reactive oxygen species, which cause damage to macromolecules and cellular membranes (Lang et al., 2016). Despite cellular impairment, low moisture foods remain non-sterile, permitting surviving pathogens to proliferate upon rehydration (Lang et al., 2016).

Various microorganisms including fungal spores like *Aspergillus*, and bacteria species like *Salmonella* can endure the drying process. Prolonged drying times to attempt to inactivate these microorganisms can lead to product discoloration, serving as an indicator of reduced vitamin and mineral content (Ratti, 2001) while also inducing lipid oxidation (Larouche et al., 2019). Therefore, blanching, a common pre-drying practice for most commercialized edible insects, assists in reducing initial microbial counts and enzymatic activity, although it might not be sufficiently intense to inactivate bacterial spores (Kamau et al., 2018; Jay, 1992; Larouche et al., 2019; Melgar-Lalanne et al., 2019; Vandeweyer et al., 2017; Zhen et al., 2020). For instance, Vandeweyer *et al.*, (2017b) found yellow mealworms subjected to blanching (100°C, 40 s) and microwave drying (maximum 80°C, 8−20 min) still retained 1.3−1.9 $\log_{10}$ cfu/g aerobic bacterial spores.

In pursuit of alternatives to thermal treatments, non-thermal treatment technologies such as high hydrostatic pressures, UV-light, pulsed electric fields (PEF), ultrasound, cold plasma and irradiation are potential options for post-processing. The aim of non-thermal treatments is to inactivate microorganisms while safeguarding the sensory attributes and nutrient values of edible insects and insect-based feeds (Zhang et al., 2019). To date, only high hydrostatic pressures (Kashiri et al., 2018; Larouche et al., 2019), and cold plasma (Rumpold et al., 2014) have been investigated with limited effectiveness observed in reducing total viable counts. Ionizing radiation technologies applicable for food irradiation that could be used within the insect production include high-energy gamma rays, x-rays and energy electron beam (Zhang et al., 2019). Ionizing radiation inactivates microorganism through direct or indirect damage of the nucleic acids (DNA) (Farkas et al., 2014). Irradiation has shown to be efficient in inactivating foodborne parasites and foodborne pathogenic bacteria in low moisture foods (Farkas et al., 2014).

2. Chapter: Pretreatments to improve black soldier fly larvae performance

A review of pretreatment methods to improve agri-food waste bioconversion by black soldier fly larvae

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2.1. Abstract

As the world population increases, food demand and agricultural activity will also increase. However, approximately 30 – 40% of the food produced today is lost or wasted along the production chain. Increasing food demands would only intensify the existing challenges associated with agri-food waste management. An innovative approach to recover the resources lost along the production chain and convert them into value-added product(s) would be beneficial. An alternative solution is the use of the larvae of the black soldier fly (BSFL), *Hermetia illucens* L., which can grow and convert a wide range of organic waste materials into insect biomass with use as animal feed, fertilizer and/or bioenergy. However, the main concern when creating an economically viable business is the variability in BSFL bioconversion and processing due to the variability of the substrate. Many factors, such as the nutritional composition of the substrate heavily impact BSFL development. Another concern is that substrates with high lignin and cellulose contents have demonstrated poor digestibility by BSFL. Studies suggest that pretreatment methods may improve the digestibility and biodegradability of the substrate by BSFL. However, a systematic review of existing pretreatment methods that could be used for enhancing the bioconversion of these wastes by BSFL is lacking. This paper provides a state-of-the-art review on the potential pretreatment methods that may improve the digestibility of substrates by BSFL and consequently the production of BSFL. These processes include but are not limited to, physical (e.g., mechanical and thermal), chemical (alkaline treatments), and biological (bacterial and fungal) treatments.

2.2. Fly larvae for recycling agri-food waste

Meeting global food demands within our planetary boundaries requires recycling nutrients currently wasted within the agri-food system (Willett et al., 2019). Today, more than 1.3 billion tons of food are lost or wasted each year (Gustavsson et al., 2011). Especially in low- and middle-income countries, a lack of managing these wastes leads to adverse public health and environmental effects (Hoornweg and Bhada-Tata, 2012). Instead, utilizing nutrients in agri-food wastes contributes to more sustainable food production for the growing global population, projected to surpass nine billion by 2050 (United Nations, 2017).

An emerging solution for recycling of agri-food wastes into value-added products for food production uses black soldier fly larvae (BSFL, *Hermetia illucens* L.) (Figure 2.1a) (Gold et al., 2018). BSFL convert many agri-food wastes (e.g. manures, agricultural crops and residues and milling side streams) (Gold et al., 2018, 2020) into protein-rich insect biomass that can efficiently substitute current livestock feed ingredients, such as soybean and fishmeal, leaving behind a compost-like residue (i.e., insect frass) for fertilizer applications (Klammsteiner et al., 2020). In addition, agri-food waste bioconversion with BSFL can reduce greenhouse gas emissions compared to biowaste composting, and produce BSFL-based feeds with a lower environmental impact than current livestock feed ingredients (Gold et al., 2018; Mertenat et al., 2019; Smetana et al., 2019, 2016). However, there are some trade-offs when pursuing the pathway of BSFL recycling of agri-food waste as these substrates do not result in high BSFL performance.

2.3. Low performance of fibrous agri-food waste

One key challenge of BSFL bioconversion is the frequently low process performance with some of the most abundant and affordably sourced agri-food wastes (Gold et al., 2018; Lalander et al., 2019). For example, the bioconversion rate, that is the amount of dry larval biomass produced per unit of waste, is 1.9 – 6% for animal manures (Gold et al., 2018; Liu et al., 2018), 5% for brewery side streams (Liu et al., 2018) and 4 – 5% for fruit and vegetable wastes (Lalander et al., 2019; Somroo et al., 2019). These bioconversion rates are low relative to those for food wastes (13 – 21%) (Lalander et al., 2019; Nyakeri et al., 2017b). BSFL development is affected by highly biodegradable macronutrients such as proteins, carbohydrates, and lipids (Trinh T. X. Nguyen et al., 2013). The low performance of some agri-food wastes has frequently been associated with their high crude fiber content containing lignocellulose, which has poor biodegradability. The lignocellulosic composition primarily consists of cellulose (~30 – 50%), hemicellulose (~20 – 35%) and lignin (~10 – 25%) (Table 2.1) (Liu et al., 2008). Despite contradictory results in the literature, where Rehman et al. (2017) reported BSFL reduced lignin and cellulose reduction in dairy manure by 31 and 50%, respectively, these lignocellulosic fractions are thought to be mostly indigestible by BSFL and negatively correlated with process performance (Gold et al., 2020; Liu et al., 2018). Since the process performance and

bioconversion of these agri-food wastes are decisive for the affordability and sustainability of BSFL-based products (Smetana et al, 2019), solutions to enhance process performance and conversion are urgently needed if these wastes will be used for BSFL production.

Table 2.1 Lignocellulosic composition of typical BSFL substrates. The classification and description of the substrates used in BSFL rearing were from (Gold et al., 2018). A breakdown of the lignocellulosic composition of each individual substrate can be found in Supplementary Material.

Substrate classification	Description	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Animal manures	Livestock excreta (e.g. poultry, swine and cow)	6 – 39[a,b]	12 – 28[a,m]	4 – 23[b,m]
Fruit and vegetable waste	Discarded fruits (e.g., apples, grapes and strawberries, and vegetables (e.g., lettuce, potatoes, cassava)	10 – 16[c,d]	7 – 9[c,d]	1 – 6[c,d]
Organic fraction of municipal solid waste	Considered a mixture of foods, fruits, vegetables, garden wastes, and soiled paper	5 – 37[e,g]	9 – 13[e,g]	10 – 19[e,f]
Milling and brewery sides streams	By-products from the milling and brewery industry, such as brewers spent grain	16 – 25[h,i]	11 – 28[j,k]	6 – 27[i,j]
Agricultural crops and harvesting residues	The residue of varying types of crops such as, sugarcane bagasse, wheat straw, and barley straw	26 – 50[n,o]	11 – 34[l,o,]	10 – 47[n,o]

[a]Sun & Cheng (2002); [b]Li et al. (2011); [c]Meneguz et al. (2018); [d]Edwiges et al. (2018); [e]Hartmann & Ahring (2005); [f]Rao & Singh (2004); [g]Zhu et al. (2010); [h]Kanauchi et al. (2001); [i]Meneguz et al. (2018); [j]Mussatto et al. (2006); [k]Shumo et al. (2019); [l]Mirko et al. (2021); [m]Chen et al. (2003) [n]Şenol (2021), [o]Ansari et al. (2021)
All values are presented as wt% in dry mass.

2.4. Enhancing BSFL performance with pretreatments

The structural characteristics of lignocellulosic substates (i.e., lignin presence, high cellulose crystallinity, water insolubility, and resistance to depolymerization) are common problems affecting the efficient valorization of many agri-food wastes (Agbor et al., 2011). Various physical, chemical, and biological substrate pretreatments have been extensively studied to enhance the conversion of lignocellulosic substrates for bioenergy yields (e.g., biogas, methane and ethanol) from anaerobic digestion and fermentation, ethanol production, (vermi)-composting and, to a lesser extent BSFL rearing performance (Carlsson et al., 2012; Hamelincket al., 2005; Kim 2013; Mosier et al., 2005). The overall bioconversion process performance, and economic viability are greatly affected by the availability and degradability of the substrate. The goal of substrate pretreatment is to degrade lignin, reduce the crystalline structure of cellulose

(Figure 2.1b), and increase pore size and surface/volume ratio, making it more suitable for digestion by larval (e.g., insect-based bioconversion) enzymes (Kim 2013; Mosier et al. 2005).

Implementation of pretreatments in BSFL production, could potentially enhance bioconversion efficiency and reliability across various agri-food wastes. However, few studies have investigated the effects of physical, chemical (Liu et al., 2021), and biological pretreatments (Isibika et al., 2019) on BSFL bioconversion. The goal of this review is to evaluate state-of-the-art pretreatment methods used for biogas production and (vermi-) composting, providing direction for future research activities. Due to the use of semi-solid substrates and the importance of microbiological processes, these waste conversion processes are probably most similar to BSFL bioconversion and thus the focus of this review. To synthesize the most relevant research for bioenergy/biogas production and (vermi-) composting, only those published in the last 20 years were considered (see Supplementary Material, for the four-step approach conducted for this review). To enable economically viable waste management and feed production, technologies considered by previous studies to have high energy demands (e.g., sonication, microwave, pulsed electric field, steam explosion), or include costly additives (e.g., commercial cellulolytic enzymes), are not discussed in detail in this review.

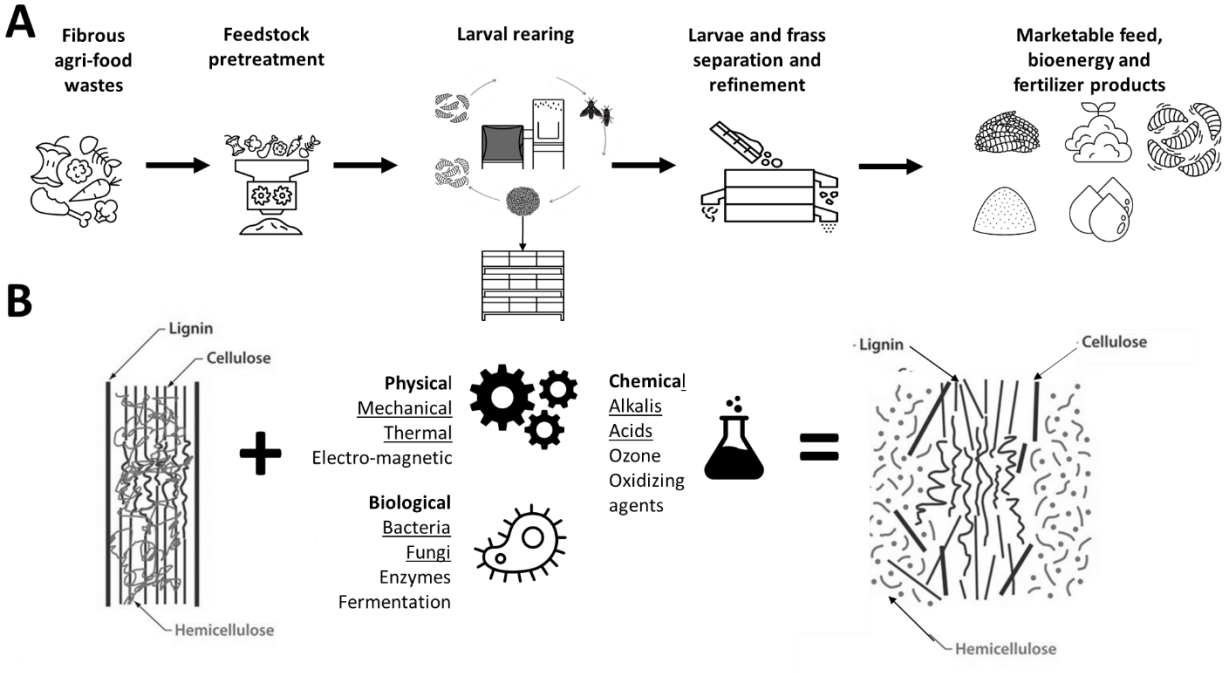

Figure 2.1. (A) Schematic of BSFL process for the production of insect-based feed, bioenergy and fertilizer from agri-food wastes and byproducts. (B) Schematic of the structural change of lignocellulosic components after physical, chemical, and biological pretreatments as part of substrate preparation. Underlined pretreatments are reviewed in this study. Adapted from Mosier et al., 2005 and Eawag/Sandec.

2.5. Physical pretreatments

Physical pretreatment is a widely applied substrate pretreatment for improving biogas production and composting. Physical pretreatment alters the lignocellulosic characteristics (e.g., reduce crystallinity, increase surface area, and increase porosity) using mechanical, thermal, and electromagnetic processes (Atelge et al., 2020). In contrast to chemical and biological pretreatments, physical pretreatments do not require additives (e.g., chemicals, microbes), but depending on the technology and the process target, they can demand energy (i.e., electricity and heat) requirements of 3 – 55 kWh/t per substrate (Bhagwat et al., 2015; Kratky and Jirout, 2011; Menardo et al., 2012).

Mechanical pretreatment can reduce substrate particle size with various choppers and mills (e.g., hammer mill, knife mill, bead mill, wet disk mill). Grinding and chopping typically involve fracturing the substrate down to diameters of <2 mm and 10 – 30 mm, respectively (Oyedeji et al., 2020). These technologies are considered more suitable for substrates with moisture contents lower than 15%, while colloid mills, extruders, and expanders are suitable for substrates with moisture contents over 15 – 20% (wet basis) (Kratky and Jirout, 2011).

Reduced substrate particle size has been shown to positively correlate with biogas or methane yield (Victorin et al., 2020), in addition to increasing cellulose degradation (Sharma et al., 1988). Mechanical pretreatments, such as grinding, extrusion, and mechanical disintegration, have already been applied in full-scale for animal manure, municipal solid waste, and agricultural crops and residues (Carrère et al., 2016). Particle size reduction, ranging from ≤2 mm to 5 cm for manures, fruit/vegetable waste, and crop and harvesting residues has shown to increase methane production/yield by 16 – 89% in comparison to untreated substrates (see Supplementary Material) (Angelidaki & Ahring, 2000; Menardo et al., 2012; Mshandete et al., 2006; Sharma et al., 1988). For example, methane production increased by 16% and 23% for cattle manure and sisal fiber, respectively, when reduced to 2 mm average particle size (Angelidaki and Ahring, 2000; Mshandete et al., 2006). The increased surface area provides greater access for microorganisms, which positively impacts methane production/yield. However, the benefit of particle size reduction is very dependent on the substrate. For example, Menardo et al. (2012) reported a methane yield increase of 57% and 19% for wheat straw and barley straw, respectively, when reduced to 5 cm, but did not observe the same effect for rice straw. Palma et al. (2019) suggested that the mechanical pretreatment efficiency may also be substrate-dependent for BSFL bioconversion, as decreasing almond hull particle size with a hammer mill from 6 to 4 mm decreased larval mass by 10%. The authors concluded that BSFL may benefit from aeration due to its larger particle size. In contrast to anaerobic digestion, BSFL based conversion is an aerobic process, requiring oxygen to function. Thus, porosity and airflow play a role, and a more compacted

substrate with lower particle size may lead to a reduced oxygen supply for BSFL. However, more research is needed to identify the optimal particle size for efficient BSFL bioconversion.

Another common physical pretreatment worth considering is thermal pretreatment, as the main goal is to increase the solubility of the organic matter, improving biodegradability and increasing biogas production and the efficiency of composting (Appels et al., 2010; Cao et al., 2019; Cesaro and Belgiorno, 2014). Thermal pretreatment is highly dependent on temperature, time, and, in some cases pressure, with temperatures above 100 °C and processing times ranging from 15 to 120 min with 2 – 9 bar pressure (Atelge et al., 2020; Carrère et al., 2010). However, these operating conditions may not be suitable for BSFL processing because of the inactivation of potentially beneficial microorganisms present in the substrate (Gold et al., 2020). For example, Isibika et al. (2019) attributed the 15% and 19% decrease in BSFL larval mass and bioconversion rate, respectively, on banana peels following pretreatment for 60 min at 120 °C at 2 bar, to release of toxic tannins. The production/release of phenolic compounds at high temperatures (e.g. >160°C) could potentially inhibit larval and microbial metabolism (Hendriks and Zeeman, 2009; Müller, 2000). In general, low temperature thermal pretreatment (e.g., <100 °C) has shown to increase methane and biogas production/yield for animal manures and agricultural crops and residues by 14 – 62% compared to untreated (see Supplementary Material) (Menardo et al., 2012; Nava-Valente et al., 2021; Şenol, 2021). However, thermal pretreatment at 70 °C for 24 hours for buckwheat hull had no significant effect on methane yield as lignocellulose degradation was not observed (Mirko et al., 2021).

Therefore, pretreatment at lower temperatures (80 – 90 °C) and longer holding times (≥60 min) could be a more suitable treatment condition of the substrate before BSFL bioconversion, in addition to achieving pasteurization of the substrate (i.e., inactivating relevant vegetative pathogens that may still be present). Similar treatment settings increased biogas production from agricultural crops and residues by 60%, when pretreated at 80 – 90 °C for 30 – 60 min (Menardo et al., 2012, see Supplementary Material). Thermal pretreatment at 90 °C for 4 hours increased the composting efficiency of animal manure mixed with agricultural crops and residues as humic substances, and stable forms of organic matter, increasing by 14 – 18% (Huang et al., 2019; Zhu et al., 2021, see Supplementary Material). Overall, thermal pretreatment at these lower temperatures has demonstrated positive effects on biogas production and composting.

2.6. Chemical pretreatment

Chemical pretreatments use oxidizing agents (e.g., H_2O_2), acids (e.g., H_2SO_4 and HCl), alkalis, and ozone to break down lignin and/or hemicelluloses while increasing the accessibility of cellulose (Behera et al., 2014; Carrère et al., 2010). The efficiency of chemical pretreatment is highly substrate-dependent and requires optimization of chemicals, dose, and treatment time for optimal results.

Acid pretreatments typically use either concentrated or diluted acids. For concentrated acids, a concentration above 30% (w/v) is usually needed and carried out at temperatures lower than 100 °C for several hours. However, a major disadvantage is that a high concentration leads to high corrosiveness, thus requiring a setup that is resistant to corrosion (Sun and Cheng, 2002). Additionally, a neutralizing agent is needed to increase the pH. For the diluted form, an acid concentration of 0.5 to 5% (w/v) can be used, but at high temperatures (120 – 215 °C) for a few minutes (Sun and Cheng, 2002). As mentioned in Section 2.5, high temperatures may not be suitable for BSFL bioconversion. Therefore, our review focuses on alkaline treatments due to their milder conditions compared to acid treatment (Kim et al., 2016) and because of their proven effect on high-lignin substrates, such as agricultural crops and residues, municipal solid waste, and animal manure (Carrère et al., 2016).

Treatment of substrates with alkaline chemicals (e.g., sodium hydroxide, lime, and ammonia) has been most widely studied because of its effectiveness in lignin degradation, increasing access to cellulose and hemicellulose for microbial decomposition (Bochmann & Montgomery, 2013; Carrère et al., 2011). A neutralizing agent, such as sulfuric acid, may be needed to reduce pH after alkaline pretreatment.

Pretreatment of lignocellulosic substrates with 1–10% sodium hydroxide (NaOH) is the most commonly used alkaline pretreatment, with typical residence times between 1 – 24 hours (Sambusiti et al., 2013; Taherdanak & Zilouei, 2014, see Suppementary Material). Agricultural crops and residues pretreated with NaOH at ambient temperature (e.g., 20 – 25 °C), have shown to increase biogas or methane production by 13 – 89% (see Supplementary Material) (He et al., 2008; Neves et al., 2006; Taherdanak and Zilouei, 2014). However, there could be a threshold where NaOH pretreatment is no longer effective, e.g., if lignin content exceeds 26% (Sun and Cheng, 2002). Liu et al. (2021) tested alkaline pretreatment (NaOH + H_2O_2 for 6 h at 30 °C) on the lignocellulosic content of rice straw before BSFL rearing. The authors observed a decrease in hemicellulose and lignin contents by 22%, and 68%, respectively, and an increase in cellulose by 25%. The authors found that following pretreatment, cellulose decomposition by BSFL was 10% higher (see Supplementary Material). Surprisingly, this did not significantly change rice straw reduction by BSFL compared to the untreated control. However, the results are still promising, given that pretreatment increased the harvested larval biomass by 32%.

Additional alkaline pretreatments use lime ($Ca(OH)_{2,}$), aqueous ammonia, or urea to maximize biogas/methane production (17 – 173%) for animal manure, municipal organic solid waste, and agricultural crops and harvesting residues (see Supplementary Material) (Antonopoulou and Gavala, 2015; Jurado et al., 2013b; Liang et al., 2014; López Torres and Espinosa Lloréns, 2008; Mirtsou-Xanthopoulou et al., 2014) . Lime pretreatment was shown to enhance biogas production with municipal organic solid waste by 173%

(López Torres and Espinosa Lloréns, 2008), but showed contradicting results with animal manure (Niasar et al., 2011). Aqueous ammonia has gained increased attention because of its effectiveness in lignin removal or modification of substrates while preserving the total carbohydrate content (Carrère et al., 2016; Mirtsou-Xanthopoulou et al., 2014). Pretreatment with 3% aqueous ammonia at 22 °C and an optimal time of 3 days was found to increase methane yield by 37 – 104% for animal manure and agricultural crops and residues (Jurado et al., 2013; Jurado et al., 2013; Mirtsou-Xanthopoulou et al., 2014). The addition of aqueous ammonia at 1% to banana peels, pretreated for 7 days, increased crude fiber by 5% and positively increased larval biomass and bioconversion by 31% and 33%, respectively (Isibika et al., 2019). However, at a dose of 0.8% larval bioconversion decreased by 1% compared to the control. Interestingly, urea pretreatment has been used to increase the nutritive value of ruminant feeds (e.g., rice and wheat straw) (van Kuijk et al., 2015) by breakdown of lignin (Yao et al., 2018). Palma et al. (2019), observed an increase in larval biomass by 10% when supplementing almond hulls with urea, changing the C/N ratio (i.e., carbon to nitrogen ratio) from 16 to 32. Ammonia and urea can increase the digestibility of lignocellulosic substrates for BSFL; however, it remains unclear whether these effects stem from improved digestibility of the substrates due to chemical fiber decomposition or microbial conversion of the added non–protein nitrogen and/or degraded fibers into protein digestible by BSFL (Isibika et al., 2019).

2.7. Biological pretreatment

Biological pretreatments add bacteria, fungi, yeasts or mixtures of microorganisms to substrates for the decomposition of lignin and/or cellulose by microbial enzymes (e.g. lignin modifying enzymes (LME) and cellulase, respectively) (Galbe and Zacchi, 2012; Atelge et al., 2020). The advantages of biological pretreatments over physical and chemical pretreatments are that they require less energy, no chemicals, and necessary microorganisms can be isolated from substrates (Mishra et al., 2018). Disadvantages include the slow hydrolysis rate and limited control over the process, possibly making it less suitable for industrial scale (Agbor et al., 2011). Various factors can influence the effectiveness of biological pretreatments, such as temperature, moisture content, incubation time, carbon and nitrogen sources, and microbial species (Sharma et al., 2019). These factors should be optimized to improve the hydrolysis rate.

Fungal pretreatments have been studied extensively for biogas production. Among the candidate fungi, white-rot fungi (e.g., *Phanerochaete chrysosporium, Pleurotus ostreatus, and Ceriporiopsis subvermispora)* possess enzymes for lignin degradation (Wan and Li, 2012). Pretreatment of agricultural crops and residues with white-rot fungi for 14 – 40 days was shown to decrease substrate lignin by 16 – 33% (Alexandropoulou et al., 2017; Mustafa et al., 2016) (see Supplementary Material). This has led to an increase in methane yield or biogas production from 6% to 154%, but has also shown to decrease methane yield by 9% (see Supplementary Material) (Alexandropoulou et al., 2017; Liu et al., 2014; Mustafa et al.,

2016; Zhao et al., 2014). As indicated by the range of results among studies, the effectiveness of the fungi is highly substrate- and species-dependent. For example, *C. subvermispora*, a wood-decaying fungus, was more effective at degrading woody substrates than herbaceous substrates (e.g., wheat straw and soybean straw) (Wan and Li, 2012). Brown-rot fungi (e.g., *Serpula lacrymans*) were more effective degraders of hemicellulose and cellulose, suggesting they are suitable for substrates low in lignin but high in cellulose (Wan and Li, 2012). Soft-rot fungi (e.g., *Ceratocystis* spp.) have shown to degrade cellulose and hemicellulose on woody substrates (Madadi and Abbas, 2017).

Results of Isibika et al. (2019) confirmed that substrate fungal pretreatment can also increase BSFL rearing performance but highlight that pretreatment time and fungi choice can highly influence the results. Pretreatment of banana peels with *Trichoderma reesei* for 7 – 21 days increased larval mass by 26 – 70% and bioconversion ratio from 14% (after 7 days pretreatment) to 61% (after 14 days pretreatment). However, pretreatment of banana peels with *Rhizopus oligosporus* decreased bioconversion rate by 7.5% when pretreated for 7 days, potentially due to the low fiber degradation of 10% compared to *T. reesie*, (40 – 53%).

Although fungi are the main source of lignocellulosic enzymes (Taha et al., 2015), many bacteria also possess enzymes that are effective degraders of lignocellulosic fractions, such as the genera *Bacillus (e.g., Bacillus subtilis)*, and *Streptomyces*, which increased biogas production for agricultural crops and residues by 11 to 42% compared to those without pretreatment (Zhao et al., 2019; Zhong et al., 2011) (see Supplementary Material). These strains may be present in the larval gut and could potentially improve the digestion of lignocellulose by isolating these strains and using them as a pretreatment for BSFL. Additionally, as discussed by Gorrens et al. (2021), the addition of dominant endogenous bacteria, such as *Enterococcus* spp. and *Providencia* spp. during BSFL rearing can positively influence the bioconversion of difficult to digest substrates into valuable biomass due to the production of lignocellulose-decomposing enzymes (Jiang et al., 2019). Similarly, pectinase activity was described by Callegari et al. (2020) for *Klebsiella* spp. isolated from BSFL.

In the last decade, interest in using bacteria on animal manure and agricultural crops and residues for BSFL rearing has increased, but as a co-treatment (i.e., bacteria are added together with larvae at the beginning of rearing) (Somroo et al., 2019; Yu et al., 2011; Zheng et al., 2012). On soybean curd residue, *Lactobacillus buchneri* increased the bioconversion rate and larval mass by 38% and 39%, respectively (Somroo et al., 2019). Additionally, Mazza et al. (2020) inoculated chicken manure with isolated pure bacterial strains from larval eggs and gut and evaluated their effect on larval performance. From the bacterial strains tested, *Kocuria marina*, *Proteus mirabilis*, and *Bacillus subtilis* improved larval mass by 15 – 19% compared to the control (without bacteria). In contrast, *Gordonia sihwensis* and *Micrococcus luteus* led to a decrease in

larval mass by up to 1% (see Supplementary Material). Substrate characteristics such as lignin and hemicellulose content and the cellulose crystallinity have shown to affect enzyme activity (Taha et al., 2015).

Future research on biological pretreatment should evaluate whether the use of white-rot fungi, *Trichoderma reesei,* or bacteria such as *Bacillus subtilis*, *Lactobacillus buchneri, Pseudomonas* spp.*, Enterococcus* spp., and *Providencia* spp. would enhance the production of bacteria that excrete cellulosic enzymes (e.g., cellulase) which have been identified in the BSFL gut (Callegari et al., 2020), thus increasing digestion of the cellulose while improving overall conversion efficiency. Taha et al., (2015) observed lignocellulosic enzymatic increase from fungi and bacteria between 3 and 12 days; therefore, a minimum pretreatment time of 3 days should be studied.

2.8. Future application of treatments in BSFL industry

Similar to composting and anaerobic digestion, individual mechanical, chemical and biological pretreatments, or a combination of these, may improve BSFL bioconversion performance with lignocellulosic substrates. As demonstrated by the variable efficiencies reported in this review and the unknown effects of treatments on BSFL, laboratory and pilot-scale studies are needed to determine transferability for industrial application. These efforts should work towards broad substrate-specific process recommendations (e.g., particle size, temperature, chemical and dose) and gaining further understanding of the underlying effects on the BSFL digestion process.

Based on this review, bioconversion efficiency could be determined by mechanical pretreatment of agricultural crops and residues, municipal organic solid waste, and animal manure (e.g., with <6 mm particle size). Because mechanical pretreatment may release water (e.g., in fruit and vegetable wastes) bound behind cell walls, substrate dewatering or addition of a drier substrate (e.g., paper) might be required to produce a suitable BSFL substrate. Thermal treatments with lower temperature treatments and longer holding times (e.g., 80 – 90 °C for >60 min) may provide positive effects for some substrates. However, this increases energy requirements, and depending on the energy source could impact the environmental benefits of BSFL products in comparison to current animal feed benchmarks such as fish meal.

Previous studies have shown improved performance with NaOH, urea, and ammonia treatments for all lignocellulosic substrates. For urea and ammonia pretreatment, doses of 1 – 3% with treatment times of less than 7 days for all lignocellulosic substrates could be effective. Future research should focus on increasing process improvements beyond those reported and assess whether chemical pretreatments have an influence on downstream processing (e.g., quality of feed and fertilizer products) to justify the additional operational resource requirements of chemical addition for BSFL rearing.

Biological pretreatments (e.g., with white-rot fungi or *Trichoderma reesei*) have shown to effectively delignify substrates and may stimulate microbial enzyme activities that will further improve digestibility by BSFL. Treatment with *Trichoderma reesei* for 14 days positively increased BSFL bioconversion. Additionally, the use of bacterial pretreatments, such as Bacillus subtilis, with a minimum pretreatment time of 3 days, could also enhance microbial enzymatic activities. However, similar to chemical pretreatment, the dose and treatment time for biological pretreatments need to be optimized, as typical pretreatment times used (20 – 30 days) may not be feasible.

For all pretreatments, efficiency gains need to be balanced with additional capital and operational resources, such as investment, labor, time, process additive cost, and energy. An economic assessment of the BSFL production chain should be conducted to evaluate the minimum process and bioconversion needed to justify the use of pretreatment for agri-food wastes. Ultimately, life cycle assessments should evaluate the effects substrate pretreatment may have across the different environmental impact categories.

3. Chapter: Chemical Pretreatment

Evaluation of ammonia pretreatment of four fibrous biowastes and its effect on black soldier fly larvae rearing performance

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3.1. Abstract

Biowaste treatment with black soldier fly larvae (BSFL, *Hermetia illucens*, L.) can promote a more sustainable food system by reusing nutrients that would otherwise be wasted. However, many agri-food wastes and byproducts are typically high in lignocellulosic fibers (i.e., cellulose, hemicellulose, and lignin), making it resistant to efficient larval and/or microbial degradation. Ammonia pretreatment could be used to partially degrade lignocellulose, making the biowaste more easily degradable by the larvae and/or microorganisms. This study evaluated ammonia pretreatment for lignocellulose degradation and its effect on BSFL performance on four fibrous biowastes: brewers spent grain, cow manure, oat pulp, and grass clippings. First, the optimal ammonia dose (1% or 5% dry mass) and pretreatment time (three or seven days) were assessed by measuring fibers after treatment and further examined using Fourier transform infrared spectroscopy (FTIR) spectra and scanning electron microscopy (SEM) images. Second, BSFL rearing performance on ammonia-pretreated substrates was assessed with a 9-day feeding experiment. Three-day pretreatment with 5% ammonia was chosen as it decreased the total fiber content by 8–23% for all substrates except cow manure. Contrary to expectations, ammonia pretreatment with all substates decreased BSFL rearing performance metrics by more than half compared to the untreated control. Follow-up experiments suggested that ammonia pretreatment had a dose-dependent toxicity to BSFL. Interestingly, three-day fermentation of cow manure and oat pulp increased bioconversion rate by 25–31%. This study shows that ammonia pretreatment is not suitable before BSFL rearing. Ammonia toxicity to BSFL and other pretreatments, such as fermentation, should be further studied.

3.2. Introduction

Black soldier fly larvae (BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), are an emerging biowaste treatment technology. BSFL can contribute to a more circular food system by upcycling nutrients that would otherwise be lost. BSFL can grow on a large variety of biowastes and agri-food byproducts, reducing mass/volume by 26–68% dry mass (DM) (Gold et al., 2018) and transforming the biowaste into a nutritious insect biomass that can be processed into high-protein meals as a (partial) replacement for soybean and fishmeal in animal diets (e.g., poultry, pigs, fish, pets) (Barragan-Fonseca et al., 2017; Mohan et al., 2022). In addition to the insect biomass, a compost-like nutrient-rich residue remains, which can be composted and used for soil fertilization (Klammsteiner et al., 2020). Additional applications for BSFL end-products include oils for biodiesel (Li et al., 2011a) and chitin for pharmaceuticals and technical applications (Vilcinskas, 2013).

Although BSFL can develop on many different biowastes, a current obstacle for the inclusion of low value biowastes into efficient BSFL treatment operations is their poor performance (e.g., longer treatment times, lower waste reduction and larval yields). For example, the BSFL bioconversion rate (i.e., unit of larvae produced per unit of waste) with animal manures is only 2–6% DM (Liu et al., 2018; Rehman et al., 2017b) in comparison to 13–21% DM with highly nutritious food waste (Lalander et al., 2019; Nyakeri et al., 2017b). This poor performance can also influence the potential environmental benefits (Smetana et al., 2016) and economic viability of the entire BSFL treatment system.

One reason for the low rearing performance can be the presence of difficult to digest lignocellulosic fibers (i.e., lignin, cellulose, and hemicellulose) in the biowaste. For example, Liu et al. found a negative correlation between larval weight and biowaste lignin content (Liu et al., 2018). Lignocellulosic fibers are notoriously difficult to digest because of their structure. Lignocellulose is composed of cellulose fibrils bundled together in a complex matrix, tightly bound by lignin and hemicellulose (Pérez et al., 2002). Lignin acts as a physical barrier creating difficulties for decomposition by larvae and/or microorganisms abundant in the biowaste/residue and larval digestive tract (Hu and Ragauskas, 2012). High contents of lignocellulose are common in biowastes, such as animal manures, agri-food byproducts and municipal organic solid wastes (Gold et al., 2018; Peguero et al., 2022). A reason for the decrease in BSFL treatment performance with lignocellulosic biowastes is likely the reduced availability of digestible nutrients (e.g., protein and digestible carbohydrates).

Biowaste pretreatments are a possible solution to make fibrous biowastes more degradable by BSFL and associated microorganisms. Pretreatments typically aim to partially remove lignin and/or hemicellulose thereby freeing cellulose for breakdown into more digestible compounds, simple sugars and glucose (Spano

et al., 1976). Pretreatments have been widely used over the last 20 years to increase the nutritional value of fibrous animal feeds for ruminants (e.g., forage for dairy/cattle), biogas and bioethanol yields, and composting efficiency. Various physical (e.g., mechanical, thermal, electromagnetic), biological (e.g., bacteria, fungi, enzymes), and chemical (e.g., alkalis, acids, ozone, and oxidizing agents) pretreatments exist (Peguero et al., 2022). Limited studies have started investigating chemical pretreatment to increase BSFL performance. Liu et al. (2021) observed that alkaline pretreatment increased larval weight by 47%.

Alkaline pretreatment is the most common chemical pretreatment because of its effectiveness in lignin and hemicellulose degradation (Antonopoulou and Gavala, 2015), but its potential is yet largely unexplored for BSFL treatment (Peguero et al., 2022). Specifically, soaking in aqueous ammonia pretreatment has been reported to be highly selective towards lignin at moderate temperatures (Kim et al., 2010). Ammonia pretreatment alters the chemical composition by splitting the ether and ester bonds between lignin and hemicellulose in addition to splitting the C–O–C bonds in lignin (Kim et al., 2010) by process of ammonolysis (Shi et al., 2020). Furthermore, ammonia pretreatment increases biowaste nitrogen, changes the C:N ratio, and potentially provides additional nutrients for microorganisms, while inhibiting mold growth (Sarnklong et al., 2010). Parodi et al. (2021) also revealed that BSFL can use some ammonium-nitrogen to build up their body mass.

Ammonia pretreatment conditions (e.g., dose, temperature, and time) and efficiency vary greatly among studies. To increase biogas and bioethanol yields, biowastes are typically soaked in aqueous ammonia solution with doses of 45–291% (ammonia mass per biowaste DM; see Supplementary Material for calculation of doses) with varying reagent concentrations (e.g., 5–32% w/w) and stored for three days to four weeks (Antonopoulou and Gavala, 2015; Kim et al., 2010; Li and Kim, 2011; Mirtsou-Xanthopoulou et al., 2014) resulting in 37–265% more biogas (Jurado et al., 2013a; Mirtsou-Xanthopoulou et al., 2014) and 73–89% more ethanol (Kim et al., 2008). To improve ruminant digestibility and nutritive quality of fibrous feeds, such as rice and barley straw, doses were approximately 3% and stored for one to seven weeks at 20–30°C (Fadel Elseed et al., 2003; Hartley and Jones, 1978; Selim et al., 2004). Ammonia pretreatment increased rice straw degradability by 24–26% DM (Orden et al., 2000) and improved *in vitro* ruminant digestibility of barley straw by 30% DM (Hartley and Jones, 1978). For BSFL, Isibika et al. (2019) pretreated banana peels with ammonia without analyzing the potential effects on the lignocellulosic fibers. Seven-day pretreatment with 1% ammonia did not affect bioconversion rate (7.2 ± 1.2 % vs 9.7 ± 3.9 %, based on volatile solids). However, in combination with a fungus (*Rhizopus oligosporus)*, seven-day pretreatment with 0.8% ammonia pretreatment increased bioconversion by 105% (7.2 ± 1.2 % vs. 14.8 ± 1.2 %, based on volatile solids) (Isibika et al., 2019).

This study aimed to assess ammonia pretreatment before BSFL treatment and its effect on the biowaste lignocellulosic composition. It was hypothesized that ammonia pretreatment would degrade lignocellulosic fibers, thereby increasing BSFL rearing performance. The biowastes used were brewer's spent grain (spent grain), cow manure, and grass clippings. In addition, oat pulp was used as a BSFL rearing substrate for the first time, contributing to further knowledge on additional BSFL rearing substrates. This research works towards the efficient utilization of low value fibrous biowastes in BSFL treatment.

3.3. Material and Methods

3.3.1. Source of biowastes and agri-food byproducts

Four different biowastes and agri-food byproducts were used: spent grain, cow manure, oat pulp, and grass clippings. The four substrates were chosen because of their varying lignocellulosic composition and high production amounts which could potentially be recycled by BSFL. Spent grain was obtained from the Brewdaz brewery (Zürich, Switzerland). Semi-solid fresh cow manure was obtained from a dairy farm (Dübendorf, Switzerland). Oat pulp was the slurry from separating the liquid from the insoluble fibers during oat milk production and was obtained from Soyana (Zürich, Switzerland). Grass clippings were lawnmower clippings from a domestic lawn (Bern, Switzerland). Following collection, substrates were portioned into plastic bags and stored at -20°C until further use.

3.3.2. Origin of black soldier fly larvae

Black soldier fly eggs were from the research colony at Eawag (Dübendorf, Switzerland) operated according to Dortmans et al. (2017). Neonates that hatched within 24 hours were reared at 28°C with relative humidity of 70% on chicken feed (75% moisture content, UFA 620, Landi, Dübendorf, Switzerland) for 6–7 days until reaching a mean individual weight of 1–3 mg DM. The larvae were then separated, manually counted, and directly used for feeding experiments with different substrates and treatments (raw, control, and ammonia-pretreated).

3.3.3. Ammonia pretreatment

The first part of the experiments focused on identifying the doses and pretreatment times (storage duration) resulting in fiber decomposition. Prior to ammonia pretreatment, substrates were thawed at 4°C. Pretreatment consisted of adding aqueous ammonia solution to reach different ammonia doses of 1% or 5% (v/w) (based on DM) to each substrate in glass containers. The calculations used for determining dose of ammonia solution (concentration 25%, Supelco, Switzerland) are included in the Supplementary Material. The substrate and ammonia were thoroughly mixed and covered with an airtight lid to avoid ammonia loss

and stored at ambient temperature for three or seven days (see Supplementary Material). At the end of the pretreatment time, the substrate pH (826, Metrohm, Switzerland) was measured and neutralized with the addition of 95–97% sulfuric acid (Sigma Aldrich, Switzerland). A control (without added ammonia) served alongside for the same storage durations as the ammonia-pretreated substrates (see Supplementary Material for an image of the setup). All pretreatments and controls were performed in triplicate. Following pretreatment, substrates were analyzed for fiber content, as described below.

3.3.4. Larval feeding experiments

Larval feeding experiments were conducted with the pretreatment dose and time, resulting in reduction of the mean total fiber content (i.e., neutral detergent fiber), which was 5% ammonia for three days for all substrates. Pretreated substrates were fed to BSFL for nine days at a feeding rate of 35 mg DM/larvae/day and a larval density of 2.5 larvae/cm^2 with four replicates per treatment (Gold et al., 2020b). Treatments for larval feeding experiments were: raw (no added ammonia), control (no added ammonia, but stored for three days), and ammonia pretreatment (5% for three days). The substrate was brought to 28°C prior to adding larvae and 110 randomly selected and manually counted larvae were added to plastic containers (7.5 cm diameter, 11 cm height) and placed in a controlled climate chamber (HPP 260 Eco, Memmert GmbH, Germany). During the feeding experiment, the temperature and relative humidity in the climate chamber were 28°C and 70% for all substrates except for cow manure, which was 28°C and 44–70%. At the end of the experiment, the larvae were manually harvested from the residues, counted, and weighed. After determining the fresh weight, larvae were inactivated at 105°C for 5 min and then dried at 60°C for two days (Rehman et al., 2017a). Residues were dried in a laboratory oven at 60°C until weight remained constant. Both the dried larvae and the residues were then weighed and stored at 4°C for further analyses. Using larval numbers and residue and larval dry weights, common rearing performance metrics, including survival rate, larval weight at harvest, bioconversion rate, and waste reduction were calculated according to Gold et al. (2020b).

3.3.5. Substrate and residue phyico-chemical analyses

Moisture content of the substrate was determined as the weight loss of three grams of wet sample after overnight oven drying at 105°C. Organic matter was calculated by subtracting the ash content from the total dried sample (100%). Ash content was determined as weight loss of three grams of dried sample after three hours in a muffle furnace (Nabertherm GmbH, Germany) at 550°C. Prior to analyzing fiber content, carbon, and nitrogen, substrates and residues were dried at 60°C until weight remained constant and milled to 1 mm (10,000 rpm, Retsch ZM 200, Germany). Fiber analyses included neutral detergent fiber (Van Soest et al., 1991), and acid detergent fiber (AOAC, 1977, index no. 973.18). The raw substrate was also analyzed for

acid detergent lignin (AOAC, 1977, index no. 973.18). Neutral and acid detergent fiber were analyzed with a Fibertherm® FT12 (Gerhardt Analytical Systems, Germany) using 0.5 gram of dried sample. Following acid detergent fiber, the remaining dried residue was soaked in 72% H_2SO_4 for three hours for acid detergent lignin determination. Equations 1–5 were used to estimate the lignocellulosic composition:

$$Total\ Fiber\ Content\ (\%\ DM) = Neutral\ Detergent\ Fiber\ (\%\ DM) \quad (1)$$

$$Sum\ of\ Cellulose\ \&\ Lignin\ (\%\ DM) = Acid\ Detergent\ Fiber\ (\%\ DM) \quad (2)$$

$$Hemicell.\ (\%\ DM) = Neutral\ Detergent\ Fiber\ (\%\ DM) - Acid\ Detergent\ Fiber(\%\ DM) \quad (3)$$

$$Cellulose\ (\%\ DM) = Acid\ Detergent\ Fiber(\%\ DM) - Acid\ Detergent\ Lignin(\%\ DM) \quad (4)$$

$$Lignin\ (\%\ DM) = Acid\ Detergent\ Lignin\ (\%\ DM) \quad (5)$$

Carbon and nitrogen were determined using 0.7 gram of dried sample with a C/N analyzer (Trumac CN, LECO Instruments, Germany). Substrate ammonium concentrations (control and 5%) were measured with 1 gram of wet sample diluted in 10–250 mL of deionized water using ammonium nitrogen reagents (0–47 mg/L, NH_4-N, Hach Lange GmbH, Switzerland) and a spectrophotometer (DR 3900, Hach Lange GmbH).

Substrate protein content was estimated by multiplying nitrogen results with substrate-specific conversion factors: 6.25 for spent grain (Rommi et al., 2018), 4.3 for cow manure (Chen et al., 2017), 5.4 for oat pulp (based on results for cereals) (Mariotti et al., 2008) and 4.6 for grass clippings (Hoover et al., 2019). All physiochemical analyses were conducted with three to four replicates.

Fourier-transform infrared spectroscopy (FTIR) was conducted in triplicate to evaluate the chemical compositional changes before and after ammonia pretreatment using approximately two milligrams of dried sample. Spectra were recorded on a Bio-Rad FTS 575 C equipped with a nine-reflection diamond disk of four-millimeter diameter (SensIR Technologies, Connecticut, United States). Scans were collected from 4000 to 400 cm^{-1} at 2 cm^{-1} resolution versus the appropriate background spectrum.

Scanning electron microscopy (SEM) was used to qualitatively evaluate changes in the surface morphology by comparing ammonia-pretreated substrates to raw and control substrates. Dried samples were metal coated with 5 nm of platinum/palladium in a metal sputter during planetary rotation. The samples were imaged at 2 kV by secondary electron detention using a SEM (SU5000, Hitachi, Germany), except spent grain which was imaged at 3 kV using a SEM (7000F, JEOL, Germany).

3.3.6. Ammonia pretreatment effect on microbial numbers

Total aerobic viable counts (TVC) were estimated for all substrates (control and 5%) to evaluate the effect of ammonia pretreatment on microbial populations present in the substrate. TVC were estimated using plate

counts from a dilution series. Five grams of sample were transferred to a sterile stomacher bag with 45 ml of sterile maximum recovery diluent (0.85% (w/v) NaCl, 0.1% (w/v) peptone; Sigma Aldrich) and homogenized with a stomacher for 2 min (Gold et al., 2020b). TVC were determined in duplicate per biological replicate (n=3–4) plating 0.1 mL of the dilution series on nutrient agar (1.5% (w/v) Agar; VWR International, Belgium; 0.8 % (w/v) Nutrient Broth, Difco, Switzerland) and incubated at 30°C for 48 hours.

3.3.7. Ammonia treatment effect on black soldier fly larvae

To evaluate possible effects of ammonia treatment on larvae performance, substrate microorganisms were inactivated by autoclaving (121°C for 15 mins; HG50, HMC Europe GmbH, Germany). Chicken feed (UFA 620, 70% moisture content) was used as a model substrate. Sterilization was confirmed by measuring TVC. Ammonia solution was then added to obtain different ammonia doses (0%, 1%, 3%, or 5% based on DM) and immediately neutralized with sulfuric acid. To avoid re-introduction of microorganisms, the containers were immediately covered. A larval feeding experiment was then conducted using the same experimental design and conditions as described above with four replicates for each ammonia dose.

3.3.8. Data analyses

FTIR data was analyzed and processed using Microsoft Excel (Version 2022, Washington, United States). All other data was analyzed using R statistical language (R Core Team 2022, version 4.2.0, Massachusetts, United States). The mean, median, standard deviation, and range of the biowaste composition, BSFL performance metrics, and microbial counts were calculated. We abstained from statistical analyses due to the small sample size ($n \leq 4$). The results were compared using mean and standard deviation ($n = 3–4$).

3.4. Results and Discussion

3.4.1. Characterization of raw biowastes and organic side-streams

Substrate nutrient and fiber composition had a large influence on pretreatment and BSFL rearing performance metrics. In this study, the substrates varied in nutrient and fiber composition (Table 3.1, Figure 3.1). Moisture contents were 74–75%, within the optimal range (e.g., 70–80%) for BSFL rearing (Dortmans et al., 2017). Cow manure was the exception, with 91% moisture content.

Table 3.1. Substrate nutrient composition as percent dry mass, moisture content in percent. Data displayed are mean ± standard deviation (n=3–4).

Substrates	Moisture Content	Protein	C/N ratio	Fat	Total fiber	Organic Matter
Spent grain	74.5 ± 0.3	24.5 ± 0.2	12.6 ± 0.1	2.9[a]	59.4 ± 0.6	95.5 ± 0.1
Cow manure	91.0 ± 0.0	9.1 ± 0.1	19.9 ± 0.2	4.4[b]	52.2 ± 1.8	81.8 ± 0.6
Oat pulp	73.9 ± 0.3	36.3 ± 0.2	8.7 ± 0.2	5-12*[c]	31.5 ± 0.7	93.4 ± 0.1
Grass clippings	74.0 ± 0.3	14.0 ± 0.4	12.7 ± 0.4	<5[d]	47.2 ± 0.3	89.6 ± 0.3

In parentheses: standard deviation.
*Values are based on oats and not oat pulp.
[a](Bava et al., 2019); [b](Gold et al. 2020); [c](Sterna et al., 2016); [d](Bender et al., 1989)

Spent grain is a heterogenous substrate consisting of husk, pericarp and seed coat layer which are high in protein and fiber (Mussatto et al., 2006). Spent grain was among the most nutritious substrates in this study with a protein content of 25% DM but high fiber content of 59% DM. Liu et al. (2018) reported similar values of 23% DM protein and 59% DM fiber. The high protein and fiber content found in spent grain can be attributed to the removal of barley starch during the mashing process, concentrating insoluble nutrients (Mussatto et al., 2006). The lignin content of 8% DM in this study was below the typical range of 12–28% DM for spent grain (Figure 3.1) (Forssell et al., 2008). Variation in the composition of spent grain can be expected because of many influencing factors, such as barley variety, harvest time, characteristics of the hops and mashing conditions (Forssell et al., 2008; Santos et al., 2003).

Cow manure was the least nutritious substrate, with a low protein content of 9% DM and organic matter content of 82% DM but a high fiber content of 52% DM. These findings are consistent with values of 11% DM protein, 81% DM organic matter, and 58% DM fiber previously reported by Gold et al. (2020a). Cow manure is typically characterized by a high fiber content because it mainly consists of undigested animal feed and bedding material such as straw (Saady et al., 2021).

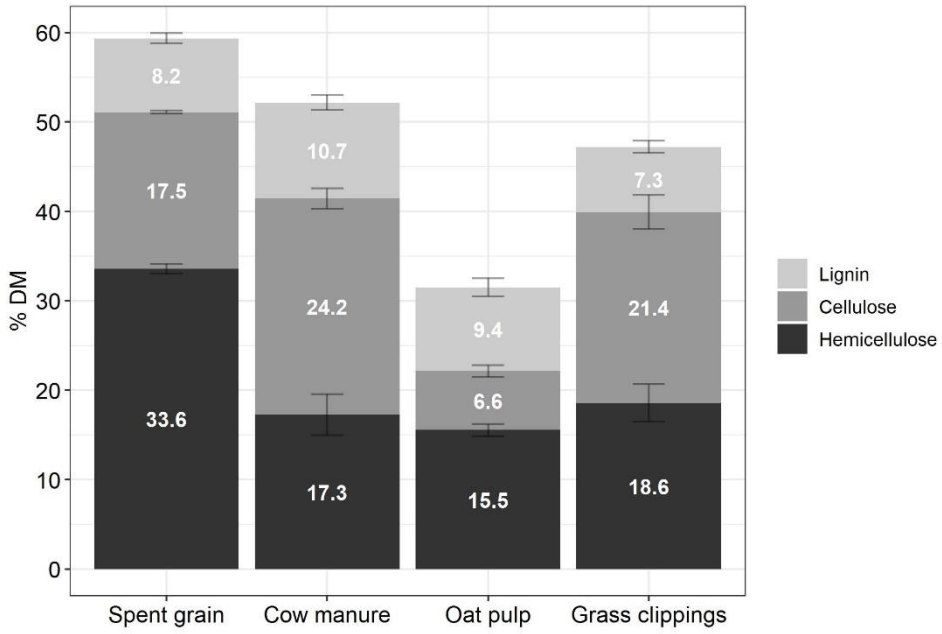


Figure 3.1. The mean lignocellulosic composition (based on DM) of the raw substrates (n=3): spent grain, cow manure, oat pulp, and grass clippings (error bars are the standard deviation).

Oat pulp was among the most nutritious substrate and had the highest protein content of 36% DM and lowest fiber content of 32% DM. The low fiber content in the oat pulp could be explained by the use of hulled oats in the oat milk production process, which would remove the largest amount of fiber found in oats (Hsu et al., 1987).

Grass clippings was in the middle range in terms of its nutritional composition compared to the other substrates. The fibers were characterized by more cellulose than lignin and hemicellulose (Figure 3.1). However, the composition of grass clippings can vary tremendously, depending on the grass type, season, and the inclusion of other materials (e.g., leaves and woody materials) (Bary et al., 2005).

3.4.2. Effect of ammonia pretreatment on substrates lignocellulosic composition

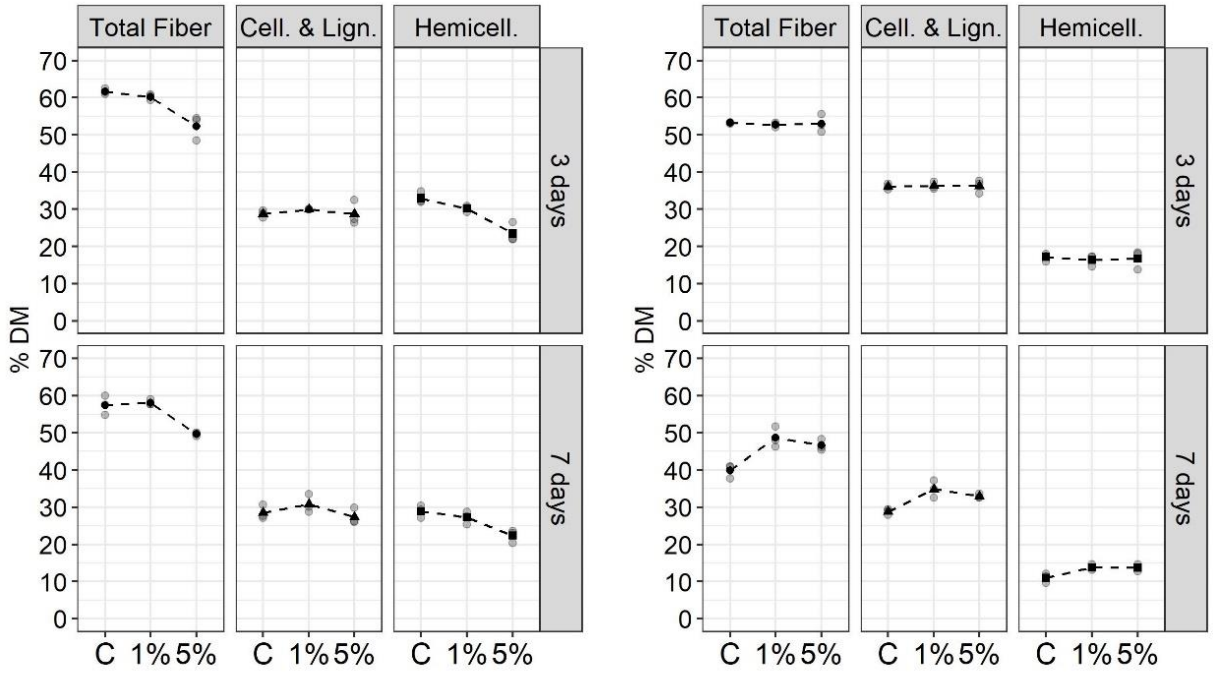
The change in substrate fiber composition is an important indicator for the effectiveness of a pretreatment. Figure 3.2 shows the lignocellulosic composition (total fibers, sum of cellulose & lignin and hemicellulose) for each substrate before (control, 3d or 7d) and after ammonia pretreatment (1% or 5%, 3d or 7d).

The pretreatment effects were compared to the control, considering the mean value and standard deviation. Overall, the effect of ammonia pretreatment on the fiber composition was low and varied greatly among the substrates. For all substrates, 1% ammonia pretreatment for three days did not have a relevant effect on the lignocellulosic composition and is therefore, not discussed further below. Additionally, 1% ammonia pretreatment for seven-days for spent grain and oat pulp did not affect the lignocellulose composition.

With spent grain (Figure 3.2a), 5% ammonia pretreatment for three and seven days decreased the hemicellulose content, from 33.0 (1.6) (control, 3d) to 23.5 (2.6) % DM (5%, 3d) and from 28.9 (1.7) (control, 7d) to 22.3 (1.8) % DM (5%, 7d). Because of the similar hemicellulose reductions by ammonia pretreatment with the two pretreatment times, three days was chosen for the larval feeding experiments. Interestingly, the hemicellulose content in the seven-day control was lower than in the three-day control (29% DM vs. 33% DM). This may indicate that microbial decomposition occurred during the longer storage time.

(a) Spent grain

(b) Cow manure



(c) Oat pulp

(d) Grass clippings

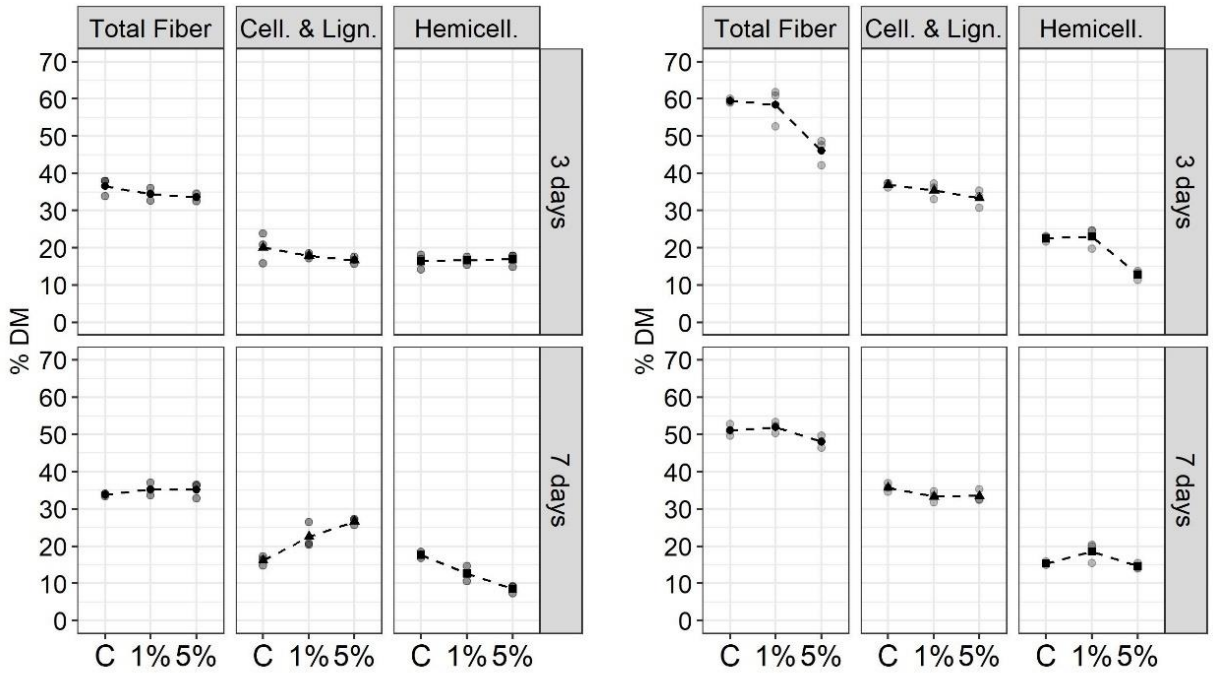


Figure 3.2. Effect of ammonia pretreatment dose (1% and 5%) and time (3 and 7 d) compared to the respective control (C) with (a) spent grain, (b) cow manure, (c) oat pulp, and (d) grass clippings. All results are given in DM. Mean (bold) and replicates (n=3) are displayed. Cell. & Lign. = sum of cellulose and lignin, Hemicell. = hemicellulose.

With cow manure (Figure 3.2b), the three-day pretreatment had little to no effect on the lignocellulosic composition. Jurado et al. (2013) also did not observe an effect on the lignocellulosic composition after pretreatment of raw animal manure with ammonia solution using a much higher dose of 291% DM for three days. Against our expectations, seven-day pretreatment increased the total fiber content, from 39.8 (1.9) (control, 7d) to 48.7 (2.8) (1%, 7d) and 46.6 (1.5) % DM (5%, 7d). This increase may be attributed to an increase in hemicellulose and cellulose & lignin contents (Figure 3.2b). Considering that the fiber content decreased from the raw substrate compared to the seven-day control (52.2 vs. 39.8% DM) indicates microbial decomposition occurring. Therefore, the higher fiber content after seven-day ammonia pretreatment relative to the control is likely not due to an increase in total fibers but due to the suppression of microbial activity by the addition of ammonia solution. Ammonia can be toxic to microorganisms and suppress microbial biowaste degradation (Weihrauch et al., 2012). Although ammonia pretreatment for three days resulted in little to no fiber degradation, 5% ammonia pretreatment for three days was chosen because of the higher fiber content observed with seven-day pretreatment compared with the control.

Ammonia pretreatment of oat pulp had little effect on the fiber composition (Figure 3.2c). Three-day pretreatment with 5% slightly decreased the total fiber content from 36.6 (2.3) (control, 3d) to 33.5 (1.0) % DM (5%, 3d) but had no apparent effect on the hemicellulose content. Contrary to the three-day pretreatment, seven-day pretreatment did not influence the total fiber content. Interestingly, seven-day pretreatment decreased hemicellulose but increased the cellulose & lignin content (Figure 3.2c). Due to the slight decrease in total fibers, 5% pretreatment for three days was selected for the larval feeding experiments.

Seven-day ammonia pretreatment had no apparent effect on the fiber composition of the grass clippings. However, 5% ammonia pretreatment for three days decreased hemicellulose from 22.5 (0.8) (control, 3d) to 12.8 (1.3) % DM (5%, 3d). Cellulose & lignin also slightly decreased from 36.9 (0.6) (control, 3d) to 33.3 (2.4) % DM (5%, 3d). Due to these decreases, 5% pretreatment for three days was chosen for the larval feeding experiment.

3.4.3. Fourier Transformer Infrared Spectroscopy (FTIR) of ammonia-pretreated substrates

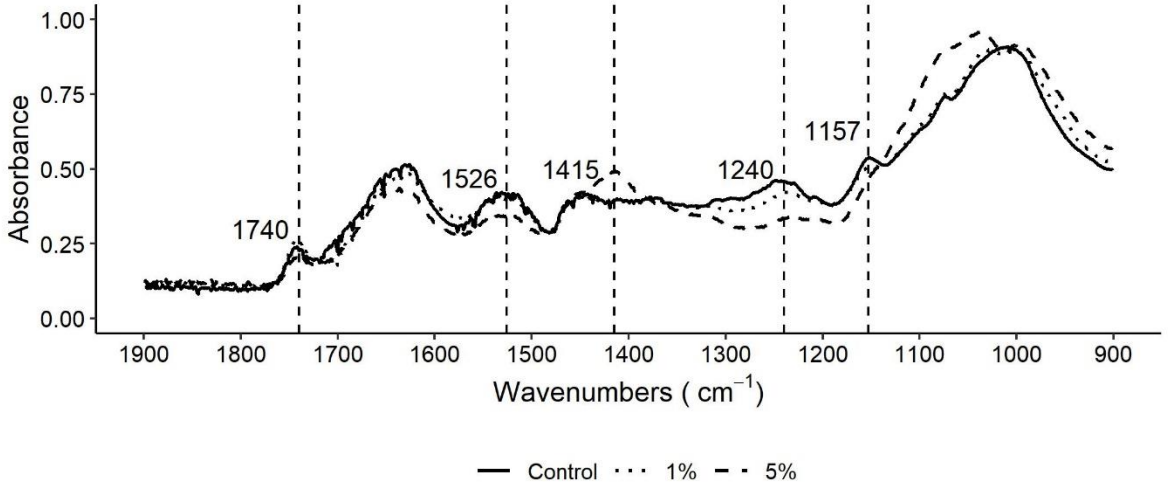
FTIR was performed to gain further qualitative insight into the chemical changes by 1% and 5% ammonia pretreatment with the most effective pretreatment time of three days. The FTIR spectra shown in Figure 3.3 were visually analyzed within the fingerprint region of 1900–900 cm^{-1} where the defined peaks are characteristic of functional groups associated with lignocellulose (Faix, 1991).

Ammonia pretreatment affected several peaks in the fingerprint region (Figure 3.3). The peak between 1740–1730 cm^{-1} is attributed to C=O acetyl group of hemicelluloses and ester bonds of the carboxyl group

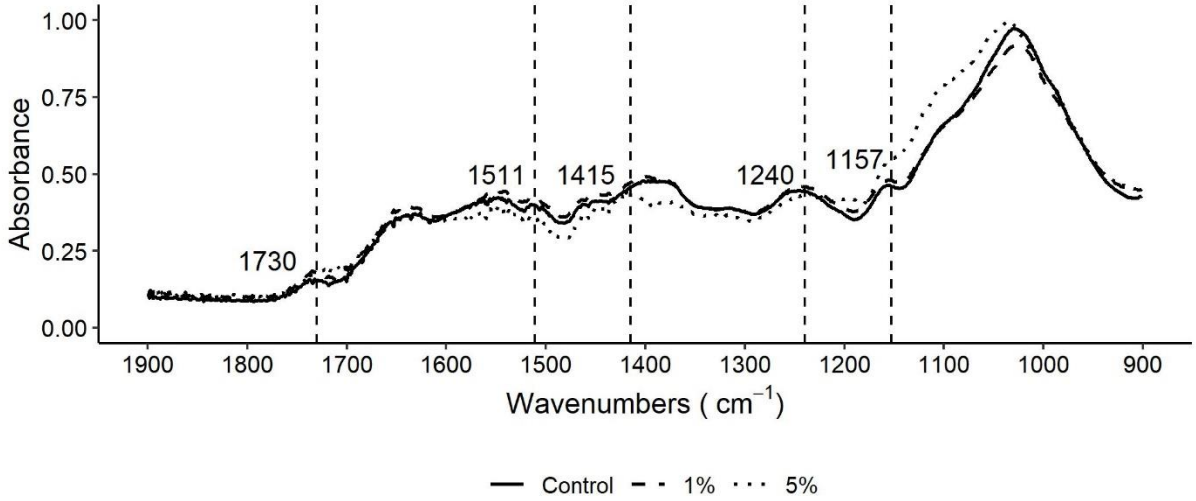
of lignin and/or hemicelluloses (El Ghali et al., 2012; Gao et al., 2020; Ravindran et al., 2018). The peak at 1526–1505 cm^{-1} is associated with the C=C bonds in the aromatic ring of lignin (El Ghali et al., 2012; Fong Sim et al., 2012; Ravindran et al., 2018). The peak at 1413 cm^{-1} corresponds to CH_2^- and $-CH_3^-$, structures for the formation of cellulosic substrates (Orozco et al., 2014). The peak at 1260–1240 cm^{-1} is associated with the C-O stretching of xylan (hemicellulose), and that at 1157 cm^{-1} is characteristic of the asymmetric stretching vibration of C-O-C in cellulose and hemicellulose (Gao et al., 2020). Additionally, the region between 1150 cm^{-1} and 1030 cm^{-1} are typical for polysaccharides, such as lignocellulose (Carballo et al., 2008) and were identified on all the substrates and treatments.

Spectra of the 1% ammonia-pretreated substrates were not visually different from the controls. This supports the fiber results where three-day 1% ammonia pretreatment had little to no effect on the lignosellusoic composition. In contrast, 5% pretreatment caused apparent changes to the chemical structure in regions characterisc of lignocelluloses. Ammonia pretreatment decreased the spectral peaks of spent grain (1740, 1526, 1240, and 1157 cm^{-1}) mainly associated with lignin and hemicellulose, oat pulp (1740, 1526, and 1157 cm^{-1}) related to lignin and hemicellulose, and grass clippings (1240 cm^{-1}) corresponding to hemicellulose. These results broadly confirmed the fiber results and provided additional information. Removal and/or reduction in peaks with spent grain, oat pulp, and grass clippings indicates hemicellulose reductions which were also found with the fiber results (Chaker et al., 2013). Additionally, the reduction of the peak at 1526 cm-1 for spent grain and oat pulp suggests an interaction with lignin, which could indicate lignin reduction. Furthermore, FTIR spectral differences in regions characteristic of hemicellulose degradation were the largest for oat pulp, where fiber analyses only showed a small reduction in total fibers, but not hemicellulose. This suggests that ammonia pretreatment also affected the chemical structure of the lignocellulosic fibers not captured in the fiber analyses, potentially making them more degradable for BSFL. All ammonia-pretreated substrates, except grass clippings, showed a peak at 1415 cm^{-1}. These findings indicate an increase in the amount of the functional group associated with cellulose.

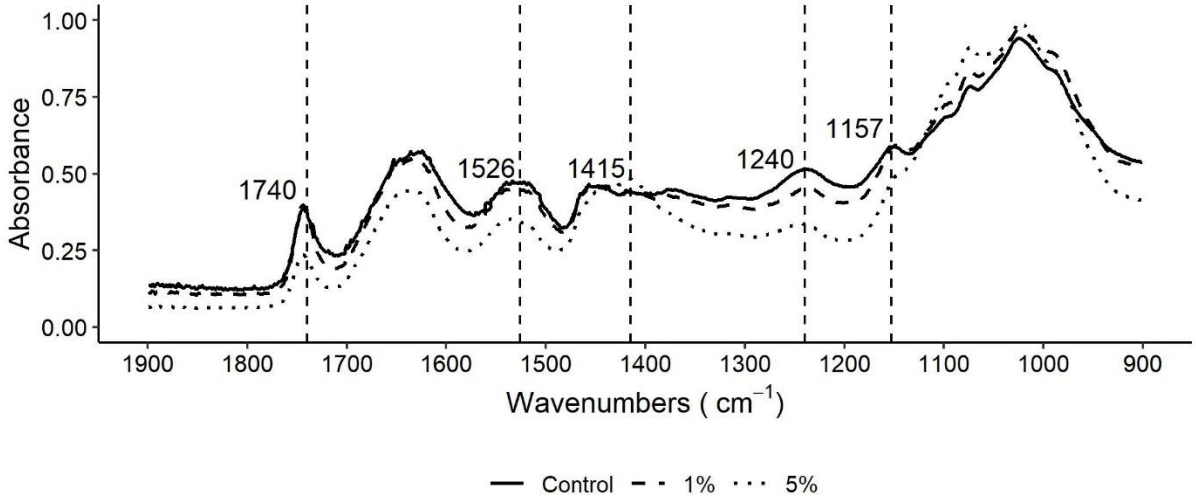
(a) Spent grain



(b) Cow manure



(c) Oat pulp



(d) Grass clippings

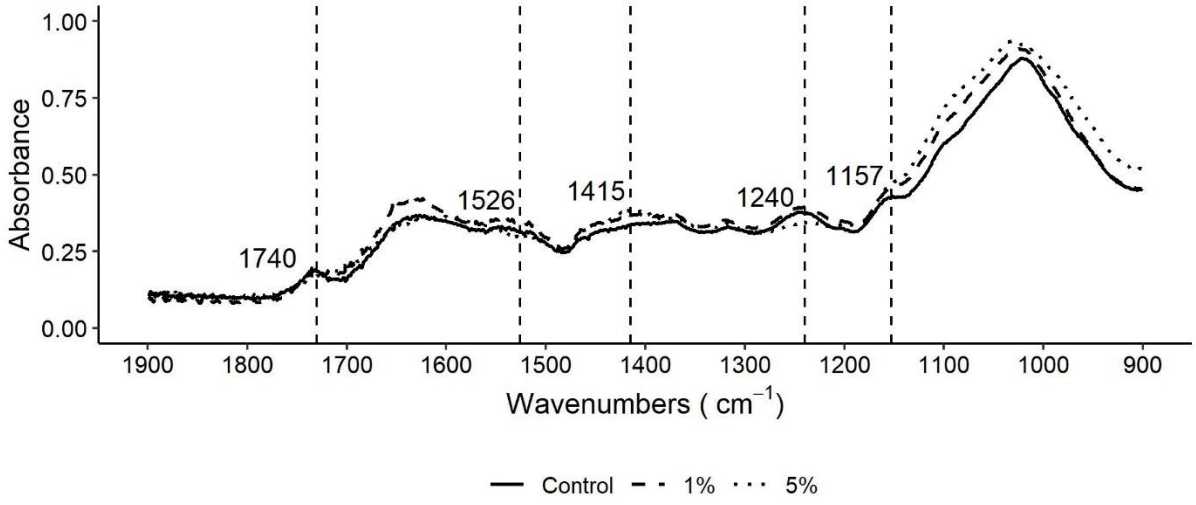


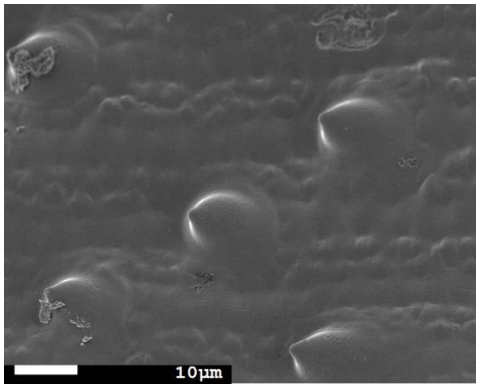
Figure 3.3. FTIR spectra of ammonia-pretreated substrates and their respective controls. Spectra displayed are the average (n=3). The peaks identified are associated with functional groups corresponding to lignin, cellulose, and hemicellulose. 1740–1730 is associated with hemicellulose and lignin; 1526–1505 is associated with lignin, 1413 associated with cellulose; 1260–1240 corresponding to hemicellulose; 1157 related to cellulose and hemicellulose; and 1150–1030 associated with polysaccharides such as lignocellulose.

3.4.4. Scanning electron microscopy (SEM) images

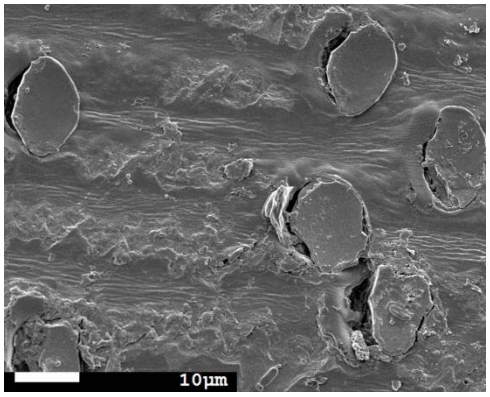
Physical changes of the substrate by the most effective ammonia pretreatment (5%, 3d) compared to the control were assessed visually by SEM (Figure 3.4). Representative SEM images were similar between the raw and control substrates; therefore, only the raw substrate is displayed here (see the control SEM images in the Supplementary Material). Ammonia pretreatment had a large influence on the surface structure of all the substrates. Before ammonia pretreatment, all raw substrates had an apparently smooth surface that could

create difficulties for larval and/or microbial decomposition of the substrate. Spent grain (Figure 4a), specifically, had several, presumably, decay-resistant silica bodies called phytoliths, on the surface. Phytoliths are known to serve as a protection for the fibers from degradation (Kim et al., 2008). Ammonia pretreatment for all substrates visibly ruptured the surface and exposed pores, potentially facilitating fiber decomposition (Kim et al., 2008). This included cow manure (Figure 4c), where fiber and FTIR spectral analyses did not suggest changes in the lignocellulosic composition. These images suggest that three-day 5% ammonia pretreatment altered the biomass structure, potentially improving the ability for larval and microbial degradation of the substrates in BSFL rearing.

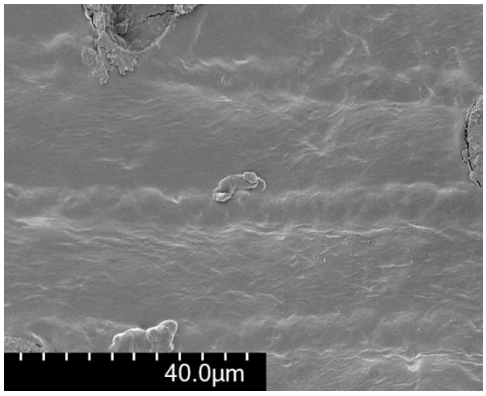
(a) Raw spent grain



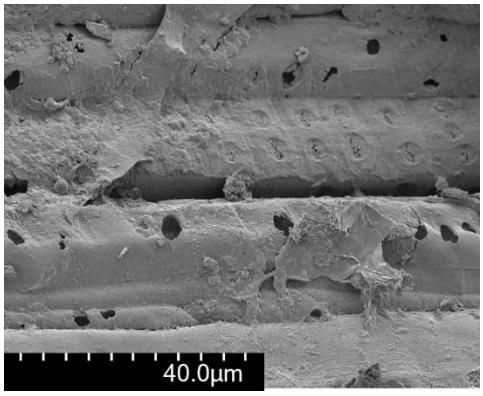
(a) Pretreated spent grain



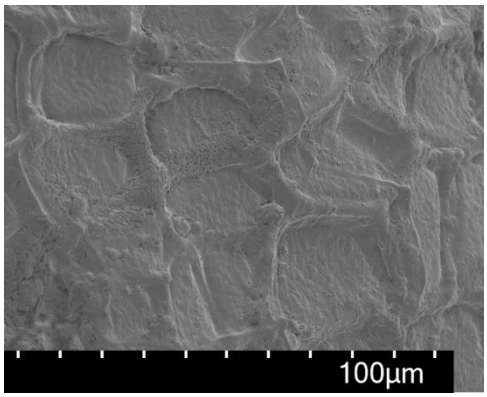
(b) Raw cow manure



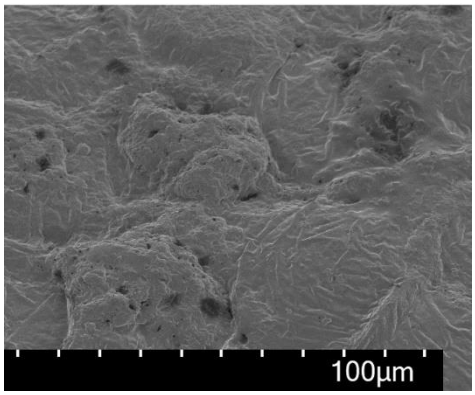
(b) Pretreated cow manure



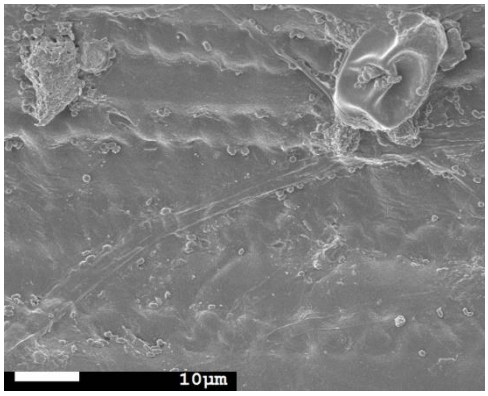
(c) Raw oat pulp



(c) Pretreated oat pulp



(d) Raw grass clippings



(d) Pretreated grass clippings

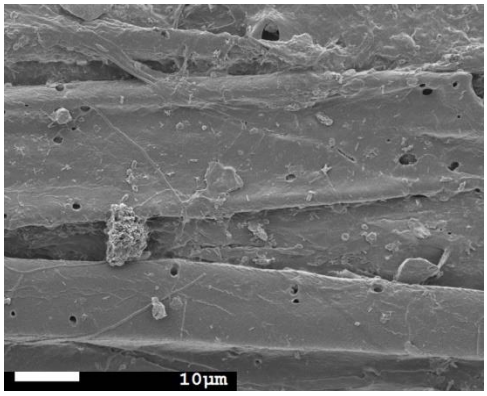


Figure 3.4. Representative scanning electron microscopy (SEM) images of the surface of the raw and ammonia-pretreated (5%, 3d) substrates.

3.4.5. BSFL rearing performance with and without pretreatment

Larvae developed on all substrates and treatments (raw, control and ammonia-pretreated) with survival rates ≥ 91% (Table 3.2). Only ammonia-pretreated grass clippings had a lower survival rate of 87%. These findings are similar to the survival rates of 90–99% on various substrates previously reported by Gold et al. (2020b).

To the best of our knowledge, this is the first time that oat pulp was used as a BSFL rearing substrate. Among the untreated substrates (i.e., raw and control), oat pulp had the best rearing performance, whereas both cow manure and grass clippings performed the worst. Oat pulp had the highest larval weight, bioconversion rate, and waste reduction of all substrates. The bioconversion rates were 15.0% DM (raw) and 18.8% DM (control), similar to 15–22% DM reported for food waste, abattoir waste, and human feces (Banks et al., 2014; Gold et al., 2020b; Lalander et al., 2019). More than half of the oat pulp was reduced and the larval weight was between 51–63 mg DM, which is comparable to 57 mg DM for BSFL reared on poultry feed, a typical high performance benchmark (Gold et al., 2020b). The rearing performance metrics of untreated spent grain were similar to those found in previous studies. For example, the bioconversion rate

with spent grain was 10% DM (control) and 11.5% DM (raw) in comparison to 9–14% DM reported by Beesigamukama et al., (2021). Both cow manure and grass clippings had the lowest larval weights and bioconversion rates. The bioconversion rates for cow manure were 3% DM (raw) and 4% DM (control). The poor performance with cow manure was expected because of its low nutrient and high fiber content (Table 3.1, Figure 3.1) and was similar to the 2–6% DM bioconversion rates previously reported by other authors (Gold et al., 2020b; Miranda et al., 2019; Rehman et al., 2017a). Although grass clippings had low bioconversion rates of 3% DM (raw) and 4% DM (control), it was reduced by approximately 30% DM. Additionally, in a preliminary study with seven-day storage, BSFL reduced untreated grass clippings by 45–65% DM and bioconversion rates were 7–8% DM (see Supplementary Material). Given that grass clippings is abundantly available and is a main component of municipal organic solid waste that is often poorly managed, grass clippings should be further explored as a rearing substrate in future research (Danial et al., 2020).

Table 3.2. BSFL rearing performance metrics on the four substrates and treatments (raw, control and ammonia-pretreated). Waste reduction with cow manure was not estimated. Data displayed are mean ± standard deviation (n=3–4).

Substrates	Treatment condition	Survival rate %	Larval weight mg DM	Bioconversion rate % DM	Waste reduction % DM
Spent grain	Raw	98.9 ± 1.1	38.4 ± 2.6	11.5 ± 0.9	50.6 ± 2.1
	Control	95.7 ± 4.2	35.8 ± 3.9	10.3 ± 1.6	58.3 ± 5.1
	Pretreated	97.3 ± 2.6	11.8 ± 2.2	3.1 ± 0.7	26.6 ± 5.9
Cow manure	Raw	91.1 ± 4.2	11.4 ± 0.6	2.9 ± 0.1	-
	Control	93.6 ± 1.5	14.1 ± 0.8	3.8 ± 0.2	-
	Pretreated	96.1 ± 2.5	6.8 ± 0.3	1.6 ± 0.1	-
Oat pulp	Raw	99.5 ± 0.9	50.8 ± 1.7	15.0 ± 0.5	59.6 ± 1.5
	Control	99.3 ± 0.9	62.9 ± 3.8	18.8 ± 1.3	66.5 ± 1.3
	Pretreated	98.2 ± 2.1	12.9 ± 2.0	3.0 ± 0.6	24.3 ± 4.4
Grass clippings	Raw	97.0 ± 0.9	13.5 ± 2.1	3.1 ± 0.7	30.2 ± 8.4
	Control	98.9 ± 0.9	17.0 ± 3.0	4.3 ± 1.0	29.5 ± 3.4
	Pretreated	87.3 ± 6.9	7.3 ± 0.5	1.1 ± 0.3	13.8 ± 4.8

Overall, BSFL performance between the raw and control was similar for all substrates. Interestingly, three-day storage without the addition of ammonia improved the larval rearing performance for cow manure and oat pulp. For example, the larval weight increased by 20–24%, and bioconversion increased by 25–31% for cow manure and oat pulp. This could be due to fermentation occurring during storage, increasing microbial degradation of the substrates, and providing accessible nutrients to the larvae. Previous studies have also reported positive effects of fermentation with and without microbial inoculants (Van Campenhout, 2021). For example, Mohd-Noor et al. (2017) found that four-week fermentation of coconut endosperm increased larval mass by approximately 79% and Wong et al. (2020) found that 14-day fermentation of coconut endosperm with the addition of bacteria increased larval mass by approximately 41% in comparison to an unfermented control. Our results for cow manure and oat pulp suggests that fermentation without added inoculum can increase rearing process performance with only three days of storage. Research should investigate the change in microbial composition during fermentation and how this may play a role in increasing larval bioconversion of substrates. The microbial composition was not assessed in this study, but a shift in the microbial community during storage may have occurred, resulting in a microbial composition more suitable for facilitating larval decomposition of cow manure and oat pulp.

Contrary to our expectations based on the fiber analyses, FTIR and SEM results, BSFL performance was the lowest in all estimated performance metrics for all ammonia-pretreated substrates (Table 3.2). For example, bioconversion rate decreased by 57%–84% with ammonia-pretreated substrates compared to the control. Waste reduction and larval weight were 53%–63% and 22–67% lower, respectively, to those of the control. This means that ammonia pretreatment with the dose and time tested in this study is not a viable pretreatment option. The reasons for this performance decrease could be two-fold: Firstly, BSFL rearing is strongly influenced by the substrate and frass microbial community. The added ammonia nitrogen could have shifted the C:N ratio to a less favorable ratio for the substrate microbial community, decreasing the microbial numbers needed for proper BSFL development. For example, Gold et al. (2020d) found that inactivating the substrate microbial community within canteen waste decreases bioconversion rate by 31%. Secondly, the added ammonia could have been directly toxic or created an environment toxic to BSFL and their intestinal microbial community. To inform future research direction for waste treatment with BSFL following ammonia pretreatment, these two reasons were investigated in follow-up experiments.

3.4.6. Ammonia toxicity to microorganisms and larvae

To evaluate the toxicity of ammonia to microorganisms, chicken feed was pretreated with 5% ammonia for three days, and microbial numbers were determined. Ammonia pretreatment reduced the microbial numbers on all substrates and the C:N ratio (Table 3.3). Microbial numbers decreased by around 3 to 4 $\log_{10}$ cfu/g substrate (Table 3.3), indicating that ammonia had an inhibitory effect on the substrate microbial

community. This inhibition could be due to the specific storage conditions or the elevated ammonium concentrations (NH_4^+-N) of 6–18 mg/g substrate in ammonia-pretreated substrates. However, these ammonia levels are much lower than 2,000–25,000 mg/l proposed to be toxic in anaerobic digestion (Yenigün and Demirel, 2013). It is difficult to conclude how the decrease in the C:N ratio by ammonia pretreatment contributed to the poor larval performance. The C:N ratios in the ammonia-pretreated substrates ranged from 6–13 (Table 3.3). Lu et al. (2021) observed a 26% decrease in larval yield when altering the C:N ratio from 18 to 10 with food waste. However, adjusting the C:N ratio can also have beneficial effects on BSFL performance. Palma et al. (2019) found decreasing the C:N ratio from 49 to 16 in almond hulls increased larval weight by 42%, suggesting that the supplementation of nitrogen has a positive effect on larval performance.

Table 3.3. Microbial counts, ammonium concentration and C:N ratio in the substrates: control and ammonia-pretreated at 5% for three days. Data displayed are mean ± standard deviation (n=3–4).

Substrates	Treatment Condition	Total viable counts log_{10} cfu/g	Ammonium (NH_4^+-N) mg/g	C:N ratio
Spent grain	Control	8.1 ± 0.3	0.04 ± 0.01	12.2 ± 0.5
	Pretreated	4.4 ± 0.5	5.7 ± 0.5	7.2 ± 0.2
Cow manure	Control	8.6 ± 0.1	3.3 ± 0.2	20.1 ± 0.6
	Pretreated	5.8 ± 0.1	18.2 ± 0.9	13.2 ± 1.6
Oat pulp	Control	7.3 ± 0.5	0.05 ± 0.00	9.1 ± 0.0
	Pretreated	4.6 ± 0.2	6.0 ± 0.5	6.2 ± 0.4
Grass clippings	Control	9.7 ± 0.4	1.7 ± 0.3	12.3 ± 0.7
	Pretreated	7.9 ± 0.5	8.6 ± 0.6	6.8 ± 0.8

A toxicity test on chicken feed was then performed to evaluate whether ammonia treatment (0, 1, 3, or 5%, 0 days) had a direct detrimental effect on the larvae. Sterilized chicken feed was used to evaluate the potential effects to the larvae without influence from the substrate microbial community. Ammonia treatment caused a small decrease in survival rates from 98.9 (1.1) on control to 89.7 (4.1) % for 5%.

BSFL rearing performance was inversely proportional to the ammonia dose (Figure 3.5). The bioconversion rate was 25.1 (2.4) % DM typical for chicken feed (Gold et al., 2020b) and decreased by 18% with 1% and 96% with 5% ammonia pretreatment. These results indicate that the ammonia pretreatment had a direct adverse effect on the larvae and was probably the main reason for the decrease in rearing performance

observed in the previous experiment with the four biowastes and not because of the reduced microbial numbers or shift in the C:N ratio. The dose-dependent detrimental effect on the larvae and/or associated microorganisms could be a result of a secondary effect generated by ammonium salts produced during the pH neutralization between ammonia and sulfuric acid. For example, ammonium sulfate can enhance the osmotic stress of the substrate, reduce microbial numbers (Müller et al., 2006) and/or cause metabolic stress on the larvae, influencing larval development (Belloni et al., 2018; Weihrauch et al., 2012). Lu et al. (2021) found similar results with BSFL, where addition of ammonium chloride (NH_4Cl) (1 g of nitrogen/100 g food waste DM) decreased the larval yield by 52% compared to the control. The authors attributed this performance decrease to the toxicity of the ammonium chloride salt to BSFL. Ammonium salts can be toxic to BSFL by compromising lysosomal proteases that are involved in intracellular degradation of proteins (Weihrauch et al., 2012). Based on their ecological niche, BSFL can be expected to be tolerant to metabolic byproducts such as ammonia, similar to other Dipteran flies feeding on biowastes such as fruit flies (*Drosophila*) (Belloni et al., 2018). However, ammonia could be especially toxic to young larvae. Mature BSFL thrive in frass with ammonia concentrations in the order of 5–9 mg/g (Fuhrmann et al., 2022; Visvini et al., 2022), similar to 6–18 mg/g in the four ammonia-pretreated substrates (Table 3). Belloni et al. (2018) found that a high concentration of ammonium chloride (13.3 g/L) led to 100% mortality of *Drosophila suzukii* larvae soon after hatching. The authors attribute this to the larvae's inability to identify ammonia as a toxin and react to the exposure, compromising the larvae's ability to metabolize ammonia and resulting in acute intoxication. This could imply that when young BSFL may be exposed to increased ammonia concentrations, their larval feeding and development time are decreased as they adapt to ammonia exposure. These results suggest that future research should study substrate/frass ammonia concentrations across the entire rearing cycle, and the toxicity of ammonia to all BSFL life stages.

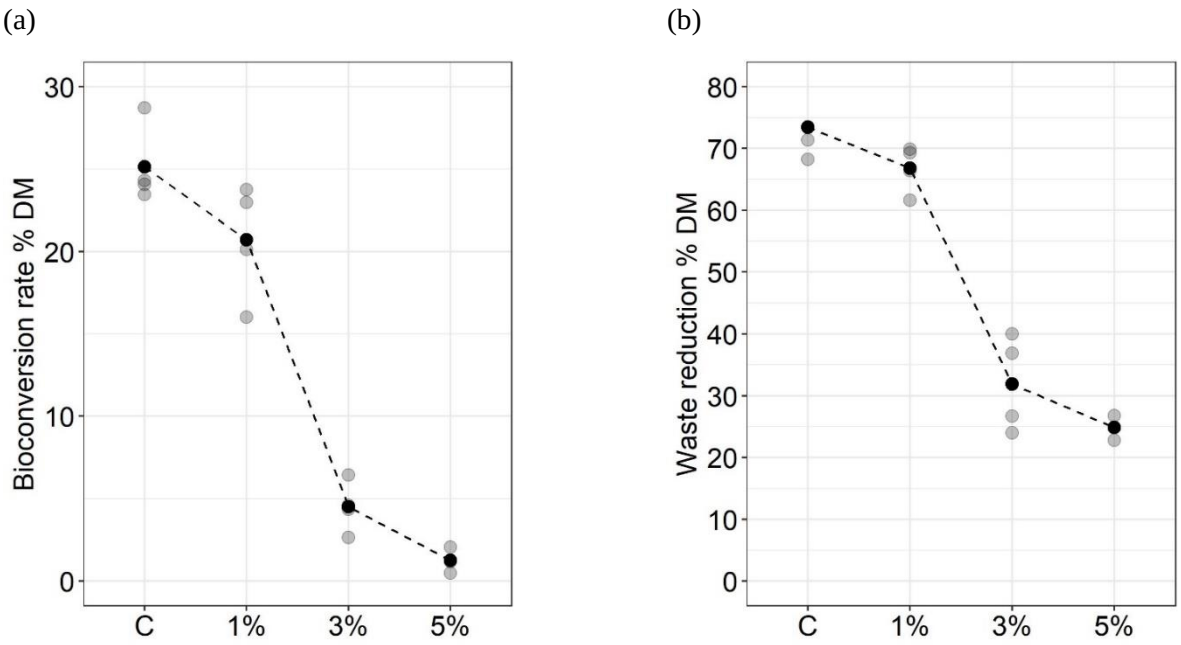


Figure 3.5. (a) Bioconversion rate and (b) waste reduction of BSFL on sterilized chicken feed at varying ammonia doses compared to the respective control (C). Mean (bold) and replicates are displayed (n=3–4).

3.5. Conclusions

The present study is the first study to systematically assess whether ammonia pretreatment reduces the lignocellulosic composition in biowastes and improves the BSFL rearing performance on fibrous biowastes. Fiber analyses, FTIR spectra, and SEM images revealed that ammonia pretreatment altered the lignocellulosic composition with the potential for increased larval and microbial decomposition. However, BSFL feeding experiments showed that ammonia pretreatment was not a suitable biowaste pretreatment. BSFL rearing performance metrics were at least halved on all substrates, likely due to the toxicity of ammonia/ammonium and/or their salts to the larvae or associated microorganisms. Future research should explore the toxicity of ammonia at different life stages of BSFL, as older larvae may be less susceptible to ammonia and could therefore allow a second and third feeding of ammonia-pretreated substrates. In addition, other alkaline (e.g., sodium hydroxide), physical, and microbial pretreatments resulting in lignocellulosic degradation should be explored as they may be more suitable for improving BSFL development. Short storage/fermentation periods of less than seven days should be explored, since this was shown to improve larval performance on cow manure and oat pulp. For any pretreatment, a life cycle and life cycle cost assessment should be conducted to evaluate its effect on the environmental and economic aspects of the BSFL production system.

4. Chapter: Physical Pretreatment

Physical pretreatment of three biowastes to improve black soldier fly larvae bioconversion efficiency

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4.1. Abstract

Black soldier fly larvae (BSFL, *Hermetia illucens* (L.)) are increasingly recognized for their efficient biowaste reduction while yielding valuable proteins and fats for animal feed and aquaculture. However, biowastes high in lignocellulosic fibers, including cellulose, hemicellulose, and lignin, present challenges for degradation by microorganisms in the biowaste and larval digestive tract as well as the larvae themselves. To address this challenge, this study investigated two biowaste physical pretreatments (thermal and mechanical) for improving BSFL processing of fibrous biowastes. Cow manure, spent grain, and grass clippings were thermally pretreated at 90°C for three durations (0.5, 1 and 4 h). Contrary to expectation, thermal pretreatment resulted in either no improvement or decreased larval performance on all substrates, regardless of treatment duration. In contrast, mechanical pretreatment of spent grain and grass clippings, involving milling with three screen sizes (0.5, 1 and 2 mm) showed promising results. Bioconversion rates on 0.5 mm-milled spent grain and grass clippings increased by 0–53% and 25–44% dry mass, respectively. Additionally, larval protein conversion increased by 41% and 23% on spent grain and grass clippings, respectively. Analysis of particle size revealed smaller particles than the initial screen sizes, potentially enabling larval ingestion. However, mechanical pretreatment did not impact fiber degradation by larval conversion, as hemicellulose decreased by 25% and 75% for spent grain and grass clippings, respectively, regardless of particle size. This study highlights mechanical pretreatment's potential in enhancing BSFL bioconversion of fibrous biowastes and the importance of understanding substrate physical properties influencing substrate microorganisms and BSFL.

4.2. Introduction

Rapid urbanization due to population growth, exceeding 9 billion people by 2050, intensifies waste management challenges as expanding cities and thriving economies contribute to increased waste generation. Currently, the world generates 2 billion tons of waste annually, of which 33% is inadequately managed (Kaza et al., 2018). Among this waste, food and green biowaste constitute the largest fraction, accounting for 44% of the total waste (Kaza et al., 2018). Poor waste management poses risks to public health, such as higher incidences of diarrhea and acute respiratory infections for those near open dump sites (UN-Habitat and United Nations Human Settlements, 2010). Innovative approaches that can reuse biowaste and generate revenue, thereby offsetting the costs to manage the waste are needed. Among these approaches, black soldier fly larvae (*Hermetia illucens*, L., BSFL) have emerged as a promising biowaste treatment technology, efficiently recycling nutrients from various biowastes into insect biomass rich in protein (32–58% dry mass, DM) and fat (15–39 % DM) suitable for animal feed and aquaculture applications (Gold et al., 2018). Furthermore, the residual byproduct left by BSFL is rich in nutrients, bioactive compounds, and beneficial microorganisms with value as soil conditioners and/or organic fertilizer (Fuhrmann et al., 2022; Poveda, 2021). By incorporating feeds derived from BSFL, there is potential to reduce reliance on environmentally concerning resources such as fish and soybean meal (Vongvichith et al., 2020).

Despite their ability to grow on a diverse range of biowastes, a major obstacle for this emerging biowaste technology is their unreliable and inefficient growth on fibrous biowastes containing lignocellulosic fibers (i.e., lignin, cellulose, hemicellulose) (Peguero et al., 2022). Consequently, this limitation can result in lower bioconversion rates (2–6% DM) compared to bioconversion rates on highly nutritious feeds (>15% DM), resulting in a reduced product yield per unit of waste, thus impacting the amount of product and revenue that can be generated. Furthermore, performance variability hampers product consistency and reliability, posing a challenge when attempting to create a profitable business from the upcycling of biowaste.

Lignocellulosic fibers are complex molecules that are resistant to microbial and/or larval degradation (Atelge et al., 2020). Lignin forms a protective barrier around cellulose and hemicellulose hindering their conversion into simple sugars through microbial degradation (Agbor et al., 2011; Atelge et al., 2020). While the BSFL digestive system is inefficient in breaking down lignin, as indicated by the negative correlation between larval weight and lignin content (Liu et al., 2018; Zheng et al., 2012), other studies have reported that BSFL reduced lignin in dairy manure and rice straw by 31% and 50%, respectively, (Liu et al., 2021; Rehman et al., 2017b). Nevertheless, by implementing physical (e.g., mechanical and thermal), chemical (e.g., alkaline and acids) and biological (e.g., bacteria and fungi) pretreatments, biowaste could be more digestible for larvae and/or microorganisms in the biowaste or larval digestive tract (Peguero et al., 2022) .

Physical pretreatments, such as thermal and mechanical require energy but offer the advantage of not requiring the use of additional compounds, such as chemicals (e.g. HCL, NaOH) commonly used for chemical pretreatment or microbial inoculums commonly used for biological pretreatment in order to modify lignocellulosic characteristics (Atelge et al., 2020). Thermal pretreatment (>100 °C, 15–20 mins, 2–9 bar pressure) can improve biowaste digestibility by enhancing the solubilization of organic matter, however these treatment conditions may not be suitable for BSFL because of the inactivation of potentially beneficial microorganisms (Peguero et al., 2022). Since biowaste microbial community contributes to the bioconversion performance (Gold et al., 2020d), lower temperatures and longer holding times (e.g. 80–90 °C for > 60 min) with a lower impact on the microbial community may be more suitable for BSFL biowaste treatment (Appels et al., 2010; Peguero et al., 2022). These treatment conditions (e.g. 80–90 °C for > 60 min) have previously demonstrated positive impacts for other bioprocessing technologies, such as 60% increase in biogas production from agricultural crops and residues and 14–18% increase in release of humic substances during composting of dairy manure (Huang et al., 2019; Menardo et al., 2012; Zhu et al., 2021).

Alternatively, mechanical pretreatment, such as milling, could improve larval performance, by reducing the biowaste particle size, increasing the surface area and bulk density of the biowastes, and potentially reducing cellulose crystallinity. These different effects could promote microbial activity and increase degradability by biowaste microorganisms (Atelge et al., 2020; Mshandete et al., 2006). Reducing particle size to $\leq$2 mm for manures, fruit/vegetable waste, and crop and harvesting residues increased methane production by 16–65% compared to the untreated control (Angelidaki and Ahring, 2000; Menardo et al., 2012; Mshandete et al., 2006; Sharma et al., 1988).

More research is needed on the effectiveness of thermal and mechanical biowaste pretreatment for different BSFL biowastes with different lignocellulosic, nutrient and microbial contents and composition as well as process conditions (e.g., temperature, holding time, particle size), which all have been shown to influence pretreatment outcomes (Menardo et al., 2012; Sharma et al., 1988; Wang et al., 1997). Few studies have examined the effects of thermal (Isibika et al., 2019; Liew et al., 2022) and mechanical (Palma et al., 2019; Yakti et al., 2023) pretreatment on BSFL biowaste treatment. For example, Liew et al. (2022) found thermal pretreatment of waste activated sludge (e.g., >75 °C, >1 h) increased BSFL larval weight by 45–292 % compared to the untreated control (Liew et al., 2022). However, one study has found that higher temperature pretreatment (e.g., 120 °C) of banana peels negatively impacted larval weight by 15 % (Isibika et al., 2019). For mechanical pretreatment, reducing particle size of wheat straw from 3 mm to <1 mm and almond hulls from 6 mm to 4 mm decreased larval weight by 32 % and 10 % respectively (Palma et al., 2019; Yakti et al., 2023). However, many studies initially reduce particle size of the selected biowastes and do not compare results to the untreated control, complicating drawing robust conclusions of the effect of particle size

reduction on BSFL biowaste processing (Laganaro et al., 2021; Liland et al., 2017; Liu et al., 2021; Palma et al., 2019).

This study assessed the impact of thermal and mechanical pretreatment on BSFL bioconversion with cow manure, spent grain and grass clippings. It was hypothesized that thermal and mechanical pretreatment would increase larvae performance. Furthermore, it was hypothesized that mechanical pretreatment would promote microbial growth and respiration in the substrate due to the particle size reduction. Overall, this research sought to contribute to biowaste management by improving the bioconversion efficiency of BSFL on low-value fibrous biowastes.

4.3. Material and Methods

4.3.1. Source of biowastes

BSFL substrates used were spent grain, cow manure, and grass clippings, previously described in detail by Peguero et al. (2023). These substrates were selected due to their high lignocellulosic composition (>45 % DM, sum of hemicellulose, cellulose and lignin) as shown in Table 4.1 (Peguero et al., 2023).

Table 4.1. Physicochemical composition of spent grain, cow manure and grass clippings (Peguero et al., 2023). Data displayed are mean ± standard deviation (n=3–4).

Parameters	Spent grain	Cow manure	Grass clippings
Moisture content	74.5 ± 0.3	91.0 ± 0.0	74.0 ± 0.3
Organic matter	95.5 ± 0.1	81.8 ± 0.6	89.6 ± 0.3
Hemicellulose	33.6 ± 0.6	17.3 ± 2.3	18.6 ± 2.1
Cellulose	17.5 ± 0.2	24.2 ± 1.1	21.4 ± 1.9
Lignin	8.2 ± 0.6	10.7 ± 0.8	7.3 ± 0.7
Protein	24.5 ± 0.2	9.1 ± 0.1	14.0 ± 0.4
TOC (%DM)	49.3 ± 0.5	42.2 ± 0.3	43.8 ± 0.5
TN	3.9 ± 0.04	2.1 ± 0.02	3.1 ± 0.1
C/N ratio	12.6 ± 0.1	19.9 ± 0.2	14.4 ± 0.4

4.3.2. Thermal pretreatment

Substrates were treated at 90°C for 0.5, 1h and 4 h and compared to an untreated control. A substrate temperature of 90°C was selected as this temperature was previously shown to increase larval performance (Liew et al., 2022). The substrate (1–2.5 kg wet mass) was distributed in similar amounts across plastic bags. Prior to immersion, the weight and initial temperature of the biowaste in each bag was recorded. The

treatment involved immersing the substrates in sealed plastic bags within a water bath filled with deionized water at 90°C. The bags filled with the substrates were attached to a perforated metal sheet, to ensure the substrate was completely submerged in the water bath (see Supplementary Material). A lid was used to minimize evaporation and maintain water temperature. To ensure thermal consistency, temperature was measured with a thermometer in one of the substrate-filled bags. The timer for the pretreatment duration was initiated once the substrate temperature reached 90°C (±1°C).

4.3.3. Mechanical pretreatment

Two mechanical pretreatment experiments were performed with grass clippings and spent grain. In the first experiment, the substrates were reduced to 2, 1 and 0.5 mm and compared to an untreated control (i.e., without pretreatment). Based on the observed effect of particle size on BSFL bioconversion rate, a follow-up experiment compared substrates with 0.5 mm-milled to the untreated substrates. Substrates were milled (6,000–10,000 rpm, ZM 200, Retsch, Germany) following freezing by dry ice. For spent grain, a 2:1 ratio and for grass clippings, a 3:1 ratio was used (substrate: dry ice). After pretreatment the substrates were stored at −20°C until further use. Images of the substrates before and after mechanical pretreatment can be found in (see Supplementary Material).

4.3.4. Substrate physical properties

Bulk density and particle size distribution were analyzed for the mechanically treated substrates. Particle size distribution on a volume-basis was determined by static light scattering by an external laboratory (Luzern University of Applied Sciences, Luzern, Switzerland) using a Beckman Coulter LS 13 320 Laser Diffraction Particle Size Analyzer with Universal Liquid Module with a maximum detection of 2000 μm and was conducted in duplicate or triplicate. Characteristic particle size distribution values (D10, D50, and D90) were determined (Table 4.2). For all three-screen sizes of the centrifugal mill (0.5, 1 and 2 mm), the particle size analysis revealed was much smaller particles than the initial screen size, likely because particles > 2000 μm were not detected or further particle size beyond the screen size took place. Considering D50 results (Table 4.2), the centrifugal mill sizes of 0.5, 1 and 2 mm, will be referred to as low, medium and high particle size throughout the remainder of the study (Table 4.2). Since the results for grass clippings between the medium and high categories were similar, the results from the medium category will not be presented in the results section.

Table 4.2. Particle size distribution of spent grain and grass clippings was analyzed for centrifugal mills: 0.5, 1 and 2 mm. The percentiles D10, D50, and D90 are used to describe the particle size distribution on a volume basis. D50 is the median particle diameter, 10% of the sample is smaller than D10 and 90% of the sample is smaller than D90. Samples could not be analyzed for particles exceeding 2000 µm.

Substrate	Centrifugal mill size (mm)	Treatment	Sample name	D10 µm	D50 µm	D90 µm
Spent grain	2.0	High	SG_{high}	83.9 ± 32.7	463.1 ± 34.4	1045.0 ± 21.2
	1.0	Medium	SG_{medium}	74.4 ± 22.3	396.1 ± 18.8	858.7 ±41.3
	0.5	Low	SG_{low}	13.17 ± 13.39	139.5 ± 7.7	425.4 ± 12.0
Grass clippings	2.0	High	GC_{high}	57.2 ± 27.6	255.1 ± 86.0	635.4 ± 82.7
	1.0	Medium	GC_{medium}	50.0 ± 3.7	249.4 ± 11.1	595.3 ± 20.8
	0.5	Low	GC_{low}	22.8 ± 0.4	157.0 ± 2.7	429.8 ± 2.2

4.3.5. Source of BSFL

The black soldier fly neonates had the same age (hatching within 24 h) and were sourced from the research colony at Eawag (Dübendorf, Switzerland) maintained according to Dortmans et al. (2017). All neonates were reared under controlled environmental conditions, with a temperature of 28 °C and relative humidity of 70%. The neonates were fed on chicken feed (60–75 % moisture content, UFA 620, Switzerland) for 5–7 days until reaching a mean individual weight of 1–3 mg DM. The larvae were then separated and transferred to the different feeding substrates and treatments, (i.e., untreated and pretreated substrates).

4.3.6. Larval feeding experiment

Larval feeding experiments were conducted with pretreated and untreated substrates with three to four replicates per treatment. In the first feeding experiments, larvae were reared in plastic containers (diameter: 7.5 cm, height:11 cm) covered with mosquito net at 2.5 larvae/cm^2 with 35 mg DM substate/larvae/day for nine days (Gold et al., 2020b; Peguero et al., 2023). In the second mechanical pretreatment experiment, larvae were reared at 35 mg DM/larvae/day (spent grain), and 23 mg DM/larvae/day (grass clippings) for six days. The substrates were brought to a temperature of 22–28°C prior to adding larvae. For each replicate, larvae were weighed, and transferred to the substrate-filled plastic containers. In the second mechanical pretreatment feeding experiment, slightly larger containers measuring 21 cm x 15 cm x 12 cm were used and covered with mosquito net. During the first two feeding experiments (thermal and mechanical), the temperature and relative humidity in the climate chamber were 28 °C and 70 %, respectively. In the second mechanical pretreatment experiment, the ambient temperature and humidity in the rearing room were 30°C and 50–70%, respectively (see Supplementary Material) and monitored using a temperature data logger (Testo GmbH, Austria), and substrate temperature was monitored using ibuttons (Mouser Electronics, Germany), and capsules (Mouser Electronics, Germany).

Larvae were manually harvested from the residues, counted, and weighed. After determining the fresh weight, larvae were inactivated at 105 °C for 5 min and then dried in a laboratory oven at 60 °C for two days (Rehman, et al., 2017). Residues were dried at 60 °C until weight remained constant. Both the dried larvae and the residues were then weighed and stored at 4 °C for further analyses. Based on larval numbers and DM of the substrate, residue and larvae, common rearing performance parameters, including survival rate, final larval weight, bioconversion rate, protein conversion efficiency, and waste reduction were calculated according to (Gold et al., 2020b).

4.3.7. Substrate, residue and larval physicochemical analyses

Moisture content and fiber were analyzed in the substrate and residue. Larvae were analyzed for moisture and protein content. All physicochemical analyses were conducted in triplicate or quadruplicate. Moisture content of the substrate was determined as the weight loss of three grams of wet sample by overnight oven drying at 105 °C. Prior to analyzing fiber content, substrates and residues were dried at 60 °C until weight remained constant and milled to 1 mm (10,000 rpm, Retsch ZM 200, Germany). Fiber analyses included neutral detergent fiber (Van Soest et al., 1991), acid detergent fiber (AOAC, 1977) and acid detergent lignin (AOAC, 1977) using 0.5 g dried sample. Neutral and acid detergent fiber were analyzed with a Fibertherm® FT12 system (Gerhardt Analytical Systems, Germany) (Peguero et al., 2023). Acid detergent lignin was analyzed with the remaining residue following acid detergent fiber which was soaked in 72 % H_2SO_4 for three hours. Nitrogen of the larvae was determined using 0.7 g of dried sample with a C/N analyzer (Trumac CN, LECO Instruments, Germany) and larval protein was calculated using a conversion factor of 4.67 (Janssen et al., 2017).

4.3.8. Microbial respiration

The first experiment demonstrated that particle size influenced larval performance which could be due to increased microbial activity due to altered physical substrate characteristics. As a proxy for microbial activity, microbial respiration (i.e., CO_2) was estimated in the second larval feeding experiment with untreated and pretreated substrates (i.e., SG_{low} and GC_{low}). The CO_2 production (ppm), including both larval and microbial respiration, was measured for 5 mins from the headspace above the substrate after closing the rearing container with airtight lid using a pre-calibrated wireless CO_2 sensor (PS-3208, Pasco, California) (Laganaro et al., 2021). Larval CO_2 production was measured with 10–15 grams of randomly sampled mixture of larvae and residue from each substrate and treatment condition. The larvae were washed with deionized water to remove substrate residues and then transferred to a 50 mL falcon tube. The CO_2 sensor was then inserted into the top of the 50 mL tube, forming an airtight seal and recorded CO_2 at a frequency of 1 Hz (Laganaro et al., 2021). The microbial CO_2 production was estimated as the difference between the

overall CO_2 production and larval CO_2 production (Bekker et al., 2021). The CO_2 production was measured daily over the six-day feeding experiment in triplicate. CO_2 production rates (CO_2 mg/min) were calculated from the slope of the linear regression of CO_2 starting from 2 minutes, when the sensor stabilized, until 5 minutes and considering the headspace volume, number of larvae and volume of the sensor (Bekker et al., 2021).

4.3.9. Effect of particle size reduction on microbial numbers

To assess the impact of particle size reduction on microbial numbers, aerobic total viable counts (TVC) (30°C, 72 h) were estimated in triplicate for untreated substrates, and SG_{low} and GCl_{ow} on day 0, 3 and 6 of the larval feeding experiment by an external laboratory (Eurofins, Switzerland) (ISO, 2013).

4.3.10. Data analyses

The data analyses were performed using Microsoft Excel (Version 2022, United States) and R statistical language (R Core Team, 2022, version 4.2.0). The mean and standard deviation of the substrate and residue composition, larvae performance parameters, substrate temperature, CO_2 measurements and microbial counts were calculated.

4.4. Results and Discussion

4.4.1. Thermal pretreatment

Larvae developed on all substrates and treatments with survival rates ≥ 93% except for the untreated cow manure which had a lower survival rate of 83% compared to treated cow manure of 93–96% (Table 4.3). These findings are similar to 89–99% reported by previous studies indicating that rearing conditions and substrates were suitable for larval rearing (Gold et al., 2020b; Peguero et al., 2023; Rehman et al., 2017a).

Contrary to our expectations, thermal pretreatment (90 °C, 0.5,1, and 4 h) of all three substrates (Table 4.3), regardless of treatment time, resulted in either a decrease in bioconversion rate and waste reduction or showed no difference compared to the untreated. Specifically, thermally pretreated substrates decreased larval weight by 19–29%, bioconversion rate by 2–29% and waste reduction by 1–57%, compared to the untreated. Our findings are contrary to those of Liew et al. (2022), who reported an increase in larval weight by 292% with thermally pretreated (90°C, 16 h) waste activated sludge. Differences between studies could be due to several factors that varied between Liew et al. (2022) and this study such as, substrate characteristics (e.g., nutrient and lignocellulosic fiber content and composition), BSFL feeding rate (35 mg DM/larvae/day in this study vs. 22 mg DM/larvae/day in Liew et al. (2022)), larval density (2.5 larvae/cm^2

in this study vs. 0.3 larvae/cm^2 in Liew et al. (2022)), duration of experiment (9 days in this study vs. 15 days in Liew et al. (2022)) and genetics.

Thermal pretreatments (<100 °C, >30 mins) have also led to increases in performance metrics of other bioconversion processes such as methane/biogas yields by 30–91% (Appels et al., 2010; Climent et al., 2007; Wang et al., 1997). These improvements have been attributed to increasing soluble proteins and soluble carbohydrates in waste activated sludge by 381–2430% and 351–704%, respectively (Appels et al., 2010; Liew et al., 2022). Due to the neutral or negative larval performance results in this study, soluble nutrients were not analyzed. Therefore, it is not possible to conclude if solubilization of nutrients took place. However, it is evident that if this occurred it did not increase larval performance.

The decrease in larval performance in this study may also be attributed to a potential reduction or alteration in microbial numbers and composition by thermal treatment which were not further investigated in this study. BSFL processing is a bioconversion process strongly intertwined with microbially mediated processes in both the biowaste and larval digestive tract (De Smet et al., 2018; Gold et al., 2018). Reduction of microbial numbers, for example by thermal treatment may have neutral, or negative effects on BSFL processing. For example, a complete inactivation of the substrate microbial population by non-thermal pretreatment of food waste decreased bioconversion rates by 30% (Gold et al., 2020d). However, thermal pretreatment of food waste (50–60 °C, 10–30 mins) and fecal sludge (80°C, 5 mins) had no effect on larval performance compared to the untreated even though TVC decreased by 2.5–4 $\log_{10}$ cfu/g and 2-$\log_{10}$ cfu/g, respectively (Peguero et al., 2021; Van Looveren et al., 2023). Therefore, there might be a threshold where a decrease in microbial numbers affects BSFL performance.

Table 4.3 BSFL rearing performance metrics on substrates treated at 90°C for three durations (0.5, 1 and 4 h) and the untreated control. Data displayed are the mean ± standard deviation (n = 3–4).

Substrates	Treatment condition	Survival rate %	Larval weight mg DM	Bioconversion rate % DM	Waste reduction % DM
Cow manure	Untreated	82.9 ± 2.8	14.1 ± 0.6	3.3 ± 0.2	6.4 ± 4.2
	0.5 hr	96.6 ± 3.7	10.8 ± 1.1	3.0 ± 0.3	7.6 ± 2.6
	1 hr	93.4 ± 6.9	10.1 ± 0.6	2.6 ± 0.3	4.2 ± 2.5
	4 hr	93.4 ± 4.7	11.8 ± 0.6	3.1 ± 0.2	6.3 ± 3.7
Spent grain	Untreated	99.5 ± 0.5	42.6 ± 1.0	12.4 ± 0.3	53.4 ± 0.7
	0.5 hr	98.6 ± 2.7	37.8 ± 1.6	10.8 ± 0.3	50.9 ± 2.0
	1 hr	99.6 ± 0.9	35.4 ± 9.0	10.1 ± 2.8	52.9 ± 0.6
	4 hr	98.2 ± 1.5	42.9 ± 1.8	12.2 ± 0.4	54.4 ± 0.8
Grass clippings	Untreated	97.3 ± 2.0	13.4 ± 0.7	3.8 ± 0.3	22.2 ± 5.5
	0.5 hr	95.2 ± 4.4	11.3 ± 1.4	3.0 ± 0.4	13.2 ± 1.7
	1 hr	94.3 ± 6.7	10.4 ± 2.6	2.7 ± 0.8	9.6 ± 3.4
	4 hr	96.4 ± 2.7	12.8 ± 0.5	3.6 ± 0.2	12.9 ± 2.8

4.4.2. Mechanical pretreatment

Substrate physical properties

Mechanical pretreatment decreased particle size for both spent grain and grass clippings (Table 4.2), with all D-values being much lower than the screen size of the centrifugal mill (0.5, 1 and 2 mm), which could be due to further particle size reduction by collision between substrate particles, between the screen and wall of the mill and because of the detection limit of particle size determination which did not include particles > 2,000 µm. The D50 of SG_{low} and GC_{low} were 140 µm and 157 µm, respectively (Table 4.2). The D50 for SG_{medium} and GC_{medium} were slightly higher than the lower particle size range as they were 400 µm and 250 µm. However, there was no difference for the D50 between GC_{medium} and GC_{high} (Table 4.2).

As expected, this particle size reduction increased bulk density for both spent grain and grass clippings (Table 4.4). The mean bulk density increased from 549 ± 78 g/L for spent grain untreated control to 895 ± 35 g/L for SG_{low}, and from 146 ±7 g/L for grass clippings untreated control to 562 ± 37 g/L for GC_{low}. Bulk density did not appear to differ between SG_{medium} and SG_{high}. Bulk density could positively or negatively affect aeration and oxygen availability for microorganisms and larvae, but limited research has investigated the impact of these parameters on larval performance. One study reported that a higher bulk density (1494 g/L) on 1-mm milled wheat straw negatively impacted larval performance (Yakti et al., 2023). However, the observed negative larval performance could be attributed to the use of the same volume of wheat straw (5.5%) regardless of the particle size, meaning that more substrate was provided to BSFL with the lower particle size and higher bulk density, potentially restricting the pore space and aeration within the substrate. When considering adding a fibrous material at different particle sizes, less quantity of the smaller particle size should be used to maintain the same mass, accounting for the higher bulk density (Raichura and McCartney, 2006). More likely, the change in pore space and aeration with particle size and bulk density could have impacted larval performance results. Palma et al. (2018) suggested that aeration can impact BSFL, with increased aeration resulting in 5-fold higher larval DM. Aeration was not measured in this study, but a widely used metric for quantifying aeration in composting, an aerobic bioconversion process most similar to BSFL processing, is free air space (Iqbal et al., 2010). Studies suggest that an optimal free air space in composting of different biowastes is 26–33% (Eftoda and McCartney, 2004; Iqbal et al., 2010; Kulcu and Yaldiz, 2007). Since aeration, bulk density and particle size can influence BSFL processing, these basic substrate parameters should be reported in future studies. Additionally, future research should investigate the range of free air space and bulk density leading to efficient biowastes treatment by BSFL.

Table 4.4. Bulk density of the spent grain and grass clippings before and after mechanical pretreatment. Data displayed are mean ± standard deviation (n=3).

Substrate	Treatment condition	Wet bulk density (g/L)
Spent grain	Untreated	548.6 ± 76.7
	2 mm	586.5 ±14.9
	1 mm	537.9 ± 22.0
	0.5 mm	895.2 ± 35.4
Grass clippings	Untreated	145.5 ± 7.2
	2 mm	338.5 ± 4.5
	1 mm	397.2 ± 22.8
	0.5 mm	561.5 ± 36.5

BSFL performance on mechanically pretreated substrates

Mechanical pretreatment had a positive impact on larval performance with spent grain and grass clippings. Similar to thermal pretreatment, survival rates were ≥ 94% and appeared to have no effect between treatments, indicating the rearing conditions, substrates and treatments were suitable for the larvae (see Supplementary Material). Interestingly, reducing the substrate particle size increased larval weight, bioconversion rate and waste reduction, for example SG_{high} and GC_{high} by 15–19%, 17–18% and 16–20%, respectively, compared to the untreated (Figure 4.1). Further reducing the particle size increased bioconversion rate on SG_{low} and GC_{low} by 53% and 44%, respectively, compared to untreated (Figure 4.1).

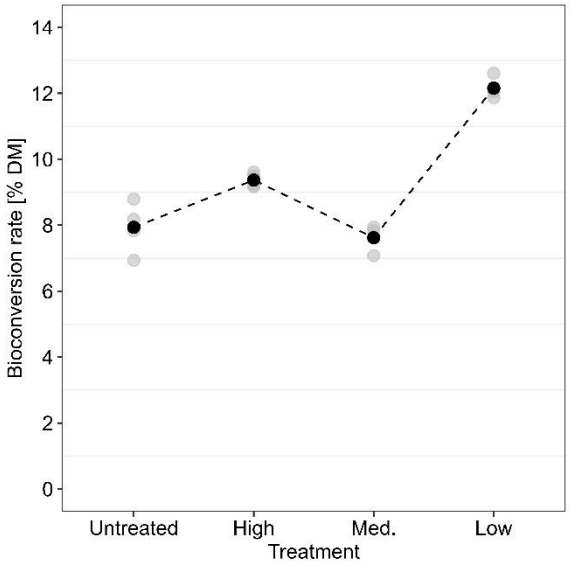
The improved larval performance with smaller particle sizes could be attributed to several factors. Firstly, the smaller particles may facilitate better microbial metabolism, thereby promoting the breakdown of organic matter by the biowaste microorganisms (Vermeulen et al., 2018). Additionally, the larvae might find smaller particles more easily digestible. Lievens et al. (2023) found that BSFL were able to ingest particles ranging from 20–110 μm, depending on the larval age and weight. Therefore, it is possible that the larvae were able to ingest these smaller particle sizes, considering that the D10 of both substrates and all treatment conditions were below 100 μm (Table 4.2), which falls within the range mature BSFL can ingest (Lievens et al., 2023).

The larval protein content were found to be comparable between spent grain and grass clippings at 32–35 %DM (see Supplementary Material), despite different substrate protein concentrations of 25 %DM and 14.0 %DM, respectively (Table 4.1). Barragan-Fonseca et al. (2018) had similar observations where substrate protein did not influence larval protein. During the lifespan of BSFL, the protein content of the mature larvae tends to decrease as the fat content increases (Liu et al., 2017). Various factors influence larval protein concentrations such as nutrient composition, larval density, stage of development and feeding rate (Beniers and Graham, 2019; Liu et al., 2017) The larval protein concentrations observed in this study (see Supplementary Material) are similar to previously reported values of 27–39% DM (initial protein values

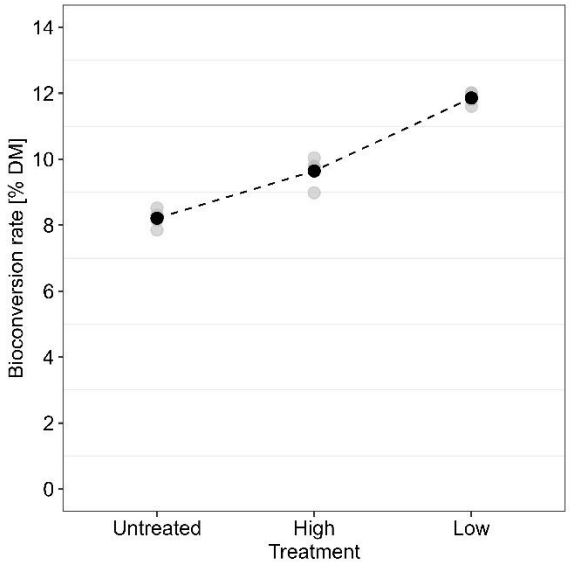
adjusted with a conversion factor 4.67 instead of 6.25) (Beniers and Graham, 2019; Nyakeri et al., 2017b; Tschirner and Simon, 2015). Similar to final larval weight and bioconversion rate, larval protein conversion was slightly higher, increasing by 40% and 24% for both SG_{low} and GC_{low}, respectively, compared to the untreated substrates (

Figure ***4.2***). This indicates that with lower substrate particle size more total protein for animal feed applications could be produced per unit of biowaste.

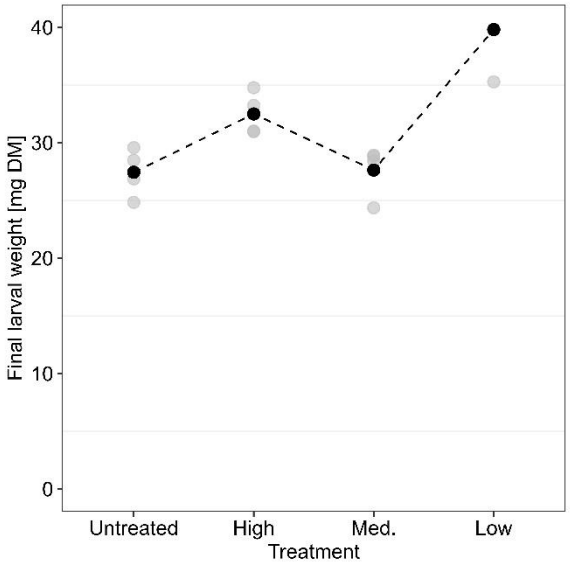
(a) Spent grain: bioconversion rate (% DM)



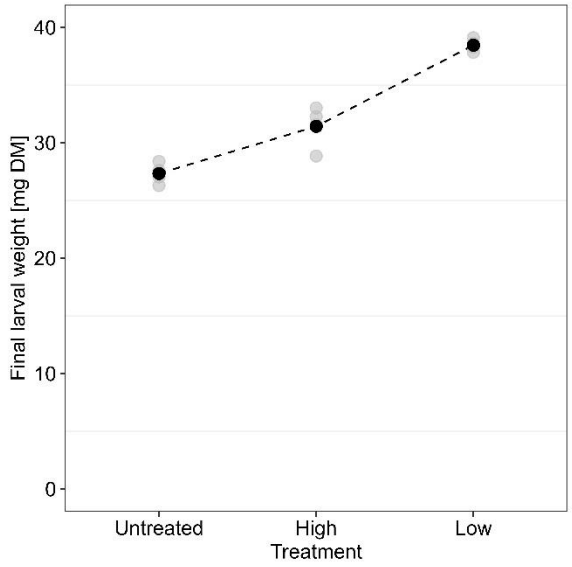
(b) Grass clippings: bioconversion rate (% DM)



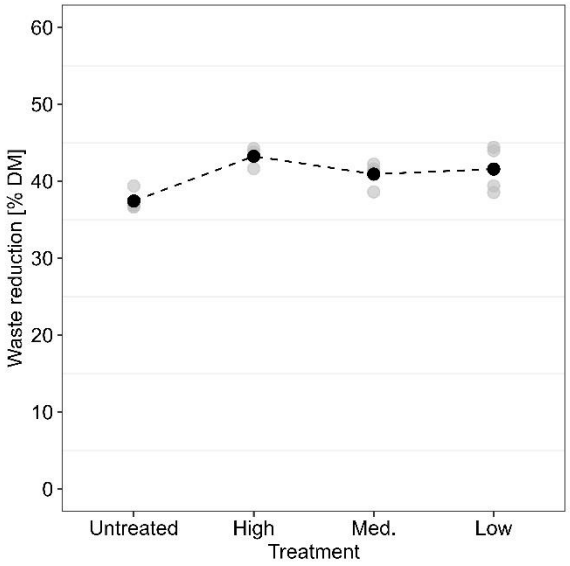
(c) Spent grain: final larval weight (mg DM)



(d) Grass clippings: final larval weight (mg DM)



(e) Spent grain: waste reduction (% DM)



(f) Grass clippings: waste reduction (% DM)

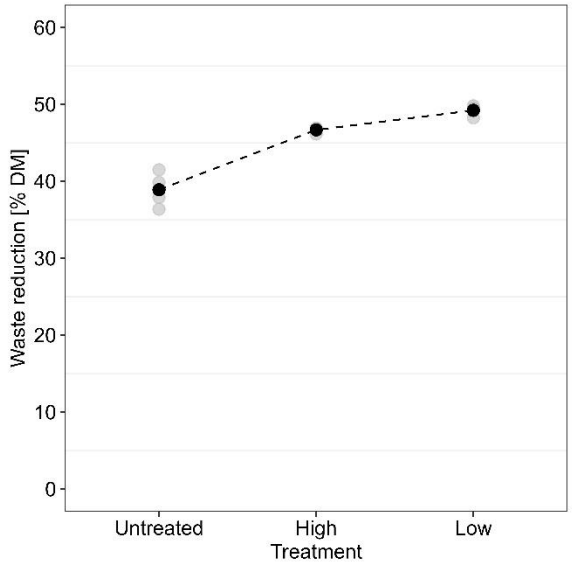


Figure 4.1. Bioconversion rate, final larval weight and waste reduction on (a,c,e) spent grain and (b,d,f) grass clippings and treatments (i.e., untreated and high, medium, and low particle size). Data displayed are mean (bold) and replicates (grey) (n = 4).

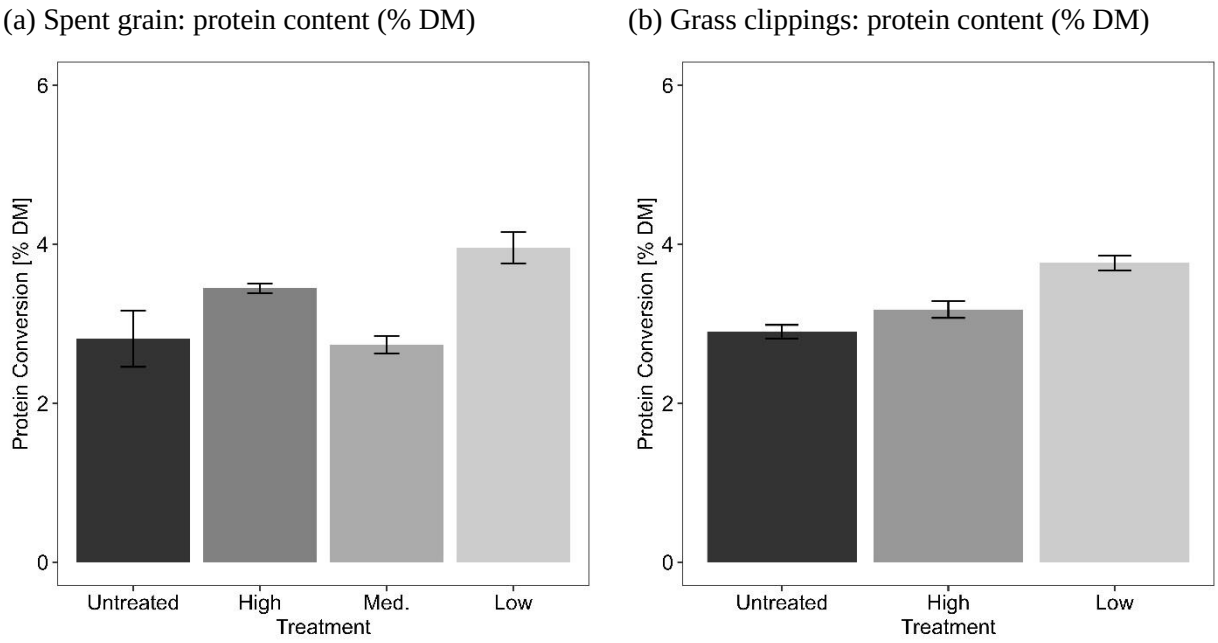


Figure 4.2. BSFL larval protein conversion on (a) spent grain and (b) grass clippings and treatments (i.e., untreated, and high, medium and low particle size). Data displayed are mean with error bars displayed as standard deviation (n=4).

Contrary to the findings in this study, Yakti et al. (2023), reported that reducing the particle size of wheat straw below 1 mm resulted in lower fresh larval weight (<80 mg wet weight/larva) compared to using wheat straw particles larger than 3 mm (110 mg wet weight/larva). This decrease in larvae performance could be attributed to a reduction of free air space by reducing particles within the substrate, decreasing aeration for the larvae and biowaste microorganisms.

The goal of particle size reduction next to direct ingestion by BSFL is to increase the surface area, thereby increasing substrate accessibility to microorganisms and potentially reducing cellulose crystallinity (Mshandete et al., 2006; Palmowski and Müller, 2000). However, in this study it did not seem that particle reduction had a direct influence on the degradation of the lignocellulosic composition during larval rearing. For instance, we observed BSFL bioconversion degraded 25 %DM of hemicellulose in spent grain and 75 %DM of hemicellulose in grass clippings, with no difference observed among the treatments (Figure S-5). The substantially higher reduction of hemicellulose seen on grass clippings compared to spent grain could indicate a different and more digestible hemicellulose composition. Hemicelluloses are complex polymers composed of various sugars with varying compositions in different biowastes (Saha, 2003). Furthermore, to understand the mechanisms behind mechanical pretreatment, the crystallinity index of cellulose and measurement of surface area should be assessed to evaluate their influence on enhanced substrate degradability.

Influence of mechanical pretreatment on microbial activity and BSFL performance

In order to elucidate potential mechanisms of the positive effects of particle size reduction on larval performance, a follow up experiment comparing untreated substrates and SG_{low} and GC_{low} measuring microbial respiration, as a proxy for microbial activity (Figure 4.4a), next to larval performance metrics and substrate/residue TVC and temperature. Our hypothesis was that particle size reduction increases microbial activity because of greater surface area and accessibility by substrate microorganisms (Palmowski and Müller, 2000), leading to higher microbial respiration for milled compared to untreated substrates.

Mechanical pretreatment increased all larval performance metrics compared to untreated (see Supplementary Material) for grass clippings, but not for spent grain. Throughout the experiment larvae grown on grass clippings had a consistently higher larval weight with GC_{low} compared to untreated (Figure 4.3a). The bioconversion rate was 23% higher for GC_{low} compared to untreated which was lower than the 44% bioconversion rate observed in the previous experiment. In contrast, performance metrics were not different between SG_{low} and untreated (see Supplementary Material) which is different to the previous experiment, where SG_{low} increased bioconversion rate by 53% compared to untreated (Figure 4.1). Interestingly, larval wet weight was initially higher with untreated spent grain up to rearing day 4, but larvae were slightly heavier with SG_{low} on harvest day 6 (Figure 4.4a). This highlights that changing the rearing container size and associated parameters can outweigh benefits of mechanical pretreatment for spent grain. For example, the larger container size and new dimensions increased the volume-based larval density from 0.06 larvae/cm^3 to 0.2 larvae/cm^3 and required reducing the rearing duration from 9 d to 6 d, two parameters that can influence larval growth outcomes (Barragan-Fonseca et al., 2018). Whereas mechanical pretreatment has demonstrated improved larval performance metrics under two slightly different rearing conditions for grass clippings, future research should investigate optimal rearing conditions after mechanical pretreatment of spent grain, such as volume-based larval density (Deruytter and Coudron, 2022).

Overall, initial microbial respiration for untreated and mechanically pretreated substrates were higher compared to microbial respiration on chicken feed (Bekker et al., 2021). This is to be expected as biowastes contain a complex organic content that can be easily degraded by a diverse and abundant microbial community (Ryckeboer et al., 2003). The contrast is from chicken feed's formulation using processed ingredients, which is less conducive to microbial activity although moisture content adjustment can increase it. For example, chicken feed with a moisture content of 45–55% reached similar microbial respiration rates by day 2, compared to untreated and pretreated substrates in this study (Bekker et al., 2021).

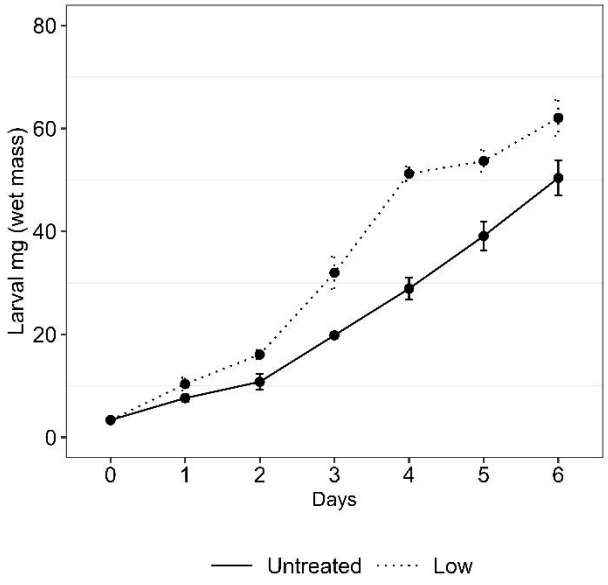
Mechanical pretreatment influenced microbial respiration dynamics for both substrates, but with opposite effects. In line with our hypothesis, for grass clippings, GC_{low} showed initially higher microbial respiration

(Figure 4.3b) than untreated on day 1 (3.5 mg CO_2/min vs. 2.0 mg CO2/min) and 3 (1.5 mg CO_2/min vs. 0.75 mg CO_2/min). From day 4, microbial respiration was higher in untreated than GC_{low} (Figure 4.3b). This initially higher microbial respiration with GC_{low} could suggest that changes in physical substrate properties by mechanical pretreatment indeed increased microbial activity. As the success of larval growth is intertwined with many microbial processes, larval could have benefited from this increased microbial activity, being one main reason for the observed increase in larval performance metrics. It was expected that microbial numbers and residue temperature would increase with higher microbial respiration, and that residue temperature was associated with microbial respiration due to the production of metabolic heat by the microbial metabolism as frequently observed in composting (Liang et al., 2003). However, residue TVC (see Supplementary Material) and temperature (Figure 4.3c) were similar between GC_{low} and untreated grass clippings. The observed residue temperature observed in grass clippings may have primarily come from the larvae, explaining the similar temperature between untreated and pretreated. As studies have shown that higher larval densities (e.g., >4 larvae/cm2) can elevate residue temperature (Li et al., 2023), future research should explore how different larval densities impact microbial activity.

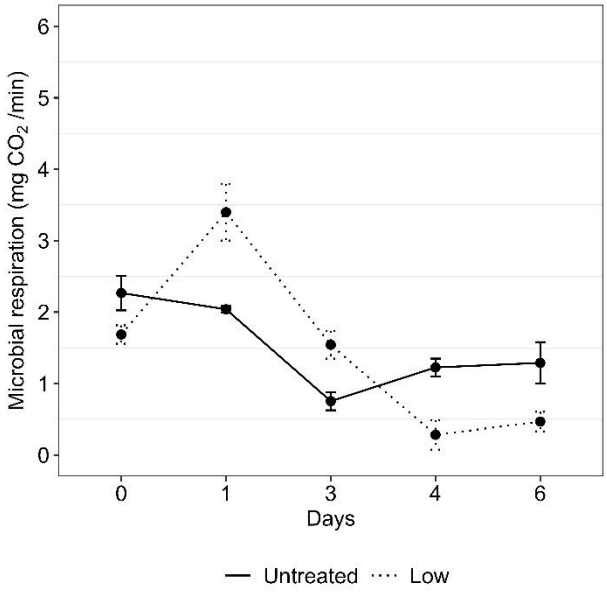
For spent grain, and opposite to our expectation, mechanical pretreatment of SG_{low} had initially lower microbial respiration (Figure 4.4b) compared to untreated on day 0 (0.6 mg CO_2/min vs. 1.6 mg CO_2/min) and day 1 (1.4 mg CO_2/min vs. 3.5 mg CO_2/min) On day 4 and particularly day 6, microbial respiration was higher in mechanically pretreated (4.5 mg CO_2/min) than the untreated substrate (2 mg CO_2/min). Spent grain is a high-moisture material that quickly self-ferments (Mussatto et al., 2006). The gradual decrease in microbial respiration for untreated spent grain could suggest that most easily accessible nutrients were likely consumed. In contrast, SG_{low} may have facilitated better nutrients availability for substrate microorganisms only by day 6 (Figure 4.4b). A limitation to this study is the absence of carbon mass balances, making it difficult to conclude on the influence of particle size reduction on nutrient utilization. Particle size reduction could impact the relative abundance of specific microorganisms (Vermeulen et al., 2018), influencing the overall biodegradation process and nutrient availability within the substrate. Residue TVC (see Supplementary Material) were similar between SG_{low} and untreated spent grain, but the residue temperature was consistently higher throughout the entire feeding experiment in the untreated spent grain (Figure 4.4c). The opposite trend observed regarding microbial respiration following mechanical pretreatment for spent grain could be due changes in physical properties limiting the provision of air for the microbial metabolism. As highlighted by the physical properties results, mechanical pretreatment drastically increases bulk density, making the material denser and thereby limiting the initial free air space or porosity available in SG_{low} compared to untreated. Consequently, mechanical pretreatment could also decrease microbial respiration and substrate temperature as observed here for spent grain (Figure 4.4b,c), which could explain the slightly lower larval weight observed up to rearing day 4 with SG_{low} (Figure 4.4a). However, it should be investigated

whether such a reduction in microbial respiration results in higher larval performance metrics in a longer feeding duration since in BSFL bioconversion microorganisms and BSFL can compete for nutrients. Therefore, it may be desired to minimize microbial respiration and maximize take-up by BSFL. To gain further insights into the interactions between biowaste microorganisms and BSFL, future research should continue to explore mass nutrient flows in relation to other physical properties including particle size, bulk density and free air space, which may play substantial roles in nutrient utilization.

(a) Grass clippings: Daily larval wet mass (mg)



(b) Grass clippings: Microbial respiration (mg CO_2/min)



(c) Grass clippings: residue temperature (°C)

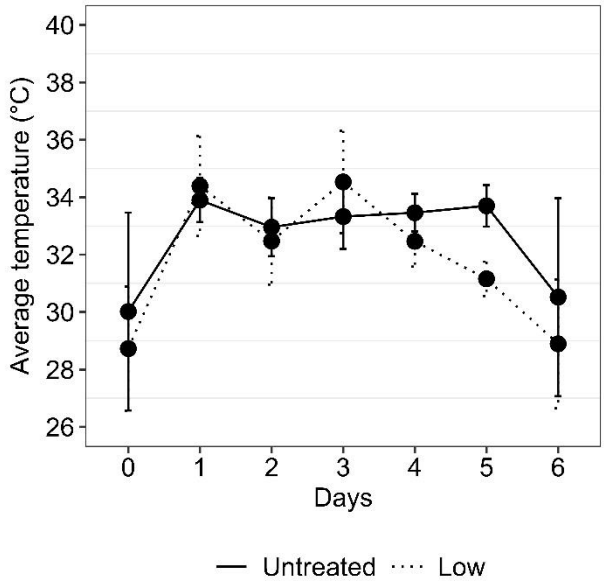
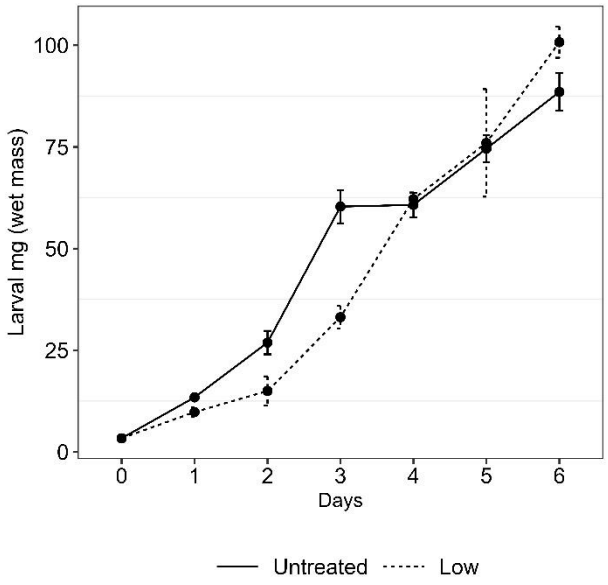
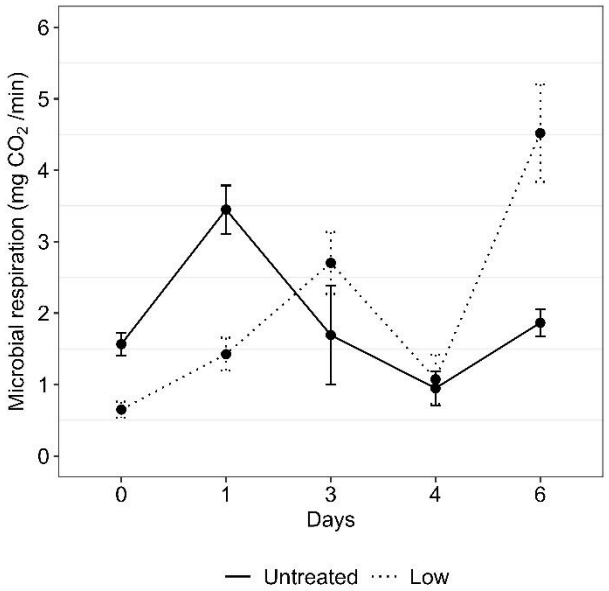


Figure 4.3. (a) Mean larval wet mass over the six-day feeding experiment; (b). Microbial respiration of grass clippings measured in mg CO2/min; (c) Substrate temperature during the larval feeding experiment. Displayed are mean with error bars displayed as standard deviation (n=3).

(a) Spent grain: Daily larval wet weight (mg)



(b) Spent grain: Microbial respiration (mg CO_2/min)



(c) Spent grain: residue temperature (°C)

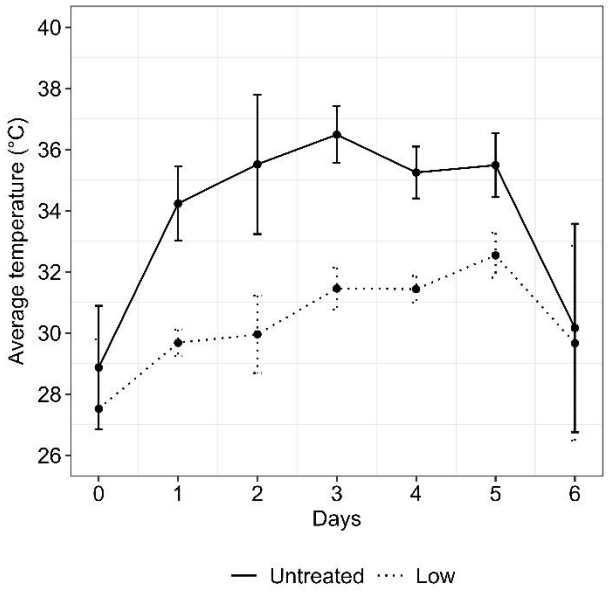


Figure 4.4. Influence of mechanical pretreatment (untreated vs. low particle size) on (a) mean larval wet mass over the six-day feeding experiment; (b) Microbial respiration of spent grain measured in mg CO2/min for days 0, 1, 3, 4 and 6; (c) Substrate temperature during the larval feeding experiment. Data displayed are mean with error bars displayed as standard deviation (n=3).

4.5. Conclusion

To conclude, the applied thermal pretreatment did not improve BSFL performance for the biowastes tested. Mechanical pretreatment for spent grain had varied effects showing increased larval performance in the smaller containers but no effect when using larger containers with shortened feeding duration. In contrast, grass clippings showed positive increase in larval performance and protein production after mechanical pretreatment in both instances. Future research should investigate the influence of larval density in relation to volume rather than surface area, as this could have affected the outcomes of mechanical pretreatment experiments. Additionally, further research should investigate physical properties and its relationship to biowaste microorganisms and BSFL, such as larval and microbial activity, microbial composition, metabolic heat and substrate temperature. Specifically, factors such as bulk density and free air space may provide valuable insights into the aeration requirements within biowastes and their impact on nutrient utilization.

5. Chapter: Product safety of whole dried insect products

Low energy electron beam to support safe whole dried insect products

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Prologue

The preceding chapters centered on the pre-processing of biowastes through pretreatments to enhance larval performance on fibrous wastes. Given that BSFL can grow on diverse biowastes contaminated with pathogens, ensuring the safety of the end-product is essential. However, even after thermal treatments used during post-processing, BSFL and yellow mealworm can still retain certain pathogens. The following chapter explores the use of a non-thermal treatment technology, low energy electron beam, to aid in the inactivation of microorganisms that may still be present.

5.1. Abstract

Product safety is a major concern when using edible insects and insect-derived products due to insects' diverse microbial community. Therefore, development of reliable post-processing treatments are required. Commonly used thermal treatments are effective against microorganisms but can have negative effects on product quality and nutritional value. Low-energy electron beam (LEEB) is an emerging non-thermal surface treatment technology for microbial decontamination of low water activity goods while preserving product quality. However, its potential application as an insect post-processing treatment has not been explored. To assess the effectiveness of LEEB treatment (250 keV and 12 kGy), three separate experiments were conducted with dried black soldier fly larvae (BSFL) and yellow mealworm (YMW). First, to assess LEEB's potential in inactivating microorganisms in insect products, LEEB treatment was conducted on dried BSFL inoculated with *Escherichia coli* K-12. Secondly, the effect of LEEB treatment on reducing naturally occurring microbial populations after microwave drying was evaluated. Finally, a six-month controlled shelf-life study (24°C, 65% RH) was conducted to assess the long-term efficacy of LEEB treatment by monitoring physical, chemical and microbiological parameters. LEEB achieved a 4-$\log_{10}$ reduction of inoculated *E. coli* K-12 on dried BSFL and was effective in reducing numbers of all microbiological parameters (aerobic and anaerobic counts) in YMW. Specifically, in non-inoculated samples, aerobic and anaerobic total viable counts (TVC) were reduced by approximately 4-$\log_{10}$ colony forming units per gram (cfu/g) in YMW. In contrast, LEEB treatment moderately reduced microbial numbers in BSFL, with aerobic and anaerobic TVC reduced by approximately 1–2-$\log_{10}$ cfu/g following LEEB treatment. Microbial counts in both BSFL and YMW remained lower than the control throughout the shelf-life. LEEB treatment did not have an influence on the peroxide value. Therefore, LEEB can be an effective and gentle processing technique to support safe dried insect products.

5.2. Introduction

Edible insects and insect-derived products have gained increased attention in recent years as potential alternatives to traditional protein sources due to their nutritionally dense profiles and potentially lower environmental impacts (Smetana et al., 2019; Spykman et al., 2021). Two of the most promising insects for food and feed are the larvae of the yellow mealworm (YMW), *Tenebrio molitor* L., and black soldier fly (BSFL), *Hermetia illucens* L. Due to their high protein content of 51−62 % (dry mass, DM) for YMW (Finke, 2002; Zhao et al., 2016) and 30−50% DM for BSFL (Gold *et al.*, 2018; Wang and Shelomi, 2017), whole insects or insect-derived products are a promising protein source for human food (Bessa et al., 2020; Zhao et al., 2016) or feed for pets (Bosch and Swanson, 2021), livestock (e.g., poultry and pigs) (De Marco et al., 2015; Hong et al., 2020) and different fish in aquaculture (Gasco et al., 2023).

YMWs and BSFL have a rich and diverse microbial community including fungi and bacteria. Freshly harvested BSFL and YMW can have total viable counts (TVCs) ranging from 8−10 $\log_{10}$ colony forming unit (cfu)/gram (Mancini et al., 2019; Raimondi et al., 2020; Stoops et al., 2016; Wynants et al., 2019; Zhen et al., 2020). These microbial communities can include heat-resistant spores and foodborne pathogens, such as *Staphylococcus aureus*, *Bacillus cereus, Clostridium perfringens, Escherichia coli, Salmonella* spp., *Listeria* spp. and mycotoxin-producing fungi (Kashiri et al., 2018; Raimondi et al., 2020; Vandeweyer et al., 2021, 2020; Wynants et al., 2019). Microbial communities in the intestinal tract of BSFL and YMW are highly variable depending on the diet they were fed as well as rearing conditions (Bruno et al., 2019b; Wynants et al., 2019). Microbiologically contaminated insects fed to livestock or consumed directly by humans can lead to foodborne illnesses (Heredia and García, 2018). Spore-forming bacteria, such as *B. cereus* and *C. perfringens* are pathogenic and highly resistant causing foodborne diseases (Delbrück et al., 2021). For example, globally, *B. cereus* represents 1–12% of cases for foodborne illnesses (Grutsch et al., 2018). To ensure prevention of such outbreaks from insect-based products, post processing steps are required.

Blanching, boiling and drying are common thermal treatments to reduce the microbial load of insects (Saucier et al., 2022; Vandeweyer et al., 2017). Although thermal treatments are effective in microbial inactivation, specific pathogens such as *Salmonella* spp. and bacterial spores are heat resistant and can remain in heat treated products (Lang et al., 2016; Zhang et al., 2018). For instance, Vandeweyer *et al.* (2017) observed 1.3−1.9 $\log_{10}$ cfu/g aerobic bacterial spores in dried YMW after blanching (100°C, 40 s) and microwave drying (maximum 80°C, 8−20 min). Multiple studies have found pathogens such as *Salmonella* spp., *E. coli* (Kashiri et al., 2018), and *B. cereus* in BSFL and YMW after drying (Grabowski and Klein, 2017; Wynants et al., 2019). Microbial inactivation and thereby product safety could be increased

by longer drying times and/or higher drying temperatures. However, this is typically associated with reductions in nutritional value, affecting product quality. Longer drying times can result in discoloration of the product which can be an indicator for reduction of vitamin and mineral contents (Ratti, 2001) and induce lipid oxidation (Larouche et al., 2019). Alternative treatments such as high hydrostatic pressures (Kashiri et al., 2018; Larouche et al., 2019), and cold plasma (Rumpold et al., 2014) have been investigated but were not effective in reducing TVCs. Therefore, it is important to identify effective treatments that can reduce microbial loads without compromising product quality (Berk, 2013).

Electron-beam, a non-thermal treatment, could be a promising technology for the decontamination of edible insects and insect-based products. The key advantage over thermal treatments is its potential to reduce microbial numbers without reducing product quality or addition of chemicals, in addition to potentially further extending the shelf-life. Electron beam has been used for various food products such as dry spices and herbs, meat and seafood or fresh produce to reduce the presence of pathogens (Clemmons et al., 2015). Similar to gamma-rays and x-rays, the main inactivation mechanism for electron beam is the damage to the microbial DNA (Hertwig et al., 2018; Moeller et al., 2008). Inactivation occurs mainly through direct interactions of electrons with microbial DNA, RNA, enzymes and membrane molecules (Hertwig et al., 2018) or indirect interactions due to the formation of free radicals (Tahergorabi et al., 2012). The penetration depth of the electron beam treatment is dependent on the kinetic energy of the electrons and the density of the treated material (Helt-Hansen et al., 2010). Electron beam can be classified into two categories based on the kinetic energy of electrons. High energy electron beam (HEEB) has a kinetic energy of > 300 kiloelectronvolts (keV) and can penetrate products up to 8–10 centimetres (i.e., density 1 g/cm^3). Low energy electron beam (LEEB) has a kinetic energy of < 300 keV resulting in a lower penetration depth of micrometres, leading mainly to surface decontamination of the product (Hertwig et al., 2018).

An advantage of LEEB compared to other ionising radiation treatments such as HEEB and gamma-ray is the lower energy produced from x-rays. This allows for compact radiation protection, reduces potential health risks and saves physical space (Schopf et al., 2022), making it feasible for use in a continuous process and integration within an existing production pipeline. Although industrial application of LEEB remains relatively new within the food industry, it has shown potential for treatment of herbs and spices (Murdoch et al., 2022). Over 50 countries have approved the use of irradiated foods, particularly in the United States (U.S.) and certain parts of Asia (Kume et al., 2009). However, maximum treatment dose measured in kiloGray (kGy) is country, product, and region specific. For example, for herbs and spices, the maximum dose in the U.S. is 30 kGy vs. 10 kGy in the European Union (EU, CFR, 2023; European Commission (EC), 2017). In regions of the world where irradiated foods are gaining increased acceptance, LEEB could be a suitable option for ensuring the microbiological safety of whole dried insect products.

The use of LEEB has yet to be investigated as a potential post treatment method for insects. Therefore, the objective of this study was to assess the efficacy of LEEB for microbial decontamination for whole dried insects, specifically BSFL and YMW, in an industrial application. This research aimed to contribute to the understanding of this technology's potential use for the insect industry in addition to providing more knowledge on using an industrial-scale LEEB unit and its effects on potentially extending the shelf-life. Thereby, this research is working towards improving the food safety of insects and insect-derived products by LEEB treatment.

5.3. Material and Methods

5.3.1. Experimental overview

The microbial treatment efficacy of the industrial LEEB unit for whole dried insect products was evaluated in three independent experiments. In experiment 1 (see section "Experiment 1"), the inactivation efficacy of LEEB treatment was evaluated on decontaminated dried whole BSFL inoculated with *E. coli* K-12, a common non-pathogenic surrogate for foodborne pathogens. Following, in experiment 2 (see section "Experiment 2"), the inactivation efficacy of LEEB treatment was evaluated on naturally contaminated (i.e., non-inoculated) microwave dried whole BSFL and YMW. In experiment 3 (see section "Experiment 3"), this was repeated with oven-dried BSFL and YMW and complemented by a six-month shelf-life test comparing control (i.e., without LEEB treatment) and LEEB-treated insects.

5.3.2. Source of insects

To mimic real-life conditions, YMW and BSFL were sourced from commercial European insect producers (Table 5.1). The insects were processed before LEEB treatment according to the commercial procedures of the insect companies (see Supplementary Material). Processing consisted of blanching and drying with different temperature and time combinations and two drying methods (i.e., oven drying and microwave drying) (Table 5.1). All insects were stored at 4 °C until use.

Table 5.1. Thermal treatment conditions of BSFL and YMW used in experiments 1–3.

Experiment	Drying method	BSFL	YMW	Biological replicates used (n=)	Source
1	Oven	Blanched: 1-2 min, 95 – 97 °C Dried: 16 h, 70 °C.	-	3	Hermetia Baruth GmbH, Germany
2	Microwave	Dried: 20 min, max. 80°C	Dried: 20 min, max. 80 °C	2	Inagro, Belgium and microwave dried by MEAM, Belgium
3	Oven	Blanched: 2 min, 75-85 °C Dried: 27 h at 80 °C	Blanched: 3 min, 95 °C Dried: 3.5 h at 90 °C	3	BSFL: Hermetia Baruth GmbH, Germany YMW: Essento, Switzerland

5.3.3. Industrial-scale LEEB unit

Whole dried insects were treated with an industrial LEEB unit (Laatu™, Bühler AG, Uzwil, Switzerland). The unit was designed for the treatment of low moisture foods (i.e., < 12% moisture content) and can continuously process up to 1,000 kg/h. However, the processing capacity was not tested in this study as the quantities used in the experiments were significantly below the commercial throughput. A schematic of the LEEB is shown in Figure 1 and was previously described in detail by Murdoch *et al.* (2022). Briefly, the product to be treated is placed in a loose form into the inlet hopper (Figure 5.1a). From the inlet hopper, the product to be treated falls onto shaking feeders, which aim to evenly distribute the product through the following treatment zone. The treatment zone contains two electron beam lamps (Figure 5.1b) which treats the product within a few milliseconds as it falls from the feeders to the outlet beyond the treatment zone, exiting the LEEB.

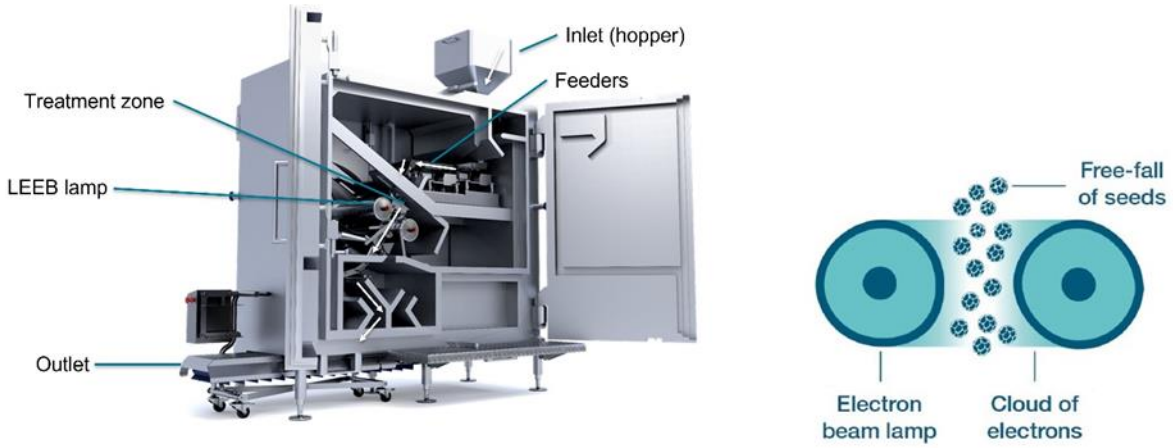


Figure 5.1. (a) Illustration of industrial scale LEEB unit and (b) schematic of the treatment zone consisting of two electron beam lamps treating the product with electrons as it passes from the feeders to the outlet (source: Bühler AG, Uzwil, Switzerland).

5.3.4. Dosimetry for LEEB treatment

The LEEB processor was characterized following ISO (2020) with radiochromic dosimetry film (B3 film, GEX Corp., Palm City, Florida, U.S.). During each experiment, routine dosimetry was performed to ensure that the beam parameters were within an expected range of performance established during beam characterization. The expected range was set at ±10 %, as the overall uncertainty (k=2) was determined at 10.6 % during B3 system calibration. To estimate the dose on the whole dried insects, cylinders (20 mm length, 4 mm width) were wrapped with B3 film and irradiated at the same beam parameters. High-speed video recording of the product flow confirmed that the cylinders and both dried BSFL and YMW had the same velocity within the treatment zone, resulting in the same treatment time within ± 4 %. The difference in velocity was not considered for measurement uncertainty, as it was less than the overall uncertainty of the B3 film during calibration. The LEEB treatment parameters were set at 250 keV at 18 mA with all other machine parameters (e.g., air flows) kept constant and ran at ambient temperature (approximately 24 °C). The resulting surface dose on the cylinders (Dμ) was 12 ± 2 kGy for 250 keV and 18 mA. These LEEB treatment parameters were used for all three experiments.

5.3.5. Estimating LEEB penetration depth of insects

The electron beams were characterized by conducting depth-dose distribution measurements on B3 film strips (18 μm thick) which were stacked in 30 layers and LEEB-treated at 250 keV and done according to ISO (2020). The LEEB depth-dose correlation was then determined by measuring the dose on each B3 film layer. The depth-dose was then calculated using the apparent density (i.e., 1.12 g/cm^3) of the B3 film. To roughly estimate the LEEB penetration depth of the insects, the apparent density (g/cm^3) was measured using an envelope and density analyser (GeoPyc 1360, Micromeritics, U.S.). The density measurement

procedure involved determining the weight of the sample, followed by placing it in a bed of DryFlo® granular medium within a cylindrical chamber. The granular medium was then carefully consolidated around the sample using a piston, that was gradually pushed into the rotating cylindrical chamber until the consolidation force (28 Newtons) was reached (Siavashani et al., 2020). This method allows for determining the density of the insect samples, including the air within the pores of the insects but excluding the air between insects. Once the insect density was obtained, a correction factor was calculated by dividing the density of the B3 film strips by the density of the insects (equation 1 shown in Supplementary Material). The depth at which 50% of the dose penetrated the B3 film was 289 μm. To estimate the depth at which 50% of the LEEB dose could have penetrated the insects, the correction factor was multiplied by 289 μm (equation 2 shown in Supplementary Material). It is important to note, these are simplified estimates to give a general indication of the potential depth-dose distribution as there is currently no direct way to measure depth-dose in insects. To give an understanding of the depth-dose in relation to how deep it could have penetrated the insects, the thickness (mm) and length (mm) of 10 insects was measured using a caliper and ruler, respectively. The thickness and length were both important physical properties to measure as the beam could have targeted the insect horizontally or vertically depending on its position during the fall through the treatment zone.

5.3.6. Experiment 1: Inactivation of a single foodborne pathogen indicator organism by LEEB

Since LEEB was not yet used for insects, the first experiment aimed to assess the inactivation efficacy of LEEB on a single microbial indicator organism on an insect product. *Escherichia coli* K-12 strain MG 1655 obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) was chosen as the model organism as it is a non-pathogenic strain and has a high radiation resistance making it a suitable surrogate for common foodborne pathogens such as *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. (Rodriguez et al., 2006). Whole dried BSFL were intensely decontaminated by HEEB treatment (10 MeV with 27 kGy, Leoni AG, Daniken, Switzerland). Decontamination was confirmed by enumeration of TVCs (detection limit: 2-$\log_{10}$ cfu TVC/g dried BSFL). Decontaminated BSFL were then inoculated with approximately 8-$\log_{10}$ cfu *E. coli* K-12/g dried BSFL as described below. The inactivation efficacy was estimated by enumerating *E. coli* K-12 on Lysogeny broth (LB) agar plates (37 °C, 24 h) before and after LEEB treatment.

Additionally, HEEB treatment was used to determine the D_{10} value, an estimate of radiation resistance for the *E. coli* K-12 strain, to assess whether it was indeed a suitable model organism for resident bacteria. The D_{10}-value is the radiation dose required for one $\log_{10}$ reduction of microorganisms at a given energy level

and is calculated from the negative inverse of the slope of a semi-logarithmic microbial inactivation curve (Blank and Corrigan, 1995). Whole dried BSFL were intensely decontaminated by HEEB treatment and then inoculated with approximately 8-$\log_{10}$ cfu *E. coli* K-12/g dried BSFL as described below. The whole dried BSFL were packaged in heat-sealed sterile plastic bags, which were then positioned flat on a tray. The insects were spread out in a single layer within the bags and HEEB treated at 0, 1, 3, 5 and 10 kGy. Following HEEB treatment, the number of *E. coli* K-12 was enumerated.

Escherichia coli K-12 was cultivated from a –80 °C glycerol stock and first grown in 10 ml of LB-broth (Sigma-Aldrich, Buch, Switzerland) in an incubating shaker for 24 hours at 37 °C at 180 revolutions per minute (rpm). Subsequently, 0.1 ml of the previous culture was propagated into 10 ml of fresh LB-broth and again incubated for another 24 hours at 37 °C and 180 rpm. After the second 24 hours, 1 ml of the culture was propagated into a sterile flask with 500 ml of LB broth and incubated overnight. To ensure no contamination, a control served alongside with 10 ml of LB-broth.

Before inoculation, 50 ml of the overnight *E. coli* K-12 culture (approximately 10^8 cfu/ml) was aseptically dispensed into ten 50 ml centrifuge tubes and centrifuged at 7.280 x g for 15 minutes at 4 °C. Following this, 49.5 ml of the supernatant was discarded. The remaining 0.5 ml of the cell suspension was vortexed. Then, 50 droplets of 100 µl of the cell suspension were inoculated onto 100 grams of sterilised whole dried BSFL, which were placed in a sterile aluminium tray and mixed using a sterile spoon. The inoculated BSFL was stored to dry under a biosafety bench at ambient temperature for approximately six hours, until the initial moisture content was reached. After inoculation, the dried BSFL had 8-$\log_{10}$ cfu *E. coli* K-12/g dried BSFL.

5.3.7. Experiment 2: Inactivation of naturally contaminated BSFL and YMW by LEEB

The second experiment assessed the efficacy of LEEB treatment on naturally contaminated whole dried BSFL and YMW after gentle microwave drying. 150 grams of dried insects were either LEEB-treated in duplicates or handled in parallel as an untreated control (i.e., without LEEB treatment).

LEEB-treated samples and controls were immediately taken to the laboratory for physical, chemical and microbiological analyses. Physicochemical analyses included moisture content and water activity of pulverised insect samples. Moisture content was calculated from the difference in weight of three grams of insect sample after overnight oven drying at 105 °C. Water activity was determined with a water activity meter at 25 °C (LabMaster a_w, Novasina, Lachen, Switzerland). Chemical analysis included primary lipid oxidation estimated with the peroxide value by an external laboratory (Eurofins, Schonenwerd, Switzerland) (AOCS, 2009).

Aerobic and anaerobic microbial numbers were enumerated on insect samples by plate counts including aerobic and anaerobic TVC, aerobic and anaerobic bacterial spores as well as yeast and moulds. For all microbial analyses, samples were plated the same day. A 60-gram insect sample was pulverised in sterilised beakers with a hand blender for one minute (Stoops et al., 2016). Then, a five-gram subsample was transferred into a sterile stomacher bag and mixed with 45 ml of sterile maximum recovery diluent (0.85% (w/v) NaCl, 0.1% (w/v) peptone; Sigma-Aldrich) and homogenized for one minute with a stomacher and then ten-fold serially diluted and plated on agar plates (Stoops et al., 2016). Aerobic TVC were enumerated on nutrient agar (30 °C, 48 h), yeasts and moulds on potato glucose and lactic acid agar (30 °C, 120 h). Aerobic bacterial spores were enumerated following incubation on nutrient agar (30 °C, 48 h) after heat shocking 2 ml of the pulverised sample mixed with max recovery diluent (80 °C, 5 min) in a water bath according to Klunder *et al.* (2012). Anaerobic TVC and anaerobic bacterial spore count (30°C, 72 h) was enumerated according to ISO (2013) in anaerobic conditions at the same external laboratory (Eurofins). The detection limit for all aerobic microbial counts was 2-$\log_{10}$ cfu/g dried insect and 1-$\log_{10}$ cfu/g dried insect for all anaerobic counts. The detailed composition of all media is included in the Supplementary Material.

5.3.8. Experiment 3: Shelf-life of LEEB-treated insect products

Experiment 3 was completed in the same manner as experiment 2 but with 500 g insects and conducted in triplicates. Following LEEB treatment, control and LEEB-treated samples were analysed for physical, microbiological, and chemical parameters (as described above). Control and LEEB-treated samples were packaged in airtight bags and stored in a dark climate-controlled chamber for a six-month shelf-life test (at 24 °C, relative humidity: 65%). Every month, insects were sampled by removing three bags from both the treatment and control. These samples were analysed for physical, and microbiological parameters as described above in triplicate. In addition, chemical analyses included primary and secondary lipid oxidation estimated with the peroxide and p-anisidine value, respectively, by the same external laboratory (Eurofins) according to AOCS (2009) and were performed for BSFL and YMW at the start of the experiment and after one, three and six months of storage. Chemical analysis for BSFL was conducted in triplicate. Since chemical analysis for YMW was only conducted for one single biological replicate per time point, the results can be found in the Supplementary Material.

5.3.9. Data Analyses

Data was analysed using R in RStudio (R Core Team 2022, version 4.2.0, United States). We abstained from statistical analyses due to the small sample size ($n \leq 3$). The results were compared using mean and standard deviation ($n = 3$).

5.4. Results and Discussion

5.4.1. LEEB penetration depth of dried insect products

Based on the measured densities of the dried insect products (see Supplementary Material), it was estimated that 50% of the LEEB dose could penetrate between 400 (oven dried) and 1000 μm (microwave) for BSFL and between 500 (oven dried) and 800 μm (microwave) for YMW (see Supplementary Material). This confirms that LEEB is mainly a surface decontamination technology. Aisala *et al.* (2021) reported the penetration depth for pumpkin seeds ranged from 295–340 μm (density 1.06–1.22 g/cm^3), while for flax seeds, it was found to be between 330–360 μm (density 1.0–1.1g/cm^3). Additionally, Gryczka *et al.* (2021) reported LEEB could penetrate the outer layer of pepper grains to 160–380 μm (density 0.9 g/cm^3). These results align with this study's findings, considering the product densities and the range of electron beam energy used (200–300 keV). In general, with the increasing density of the material, the penetration depth decreases (Ghomi et al., 2005). Given that BSFL and YMW are around 2 mm in length and 2–4 mm in thickness, the majority of the LEEB dose did not likely penetrate into the larval digestive tract that includes most microorganisms (Bruno et al., 2019b) but only penetrated up to a certain layer. Further research should investigate whether LEEB can penetrate the larval exoskeleton and penetrate the digestive tract to contribute to a more comprehensive understanding of the depth-dose distribution of LEEB on edible insects. A previous study with peppercorns highlighted that the ability of LEEB to penetrate different materials is highly variable, with LEEB penetrating the thickness of the external layer of the white peppercorn but not the black peppercorn (Gryczka et al., 2021). This may also be the case for edible insects. Interpretation of the LEEB penetration depth requires caution due to the many influencing factors, particularly for insects, such as, size, shape, density and other surface properties (Gryczka et al., 2021). Further research should explore the best approach for understanding LEEB dosimetry of different products as there are still limitations to LEEB depth-dose distribution measurements.

5.4.2. Experiment 1: Inactivation of single foodborne pathogen indicator organism by LEEB

This is the first study to assess the efficacy of non-thermal LEEB treatment on inactivating microbial numbers in whole insect products. Our first experiment assessed the efficacy of LEEB treatment on a single bacterium, *E. coli* K-12. Before LEEB treatment, the radiation resistance of *E. coli* K-12 was quantified by an inactivation curve from HEEB treatment (shown in Supplementary Material). Based on the inactivation curve, the D_{10} of *E. coli* K-12 was 0.90 kGy (r^2= 0.99), which is consistent with the previously reported value of 0.88 kGy by Rodriguez *et al.* (2006). These findings are also similar to the reported D_{10} of *E. coli* K-12 for mung bean, clover, and fenugreek seeds, which were 1.11, 1.21, 1.4 kGy, respectively (Fan et al., 2017). In contrast, on fresh-cut cabbage, the D_{10} value was calculated at 0.56 kGy (Grasso et al., 2011),

while on blueberries it was even lower at 0.37 kGy (Kong et al., 2014). This difference in D_{10} value highlights how different materials, such as fresh produce versus low water activity goods, can influence the radiation resistance of the microorganisms. This suggests, that dried BSFL may have a more similar radiation resistance to low water activity goods, such as seeds, herbs and spices. Given the high radiation resistance of *E. coli* K-12 inoculated on dried BSFL, this strain was used in the following LEEB inactivation trial as a good surrogate for common foodborne pathogens, such as *Salmonella* or *E. coli*.

LEEB (250 keV, 12 ± 2 kGy) treatment of *E. coli* K-12 inoculated at a concentration of approximately 8-$\log_{10}$ cfu/g whole dried BSFL led to a 4-$\log_{10}$ reduction (shown in Supplementary Material). The initial high level of *E. coli* K-12 inoculated on the dried BSFL, suggests that dried BSFL have a rough surface similar to red clover and fenugreek seeds (Fan et al., 2017). Surface morphology and size of the material can influence initial inoculation levels, as seeds of mung beans had lower inoculated *E. coli* K-12 concentrations of around 6-$\log_{10}$ cfu/g due to their smooth surface and larger size (Fan et al., 2017). Although a high reduction of *E. coli* K-12 was observed on inoculated dried BSFL, complete inactivation was not achieved. These findings are similar to Fan *et al.* (2017) who reported complete inactivation of *E. coli* K-12 was not achieved at doses of 4–12 kGy and 200 keV for fenugreek, mung bean and red clovers seeds. The author attributed this to a potential tailing effect, where bacterial inactivation does not follow a linear pattern but levels off at higher doses. The tailing effect has been observed for *E. coli* O157:H7 subjected to HEEB on lower water activity foods (Black and Jaczynski, 2008). However, LEEB is not intended as a sterilization technology, but rather a surface decontamination technology.

Therefore, given the effectiveness of LEEB treatment in reducing *E. coli* K-12, this suggests LEEB could be an effective method in reducing bacterial contamination on BSFL. Previous studies have shown that dried BSFL could still have *E. coli* concentrations ranging from 3–6-$\log_{10}$ (Kashiri et al., 2018; Saucier et al., 2022), while Kashiri *et al.* (2018) found *Salmonella* at 6-$\log_{10}$. Regulations on microbiological safety of insect-based products intended for animal feed state that *Salmonella* should be absent in 25 grams in the final product (European Commission (EC), 2011). Additionally, IPIFF (2019) recommends 1-$\log_{10}$ cfu/g of *E. coli* as the targeted limit. Therefore, LEEB could be effective in achieving the regulations and targeted limit for *Salmonella*, and *E. coli*, because of their lower radiation resistance compared to *E. coli* K-12. However, to confirm this, additional LEEB tests should be conducted with the pathogen inoculated at similar concentrations that have been previously identified in BSFL (Kashiri et al., 2018; Saucier et al., 2022). Based on the results, the same LEEB process parameters were used for experiments two and three.

5.4.3. Experiment 2: Inactivation of naturally contaminated BSFL and YMW by LEEB

BSFL

Initial microwave dried BSFL had a moisture content of 6% and water activity below 0.6 (see Supplementary Material) and therefore microbial growth was unlikely to occur (Bonazzi and Dumoulin, 2011). As expected, LEEB treatment had no influence on the water activity and moisture content, as all trials were conducted at ambient temperature. Microwave dried BSFL had no detectable anaerobic TVC, anaerobic bacterial spores, and yeast and moulds. These results are to be expected, as the heat generated from microwave drying primarily achieves microbial inactivation by protein denaturation within the cells, independent of the cell wall structure (Alp and Bulantekin, 2021; Woo et al., 2000). However, aerobic TVC and bacterial spores were 5.5 and 5 $\log_{10}$ cfu/g dried BSFL, respectively, suggesting that a large portion of TVC were bacterial spores (Table 5.2). TVC were consistent with previous studies that reported concentrations ranging from 5–5.5 $\log_{10}$ cfu/g after boiling and drying (Campbell et al., 2020; Saucier et al., 2022) but lower than the 7-$\log_{10}$ cfu/g dried BSFL reported by Kashiri *et al.* (2018). Such findings are to be expected as bacterial spores exhibit high heat resistance. Several bacteria belonging to the orders Bacillales and Clostridiales can survive adverse environmental conditions by forming spores (Delbrück et al., 2021). Within this range (5–8 $\log_{10}$ cfu/g) foodborne diseases can be caused by *B. cereus* (EFSA, 2005), thus indicating the need to ensure these concentrations are reduced.

LEEB treatment reduced aerobic TVC and bacterial spores by 2-$\log_{10}$ (Table 5.4). This demonstrates the efficacy of LEEB treatment in reducing microbial numbers in low water activity materials. However, the treatment was unable to reduce microbial numbers below the detection limit. Because LEEB treatment is likely most effective on or just below the surface, this could be due to the microorganisms in the larval digestive tract that were not affected by LEEB due to the limited penetration depth of the treatment. Additionally, different bacterial spores have varying levels of resistance to irradiation (Zhang et al., 2018). For example, the spore-forming pathogen *B. cereus* was found to be more resistant than *B. subtilis* (De Lara et al., 2002). Although the bacterial spores were not identified in this study, *B. cereus* was previously detected in BSFL and could have been present (Wynants et al., 2019). Further research should investigate whether varying LEEB process parameters can lead to complete inactivation of bacteria in BSFL.

The fat-rich BSFL (15–39% DM) (Gold et al., 2018) were also evaluated for primary lipid oxidation (Table 5.2). This is because lipid oxidation can reduce nutritional value, cause rancidity, and potentially pose human health risks, and is a crucial parameter when evaluating product quality (Johnson and Decker, 2015). Before LEEB treatment, BSFL had approximately 8 milliequivalents of active oxygen/kg fat (meq./kg fat), which is consistent with previous studies reporting peroxide values between 3–11 meq./kg fat (Hurtado-

Ribeira et al., 2023; Kathumbi et al., 2022; Tome et al., 2021). Interestingly, LEEB treatment did not appear to influence peroxide value, which could be attributed to the short treatment time. LEEB treatment has the potential to promote lipid oxidation through production of free radicals and/or reactive oxygen species reacting with unsaturated fatty acids (Barden and Decker, 2016). However, no lipid oxidation in BSFL after LEEB treatment may be due to the higher concentration of saturated fatty acids compared to unsaturated fatty acids in BSFL. The fatty acid profile of BSFL is highly variable but typically has 45−75% saturated fatty acids and 7–32% mono- and polyunsaturated fatty acids (Ewald et al., 2020; Hurtado-Ribeira et al., 2023; Kroeckel et al., 2012), with the latter being more susceptible to oxidation. In case of edible oils, the fatty acid composition is considered a strong predictor of oxidation stability, particularly at the early oxidation stage (Yun and Surh, 2012). The fatty acid profiles of BSFL can differ depending on the rearing substrate used, thus potentially influencing the oxidation stability (Ewald et al., 2020). Therefore, the effect of LEEB treatment on lipid oxidation needs to be further evaluated individually for different rearing substrates as various factors such as, fatty acid profile, the product type, presence of antioxidants and storage conditions can affect it (Aisala et al., 2021). Overall, the peroxide value for BSFL was below a threshold peroxide value established for animal fats (max 10 meq/kg) for human consumption (FAO and WHO, 2001) but was above the threshold peroxide value for fish oils (5 meq.kg/fat) (FAO and WHO, 2017). However, since the microwave dried BSFL had a peroxide value on the higher end compared to previous studies, this could mean the product would oxidize more quickly. As the peroxide value is affected by various factors it could be further reduced by optimizing killing and BSFL post-processing as well as defatting methods (Hurtado-Ribeira et al., 2023; Larouche et al., 2019; Zhen et al., 2020).

YMW

Similar to BSFL, YMW had a moisture content of 6% and water activity below 0.6 (see Supplementary Material) which saw no effect from LEEB treatment. Before LEEB treatment, aerobic and anaerobic bacterial spores, and yeast and moulds were not detected in dried YMW. YMW had 4-$\log_{10}$ aerobic TVC and 3-$\log_{10}$ anaerobic TVC cfu/g dried YMW (Table 5.2). Aerobic TVC were four-fold higher compared to those reported by Vandeweyer *et al.* (2017) who reported 1-$\log_{10}$ cfu/g blanched and microwave dried YMW (20 min). The difference may be attributed to the effect of blanching, which has been shown to reduce microbial counts compared to processing without blanching. Interestingly, aerobic and anaerobic TVC were similar to one another in this study which is consistent with a previous study by Vandeweyer *et al.* (2020), where aerobic and anaerobic TVC for YMW were found to be comparable. This suggests that a substantial portion of TVC in YMW consists of facultative species, which are capable of thriving under both aerobic and anaerobic conditions. More specifically, *Bacillus* spp. are known to be facultatively anaerobic (Sperber and Doyle, 2009). While bacterial spores were not detected in this study, this could be a result of the

relatively high detection limit (i.e., 2-$\log_{10}$ cfu/g dried YMW). For example, Vandeweyer *et al.* (2017) reported bacterial spore counts ranging from 1.3−1.9 $\log_{10}$ cfu/g dried YMW which are below the detection limit of this study. Additionally bacterial spores can be quite variable between rearing batches. LEEB treatment reduced aerobic and anaerobic TVC below the detection limit (Table 5.2) indicating that LEEB treatment led to a 2-$\log_{10}$ reduction for aerobic and anaerobic TVC in low water activity YMW after drying (Table 5.4).

Similar to BSFL, YMW are typically rich in fat (30−40 % DM) and were analysed for their peroxide value before and after LEEB treatment (Dreassi et al., 2017; Ravzanaadii et al., 2012; Son et al., 2020). Similar to BSFL, LEEB appeared not to affect the peroxide value for YMW. The peroxide value of YMW before and after LEEB treatment was 2.2 and 2.6 meq./kg fat (Table 5.2), respectively. This is similar to 1.6–3.5 meq./kg fat reported previously (Lenaerts et al., 2018; Son et al., 2020). In general, the high unsaturated fatty acid content of YMW (e.g., 66−77%) could increase susceptibility of fat oxidation. Additionally, the treatment process can have a high influence on the oxidative stability of the product, with freeze-dried YMW having higher peroxide values of 19−125 meq./kg fat (Jeon et al., 2016; Lenaerts et al., 2018). However, fat oxidation may also be partially inhibited by natural antioxidants such as vitamin E. YMW contain approximately 144 mg/kg fat of tocopherol, a form of vitamin E (Son et al., 2020). Such antioxidants can prevent free radicals from attacking fatty acids causing oxidation (Barden and Decker, 2016). Further studies are required to fully understand the effect of LEEB treatment on fatty acid composition, antioxidant profiles and sensory attributes since YMW are often intended for human consumption. Overall the peroxide values for YMW in this study fell well below the limits established for animals fats (10 meq./kg fat) as well as fish oils (5 meq.kg/fat) (FAO and WHO, 2017).

Table 5.2. Microbial counts and primary oxidation (peroxide value) of microwave dried whole BSFL and YMW before and after LEEB treatment. Data displayed are mean values and range (n=2).

Insect	Treatment	Primary lipid oxidation	Microbial counts (log_{10} cfu/g dried insect)				
		Peroxide value meq./kg fat	Aerobic TVC	Anaer. TVC	Bacterial Spores	Anaer. bacterial spores	Yeast & Moulds
MW dried BSFL	Control	8.1 ± 0.2	5.5± 0.6	<1	4.8 ± 0.2	<1	<2
	LEEB-treated	8.8 ± 1.7	3.5 ± 0.1	<1	3.2 ± 0.2	<1	<2
MW dried YMW	Control	2.2 ± 0.3	3.7 ± 0.1	2.8 ± 0.5	<2	<1	<2
	LEEB-treated	2.6 ± 0.2	<2	<1	<2	<1	<2

[1]MW: microwave dried; BSFL: Black soldier fly larvae; YMW: yellow mealworm; LEEB: Low energy electron beam; TVC: Total viable counts; Anaer.: anaerobic

5.4.4. Experiment 3: Shelf-life of LEEB-treated insect products

BSFL

Initial water activity before and after LEEB treatment was around 0.3 and moisture content was below 3% (see Supplementary Material). Similar to experiment 2, LEEB treatment did not influence these physicochemical parameters.

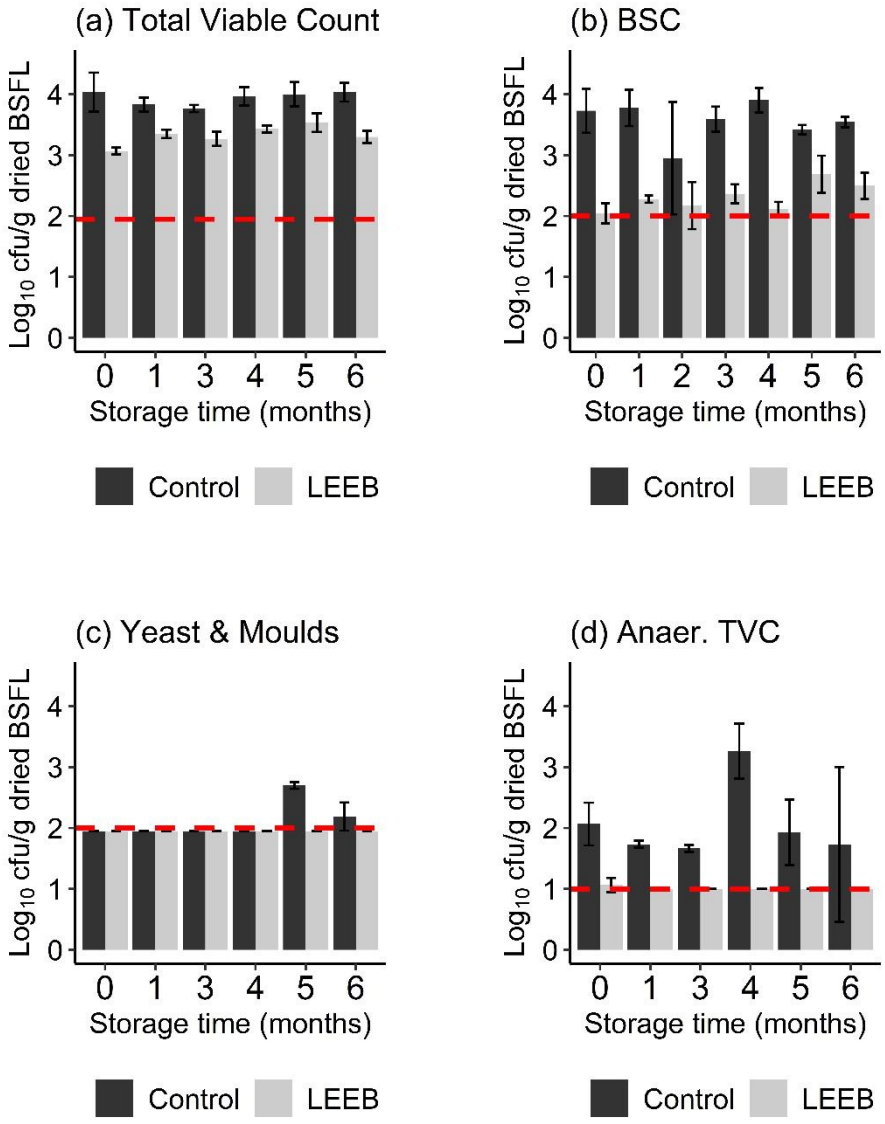
Initial microbial counts were above the detection limit for all parameters except for yeast and moulds (Figure 5.2). In comparison to experiment 2, which only detected aerobic TVC and bacterial spore counts, in experiment 3, aerobic and anaerobic TVC and aerobic and anaerobic bacterial spore count were detected. The initial aerobic TVC (i.e., 4-log_{10} cfu/g dried BSFL) was slightly lower than in previous studies, where TVC ranged between 5–5.5 log_{10} cfu/g dried BSFL (Campbell et al., 2020; Saucier et al., 2022). Additionally, aerobic TVC was lower (i.e., 4-log_{10} cfu/g dried BSFL) compared to the results of experiment 2. However, overall results in experiment 3 had more microbiological parameters detected than in experiment 2.This could be due to many factors including different BSFL rearing conditions (e.g., substrate, duration, larval densities, product cleaning) resulting in a higher initial microbial load before post-processing, in addition to the different drying methods (microwave vs. oven) and thermal conditions (Table 5.1. Thermal treatment conditions of BSFL and YMW used in experiments 1–3.), as well as potentially different product storage between experiment 2 and 3. However, systematic information on these factors was not recorded. In accordance with EU regulations for BSFL intended as animal feed, there is not a limit for aerobic TVC but it is required that producers ensure the absence of *Salmonella*, with a maximum

allowable limit of 2.5-$\log_{10}$ cfu/g for *Enterobacteriaceae* (EC, 2005). A limitation to this study, is that these specific microorganisms were not identified.

LEEB treatment decreased all microbiological parameters below the detection limit, except for TVC (3-$\log_{10}$ cfu/g dried BSFL) (Figure 5.2). Specifically, there were reductions of at least 1-$\log_{10}$ for aerobic and anaerobic TVC, and 1-$\log_{10}$ for anaerobic bacterial spore counts (Table 5.4). The largest reduction was observed in aerobic bacterial spore counts by 2-$\log_{10}$ (Table 5.4). Surprisingly, LEEB was able to reduce aerobic bacterial spore counts to the detection limit, but aerobic TVC still remained around 3-$\log_{10}$ cfu/g dried BSFL. As mentioned in experiment 2, it is important that aerobic bacterial spore counts remain below the 5–8 $\log_{10}$ cfu/g, as this is the concentration responsible for foodborne diseases (EFSA, 2005). LEEB treatment had a higher $\log_{10}$ reduction of aerobic TVC in experiment 2 compared to experiment 3 (2-$\log_{10}$ vs 1-$\log_{10}$) (Table 5.4). Trinetta *et al.* (2011) found that LEEB treatment of different seeds resulted in different levels of microbial inactivation efficiency of *Salmonella*. These findings suggested that the treated surface morphology influenced the inactivation efficiency, demonstrating that a complex surface structure can reduce the antimicrobial effectiveness of LEEB. The difference in reduction of TVC in experiment 2 compared to 3 could be due to the surface structure differences between microwave-dried BSFL and oven-dried. Upon visual inspection, the oven-dried BSFL appeared more shriveled with more crevices (see images in Supplementary Material) in addition to having a higher density compared to the microwave dried BSFL, which lowers the LEEB penetration depth. Furthermore, the low reduction of aerobic TVC could be due to the initial intense (80 °C, 27 h) thermal treatment before LEEB treatment resulting in low initial microbial counts and a low water activity of 0.3. Vegetative cells in a dehydrated state can become more resistant to ionising radiation, comparable to that of spores (Barkai-Golan and Follett, 2017). This is because when cells are in a dehydrated state, the water within the cells becomes immobilized and the cellular metabolism is slowed down, thus limiting the movement of the cells and potentially reducing overall damage caused by radiation (Barkai-Golan and Follett, 2017). Overall LEEB treatment was effective in reducing initial microbial counts compared to the control. However, further research should investigate the impact of LEEB treatment on dried BSFL using a lower drying temperature (e.g., 60°C) and shorter treatment time.

Throughout the entire shelf-life study, microbial counts were lower in LEEB-treated BSFL than in the non-LEEB-treated control (Figure 5.2). LEEB-treated anaerobic microbial counts remained at or below the detection limit (1-$\log_{10}$ cfu/g dried BSFL). Yeast and mould which typically indicate spoilage was only detected in low counts (3-$\log_{10}$ cfu/g dried BSFL) in the control after five months of storage, whereas for LEEB-treated samples they remained at or below the detection limit throughout the shelf-life study. Currently the EU regulation does not limit yeast and moulds but to obtain a high-quality certification of BSF as animal feed < 6-$\log_{10}$ cfu/g of yeast and moulds is required (GMP + International, 2020) which was

achieved with and without LEEB treatment. Overall, the findings suggest that LEEB treatment improved the shelf-life of BSFL as microbial counts remained at or below the detection limit.



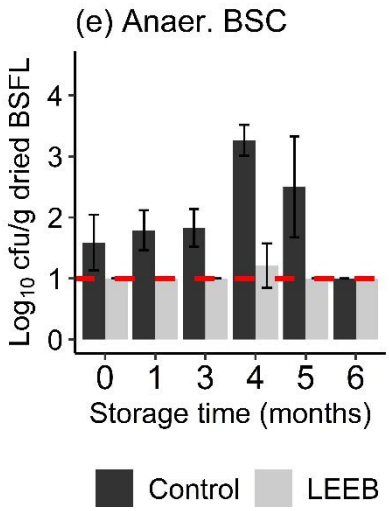


Figure 5.2. Microbial counts of control (i.e., oven-dried) and LEEB-treated BSFL samples over 6 months storage time. Control is blanched followed by drying without LEEB treatment. BSC: bacterial spore count, Anaer. TVC: anaerobic total viable count, and Anaer. BSC: anaerobic bacterial spore count. Data displayed are mean values and standard deviations shown as error bars (n=3). Samples where colonies were not observed are graphed as the detection limit (shown as red dashed line). For aerobic microorganisms, detection limit was 2-$\log_{10}$ cfu/g dried insect and for anaerobic was 1-$\log_{10}$ cfu/g dried insects. Results in month two are not displayed due to sampling error.

Peroxide value in experiment 3 (2 meq./kg fat, Table 5.3) were lower than in experiment 2 (8 meq./kg fat, Table 5.2) and below the limit for animals fats (10 meq./kg fat) and fish oils (5 meq.kg/fat) (FAO and WHO, 2017). LEEB treatment increased the peroxide value by 2 meq./kg fat, compared to the control. One possible explanation for the observed difference compared to experiment 2 could be the variation in the initial unsaturated fatty acid profile, which is influenced by factors such as the larvae's feed composition. However, since the fatty acid profile was not measured, it is difficult to draw a definitive conclusion. The p-anisidine value remained lower than the peroxide value. Within the first month of the shelf-life study, the initial peroxide and p-anisidine value in the LEEB-treated BSFL did not change. In contrast, the control peroxide value appeared to increase (Table 5.3). After three months of storage, the peroxide and p-anisidine value increased in both the control and LEEB-treated BSFL, exceeding the limit for animal-based fats (10 meq./kg fat). The similar results between control and LEEB-treated BSFL suggest that LEEB treatment has little influence on long-term lipid oxidation.

Table 5.3. Primary (peroxide value) and secondary (p-anisidine value) lipid oxidation were monitored during the oven-dried BSFL shelf-life at four different time points. Data displayed are mean values and standard deviation (n=3).

Storage Time (months)	Treatment	Peroxide value (meq./kg fat)	p-Anisidine value (-)
0	Control	2.2 ± 0.5	1.5 ± 0.1
	LEEB	3.9 ± 1.0	2.3 ± 0.7
1	Control	6.0 ± 2.5	1.9 ± 0.1
	LEEB	3.5 ± 1.3	2.1 ± 1.2
3	Control	12.0 ± 0.0	3.8 ± 1.0
	LEEB	11.7 ± 0.6	4.2 ± 0.2
6	Control	15.0 ± 0.0	6.8 ± 1.0
	LEEB	14.3 ± 0.6	6.6 ± 0.6

Table 5.4. Comparison of microwae average (experiment 2, n=2) and oven dried average (experiment 3, n=3) $\log_{10}$ reduction of initial microbial load after LEEB treatment for both BSFL and YMW.

	BSFL		YMW	
Experiment	**2**	**3**	**2**	**3**
TVC	2.0	0.96	1.7	4.1
Bacterial spore count	1.6	1.7	0.0	0.82
Yeast & moulds	0.0	0.0	0.0	0.0
Anaerobic TVC	0.0	1.0	1.7	3.9
Anaerobic bacterial spore count	0.0	0.6	0.0	0.86

YMW

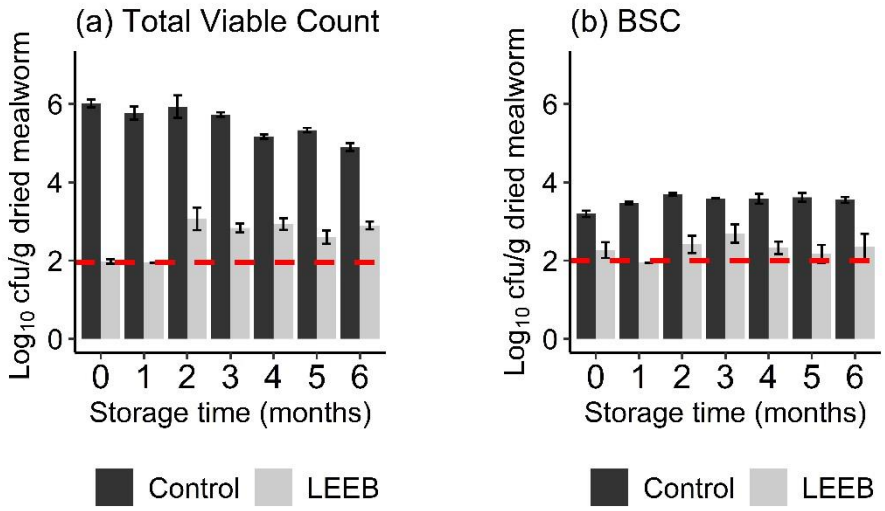
The initial water activity of dried YMW before LEEB treatment was 0.4 and the moisture content was below 6% (see Supplementary Material). While water activity remained below 0.6, it did increase to 0.5 after four months of storage in both the control and LEEB-treated YMW (see Supplementary Material).

Aerobic TVC in YMW were approximately 6-$\log_{10}$ cfu/g dried YMW (Figure 5.3). This is high relative to previously reported results after a thermal treatment (e.g., 1–4 $\log_{10}$ cfu/g dried YMW) (Mancini et al., 2019; Vandeweyer et al., 2017) and were higher than what was observed in experiment 2 (e.g., 4 $\log_{10}$ cfu/g dried YMW, Table 2). However, TVCs were slightly lower than what is typically observed in fresh YMWs at harvest (8–9 $\log_{10}$ cfu/g fresh YMW) (Caparros Megido et al., 2017; Stoops et al., 2016; Wynants et al., 2017). Anaerobic TVC were in a similar range to aerobic TVC, indicating facultative anaerobes as discussed in experiment 2 (Table 5.2). Aerobic bacterial spore counts were around 3-$\log_{10}$ cfu/g dried YMW (Figure 5.3).

Similar to experiment 2 with microwave dried YMW, LEEB treatment decreased all microbiological parameters to at or below the detection limit, except anaerobic TVC which was reduced to 2-$\log_{10}$ cfu/g dried YMW (Table 5.4, Figure 5.3). The largest decrease was observed for aerobic and anaerobic TVC, both

resulting in a 4-$\log_{10}$ reduction (Table 5.4). Because of the naturally higher initial microbial load in experiment 3, LEEB was overall more effective in reducing microbial numbers in the oven dried YMW compared to microwave dried YMW used in experiment 2 (Table 5.4). Contrary to BSFL, drying method does not seem to heavily impact the surface morphology YMW, which could be why LEEB treatment was similar within experiment 2 and 3. When intended for human consumption, IPIFF (2019) recommends less than 3-$\log_{10}$ cfu/g of aerobic TVC. Therefore, LEEB was effective in both experiments in reducing aerobic TVC to below the acceptable limit.

Throughout the six-months shelf-life study, LEEB treated YMW had lower counts in all microbiological parameters than the untreated control. Within the first two months of storage, aerobic TVC and bacterial spore counts for LEEB-treated YMWs remained at or below the detection limit, with anaerobic bacterial spore counts remaining at or below for the first three months. After two months of product storage, higher numbers were observed for aerobic TVC for LEEB-treated samples and remained around 3-$\log_{10}$ cfu/g dried YMW for the entire shelf life. This could be explained by a potential slight recovery of survived microbial cells, which were not culturable shortly after LEEB treatment. Further studies on such cell fractions are needed to verify this hypothesis. All microbial counts of the control remained stable throughout the 6-months shelf-life study with aerobic and anaerobic TVC ranging from 4–6 $\log_{10}$ cfu/g dried YMW. Yeast and mould counts was detected at 5-$\log_{10}$ cfu/g dried YMW in the control after two months of storage indicating some spoilage, as its close the limit set for animal feed of 6-$\log_{10}$ cfu/g (GMP + International, 2020). In contrast, LEEB-treated yeast and moulds remained at or below the detection limit throughout the shelf-life study.



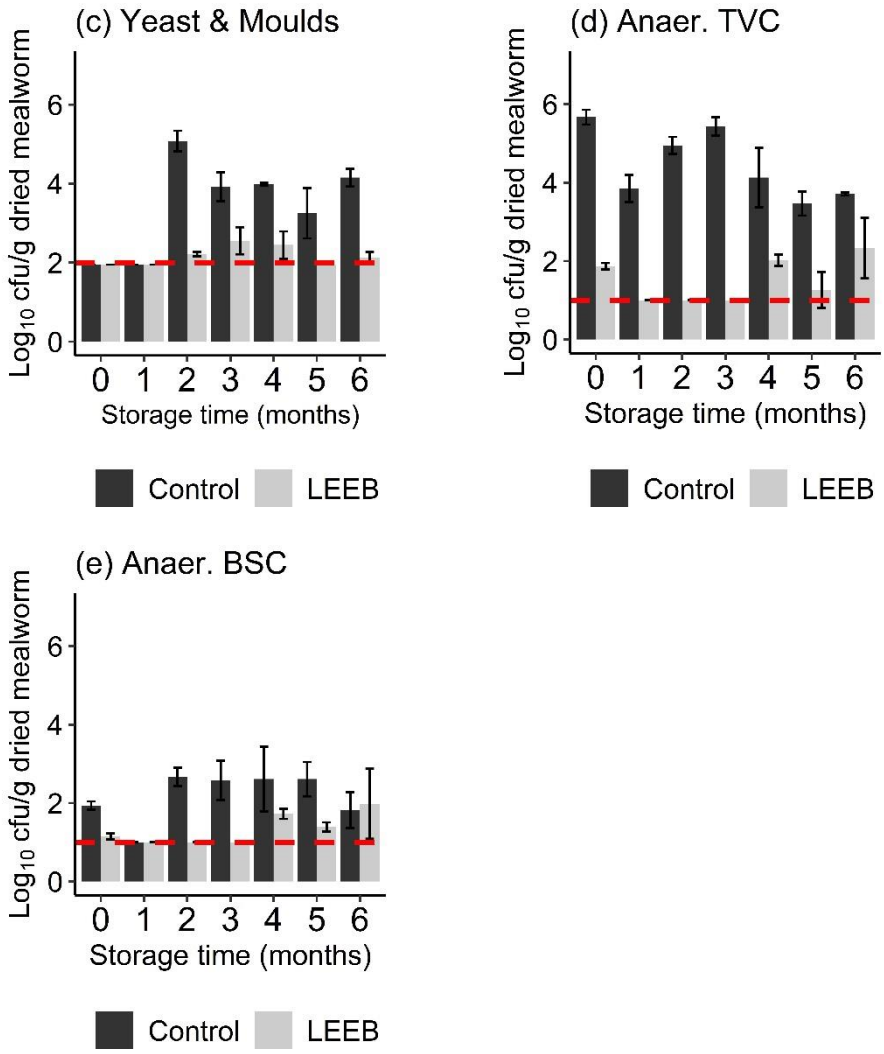


Figure 5.3. Microbial counts of control (i.e., oven dried) and LEEB-treated mealworm samples over 6 months storage time. Control is blanched followed by drying without LEEB treatment. BSC: bacterial spore count, Anaer. TVC: anaerobic total viable count, and Anaer. BSC: anaerobic bacterial spore count. Data are displayed with mean values and standard deviations shown as error bars (n=3). Samples where colonies were not observed are graphed as the detection limit (shown as red dashed line). For aerobic microorganisms, detection limit was 2-$\log_{10}$ cfu/g dried insect and for anaerobic was 1-$\log_{10}$ cfu/g dried insects.

5.5. Conclusions

LEEB treatment was effective in reducing microbial numbers in BSFL and YMW following thermal treatment, leading to an improved shelf-life. Further research should investigate the effect of LEEB treatment on BSFL with a higher initial microbial load. Despite a slight increase in lipid oxidation after LEEB treatment, the initial peroxide value for BSFL and YMW was still considered relatively low, meeting the target suitable for animal-based fat oils. Nevertheless, further research is necessary to investigate the shelf-life of microwave dried insect products, particularly in terms of lipid oxidation as this has yet to be conducted. Overall, these findings suggest that LEEB could be a complementary post-processing step to a gentler and more efficient thermal step, especially among regions of the world where irradiation is gaining increased interest.

6. Chapter: Conclusions and Future Perspective

6.1. Conclusion

BSFL holds great potential to contribute to a more circular economy by upcycling nutrients in waste and serving as an alternative feed protein source for pigs, poultry, fish, and pets. To minimize their environmental impact compared to traditional protein-feed sources, BSFL should focus on substrates not intended for food or feed. This involves using underutilized low-value fibrous biowastes such as, animal manures, yard waste (e.g., grass clippings) and agri-food wastes (e.g., vegetable and fruit pomace/peels). The primary objective of this thesis was to investigate pretreatment methods for enhancing BSFL rearing performance by unlocking nutrients in fibrous biowastes that are not as easily accessible by larvae and microorganisms in the biowaste and/or larval digestive tract. Recognizing that these biowastes are contaminated with pathogens, this research also aimed to explore a non-thermal treatment technology to support product safety, without compromising product quality and potentially extending the shelf life of both edible insects and insect-based feed. This doctoral thesis focused on two aspects of the BSFL production chain pre-processing of biowastes and post-processing of the dried products. The following graphical summary presents how these strategies could be implemented within the production chain (Figure 6.1).

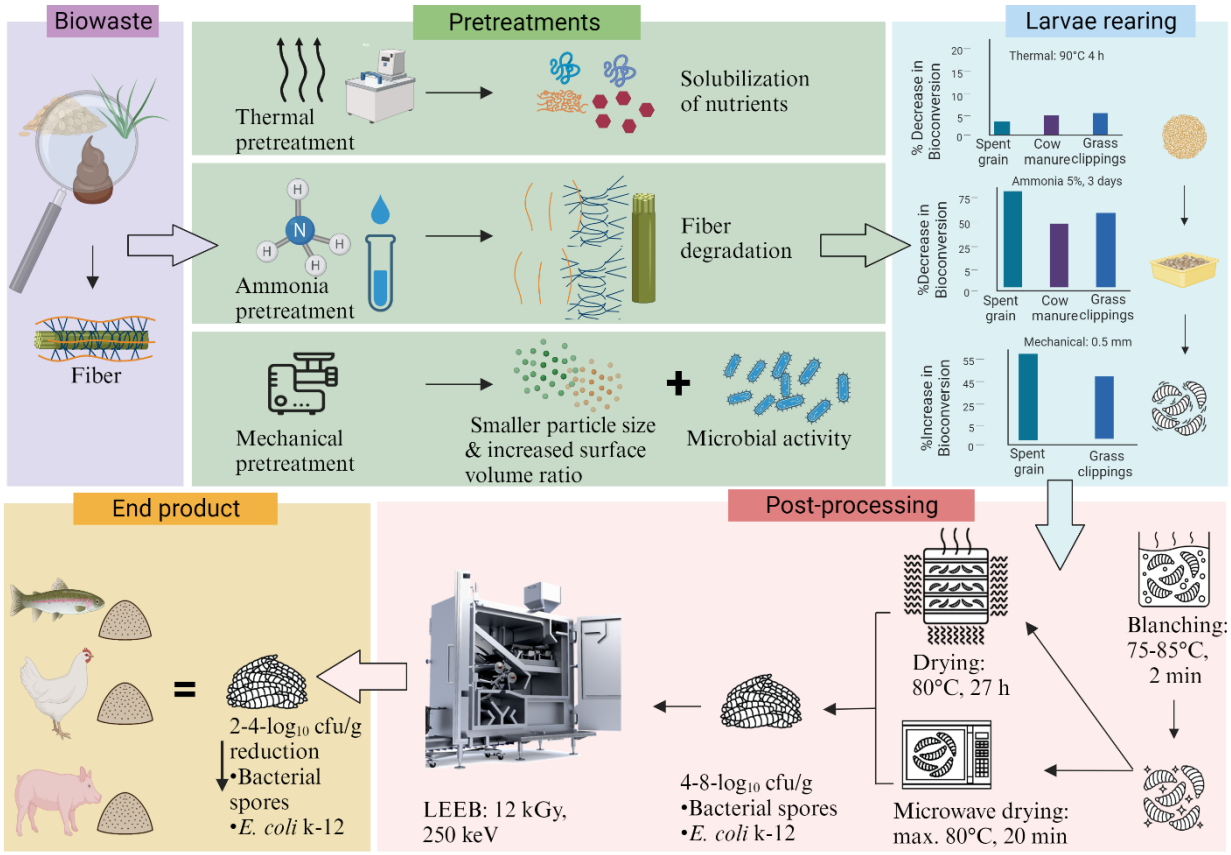


Figure 6.1. Use of biowaste pretreatments and LEEB within the BSFL production chain (created by Biorender.com, icons from Eawag/Sandec).

To improve the performance of BSFL on fibrous biowastes, **Chapter 2** was a review that evaluated and identified pretreatments that have been used in similar bioprocessing technologies such as, composting and anaerobic digestion for biogas production. The objective was to determine the transferability of these pretreatments for BSFL industrial application. Given the varying efficiencies reported in this review for improving compositing efficiency and biogas production and the uncertainties surrounding treatment effects on BSFL, laboratory and pilot-scale studies using different biowastes and pretreatments were suggested. Such studies would provide insights into which treatments hold promise for advancing BSFL production. Therefore, recommendations proposed, involved factors such as particle size, temperature/time, chemical and dose. Subsequently, the thesis aimed to practically assess some of the outlined recommendations from the review on specific biowastes. **Chapter 3,** focused on systematically assessing whether ammonia pretreatment reduces the lignocellulosic composition in biowastes and improves the BSFL rearing performance on fibrous biowastes. Although modifications in the lignocellulosic composition were evident through fiber analyses, subsequent BSFL feeding experiments demonstrated that ammonia pretreatment was not a viable strategy for any of the biowastes used. This outcome was attributed to the toxicity of ammonia/ammonium and/or their salts to the larvae or associated microorganisms. **Chapter 4** then

investigated the use of thermal and mechanical pretreatment. The applied thermal pretreatment did not improve BSFL performance on the tested biowastes. In contrast, mechanical pretreatment of spent grain had varied effects with positive results in smaller containers but no effect when using larger containers with shortened feeding duration. In contrast, grass clippings enhanced larval performance in both scenarios. The positive outcome was due to the possibility of the larvae ingesting the smaller substrate particles.

Efficiency gains resulting from pretreatment methods must be carefully weighed against associated capital and operational demands, including process additive costs, and energy consumption. Nevertheless, to determine economic viability, a comprehensive economic assessment of the BSFL production chain should be conducted when a pretreatment improves BSFL performance. This evaluation should aim to establish the minimal process and bioconversion needed to validate the incorporation of pretreatment. Additionally, sustainability assessments should be used to evaluate the varying environmental, social, and economic impacts from substrate pretreatments.

Although enhancing BSFL performance on biowastes is important for the environmental sustainability of BSFL production, remaining barriers to market entry exist, such as overall safety of the product and production processes. Therefore, ensuring that these products are safe is equally essential. Even when using common thermal treatments, the presence of pathogens has still been detected for yellow mealworm and BSFL. Ensuring the inactivation of heat-resistant pathogens such as *Bacillus cereus* is essential to prevent their introduction into the food chain, causing foodborne diseases. However, prolonged drying times would lead to product deterioration, highlighting the need for treatments that can preserve product quality. **Chapter 5** explored an emerging non-thermal treatment technology, low energy electron beam for dried BSFL and yellow mealworm. This work found that LEEB treatment was effective in reducing microbial numbers in BSFL and YMW after drying. Despite a slight increase in lipid oxidation after LEEB treatment, the initial peroxide value for BSFL and YMW was still considered relatively low, meeting the target suitable for animal-based fat oils. Overall, these findings suggest that LEEB could serve as a complementary post-processing step for gentle decontamination of low moisture goods.

6.2. Future Perspective

Although two of the three biowaste pretreatments investigated were not effective in enhancing BSFL performance, the use of biowaste pretreatments should not be dismissed, considering the diverse range of biowastes and unexplored pretreatment possibilities. While ammonia pretreatment led to a 50% decrease in BSFL development, determining whether toxic affects arose from ammonium salts or ammonia itself remains inconclusive. Therefore, future research should explore the toxicity of ammonia at different life stages of BSFL, as older larvae may be less susceptible to ammonia. A re-evaluation of ammonia without

acid neutralization should be conducted, especially given BSFL's capacity to grow on substrates with high pH levels. Moreover, should ammonia alone (i.e., without addition of any acid) lead to increased BSFL growth, subsequent research should explore whether ammonia contributes to pathogen/microbial count reduction in both the substrate and freshly harvested BSFL, thereby functioning as pre-hygienization step. Additionally, alternative alkaline (e.g., sodium hydroxide), and microbial pretreatments for lignocellulosic degradation should be explored as they may be more suitable for improving BSFL development. Investigating brief storage/fermentation periods days should be studied, given their demonstrated potential to enhance larval performance.

Lower temperature and treatment times (e.g., 80°C for 5 mins) could be further explored as this thesis only investigated a higher temperature of 90°C for 0.5 –4 hours. Various biowastes should be investigated using thermal pretreatment as varying effects from pretreatment could be found. The lower temperature and treatment times could serve as a pre-hygienization step. Therefore, evaluating microbial concentrations of freshly harvested BSFL should be conducted after rearing on the pretreated substrate.

Given the positive effects demonstrated with mechanical pretreatment, there is a need for more extensive exploration of physical properties of substrates, such as bulk density, particle size and free air space. It is necessary to understand how these physical properties influence the synergistic relationship between the substrate microorganisms and BSFL. Studies are beginning to investigate this relationship by monitoring microbial and larval respiration (e.g., CO_2 emissions) and carbon utilization. Although this offers valuable data, there are still challenges in accounting for all carbon transformations. In this context, the utilization of stable isotopes could be a powerful tool. Stable isotopes are commonly used within other processes for example with animal studies for human nutrition. Isotopes such as carbon-13 and nitrotgen-15 could be introduced into the substrate to trace the markers as they are absorbed, metabolized, and incorporated into various body tissues. Few studies have investigated this use, as it could provide information on nutrient assimilation dynamics and metabolic pathways within the BSFL rearing process. Another interesting research direction could examine the manipulation of the substrate's physical or chemical properties and impact on greenhouse gas emissions and substrate microbial community. One study found that adjusting the substrate pH had substantial differences in methane (CH_4), CO_2, and ammonia (NH_3) emissions. Correlating these emissions with microorganisms in the substrate could give insights into the interplay between microbial composition shifts, emissions, and their influence on larval performance.

Proposals for refining the methodology of larval feeding experiments are further outlined. Among the key aspects that warrant re-evaluation and standardizing within the BSFL research community are larval densities in relation to volume and duration of larval feeding experiments. To begin with, future research

should assess how larval growth is impacted when using larval density in relation to volume rather than surface area. As BSFL feed within their substrate, evaluating larval density based on volume encompasses the entire space available for the larvae. Moreover, adopting volume-based larval density assessment could facilitate more relevant cross-study comparisons, as many studies use different trays with different heights, which are currently unaccounted for. Height of the container changes height of the substrate which could also impact the larval performance results. Regarding the experiment's duration, different approaches exist among studies. For example, in this thesis, the feeding experiment duration adhered to a predefined duration, whereas other studies await the emergence of the first prepupae or the point when 50% reach prepupal stage. An alternative approach could involve continuous daily monitoring of larval growth, with the experiment concluding once the peak growth of larvae is observed. This suggestion is rooted in our observation from the mechanical pretreatment experiment where larval growth on untreated spent grain was higher for most of the experiment's duration. Only on the sixth day did the 0.5 mm treatment catch up to the untreated. Since the untreated may have been depleted of nutrients, the weight would have started to decrease while 0.5 mm was beginning to increase. The absence of daily measurements makes it difficult to draw a definitive conclusion of how a treatment is performing against one another.

Regarding research on product quality of edible insects and insect-based feeds, more extensive long-term shelf-life studies should be conducted to gain insight on the product's stability over time. For example, investigating the shelf-life of microwave dried insect products, particularly in terms of lipid oxidation as this has yet to be thoroughly examined. For microbiological safety of edible insects and insect-based feeds, further research could also investigate pretreatments. This approach entails exploring how various pretreatments such as thermal, acidification/or fermentation of the substrate contribute to enhancing the safety profile of the final product. For example, acidification could reduce pathogens in the substrate, and then monitoring whether this influences the microbial concentration of the insects after harvest. This could be coupled with a Hazard Analysis and Critical Control Points approach which could be implemented to identify hazards that may cause the product to be unsafe. Specifically, identifying points along the BSFL production chain where contamination could be introduced.

7. Supplementary Material

Chapter 2:

The review followed a semi-systematic four-step approach (see Figure S-2.1) and used the searches below. Articles were reviewed for relevant technologies that could be applied for black soldier fly larvae production. Within the last decade, pretreatments for enhancing biogas production have significantly increased. Figure 1 demonstrates the approach used in including articles for this review.

Scopus. (2021). (TITLE-ABS-KEY (pretreatment) AND TITLE-ABS-KEY (anaerobic AND digestion) OR TITLE-ABS-KEY (biogas AND production)) AND PUBYEAR > 1999 AND (LIMIT-TO (DOCTYPE , “ar”) OR LIMIT-TO (DOCTYPE , “ch”))AND (LIMIT-TO (LANGUAGE , “English”)) www.scopus.com

Scopus. (2021). (TITLE-ABS-KEY (pretreatment) AND TITLE-ABS-KEY (compost) OR TITLE-ABS-KEY (vermicompost)) AND PUBYEAR > 1999 AND (LIMIT-TO (LANGUAGE , “English”)). www.scopus.com

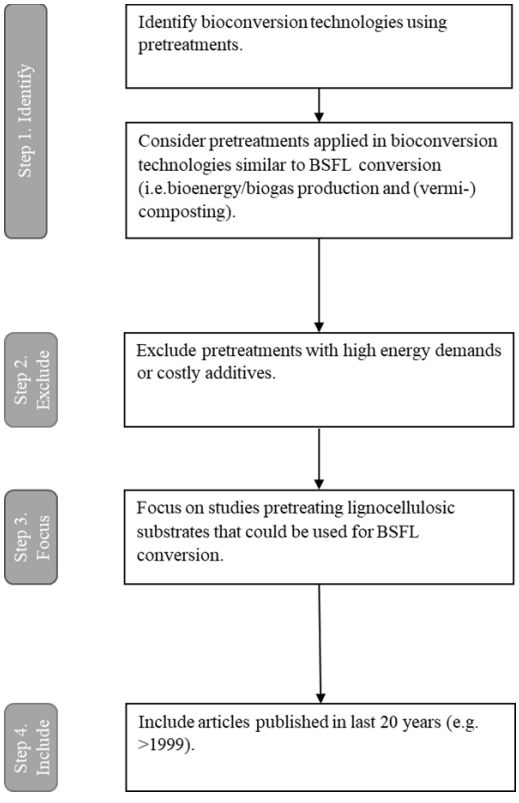


Figure S-7.1. Flow chart on the approach used to exclude or include papers for this review.

Table S-7.1. Complete breakdown of each substrate within each category. All values are presented as weight (wt)% in dry mass.

Classification	Substrate	Cellulose	Hemicellulose	Lignin	References
Animal manure	Dairy manure	38.6	18.3	23.2	(Li et al., 2011b)
	Dairy manure	31.7	16.8	13.6	(Rehman et al., 2017)
	Cattle manure	14.2-27.4	12.2-21.4	6.1-13.0	(Chen et al., 2003)
	Dairy manure	26.9	14.7	15.5	(Rehman et al., 2019)
	Dairy Manure	31.7	16.7	13.5	(Rehman, Rehman, et al., 2017)
	Poultry manure	7.7-12	16.4-21.5	4.1-7.2	(Chen et al., 2003)
	Poultry manure	14.4	20.5	4.5	(Rehman et al., 2019)
	Chicken manure	14.5	21.7	7.8	(Rehman et al., 2017)
	Chicken manure	-	17.2	-	(Shumo et al., 2019)
	Swine manure	6	28	-	(Sun & Cheng, 2002)
	Swine manure	13.2-13.9	12.2-21.4	6.1-13.0	(Chen et al., 2003)
Agricultural crops and residues	Rice straw	35.0	16.7	23.34	(Ye et al., 2013)
	Rice straw	32.6	27.3	18.4	(Zheng et al., 2012)
	Rice straw	38.3	21.3	12.5	(Dai et al., 2018)
	Rice straw	34.2	28.8	19.4	(Patowary & Baruah, 2018)
	Wheat straw	38.2	21.2	23.4	(Mosier et al., 2005)
	Wheat straw	33.5	22.6	20.9	(Wan & Li, 2011)
	Sugarcane bagasse	29.7	18.3	15.3	(Liu et al., 2017)
	Sugarcane bagasse	26-50	24-34	10-26	(Ansari et al., 2021)
	Barley straw	35.4	28.7	13.1	(Liu et al., 2017)
	Walnut shells	25.6	23	46.7	(Şenol, 2021)
	Buckwheat hull	45.1	10.5	28.1	(Mirko et al., 2021)
Milling and Brewery Sides Streams	Brewer's spent grain	25.4	21.8	11.9	(Kanauchi et al., 2001)
	Brewer's spent grain	-	11.3	-	(Shumo et al., 2019)
	Brewer's spent grain	16.32	22.17	6.21	(Meneguz et al., 2018)
	Brewer's spent grain	16.8	28.4	27.8	(Mussatto et al., 2006)
Organic fraction of Municipal solid wastes		5	13.1	18.5	(Hartmann & Ahring, 2005)
		17.5	10.7	9.6	(Rao & Singh, 2004)
		37.4	9.1	10.5	(Zhu et al., 2010)
Fruit and vegetable waste	Discarded fruits (e.g. apples, grapes, and strawberries, and vegetables (e.g. lettuce, potatoes, cassava)	15.7	8.6	5.9	(Edwiges et al., 2018)
	Celery, oranges, and peppers	9.76	6.75	1.29	(Meneguz et al., 2018)

Table S-7.2 Effect of mechanical and thermal pretreatment on lignocellulosic biomass.

Substrate classification	Substrate	Bioconversion technology	Pretreatment conditions	Lignocellulose composition affected	Effect	Reference
			Mechanical			
Animal manure	Cattle manure	Biogas production	0.35 & 2mm	Not reported	Methane production: + 16 to 20%	(Angelidaki & Ahring, 2000)
Fruit and vegetable waste	Cauliflower leaves, banana peels	Biogas production	6, 1.0. 0.4, 0.088 mm	Cellulose: cauliflower leaves: - 43 to 47%, banana peels: -39 to 43%	Methane production: cauliflower leaves: no sig. effect, banana peels: +38 to 50%	(Sharma et al., 1988)
Agricultural crops and residues	Almond hulls	BSFL	4, 6 mm	Not reported	Larval mass: -10% from 6 to 4 mm	(Palma et al., 2019)
Agricultural crops and residues; forestry residues	Wheat straw, rice straw, mirabilis leave, ipomoea fistulosa leaves, dhub grass	Biogas production	6, 1.0. 0.4, 0.088 mm	Cellulose: wheat straw: -31 to 38%, rice straw: -42 to 48%, mirabilis leaves: -37 to 39%, ipomoea leaves: -44 to 47%, dhub grass: -24 to 27%	Methane production: wheat straw, rice straw & dhub grass: +40 to 89%, mirabilis leaves & ipomoea fistulosa leaves: no sig. effect	(Sharma et al., 1988)
Agricultural crops and residues	Sisal fiber waste	Biogas production	2 mm	Neutral detergent fiber: -70%	Methane production: +23%	(Mshandete et al., 2006)
Agricultural crops and residues	Wheat straw	Biogas production	5 cm, 0.2 cm	Not reported	Methane yield: +57 to 83.5%	Menardo et al., 2012)
Agricultural crops and residues	Barley straw	Biogas production	5 cm, 2 cm, 0.5 cm	Not reported	Methane yield: +19 to 54%	Menardo et al., 2012)
Agricultural crops and residues	Rice straw	Biogas production	5 cm	Not reported	Methane yield: no sig. effect	Menardo et al., 2012)
Agricultural crops and residues	Maize stalks	Biogas production	2 cm, 0.2 cm	Not reported	Methane yield: no sig. effect	Menardo et al., 2012)

			Thermal			
Animal manure	Dairy manure	Compost	90 °C for 4 hours	Cellulose: -41%, Hemicell.: -69%, Lignin: -23% after composting (60 days)	Temp peak: + 6 days earlier, Accelerated composting time: +10 days, humic substance: +14%	(Zhu et al., 2021)
Animal manure/ Agricultural crops and residues	Pig manure & rice straw	Compost	90 °C for 4 hours	Not reported	Humic substance: +18% (increasing composting efficiency and quality)	(Huang et al., 2019)
Animal manure	Cattle manure	Biogas production	68 °C for 36, 108, and 168 hours	Not reported	Methane yield: +24 to 56%	(Nielsen et al., 2004)
Human manure	Waste activated sludge	Biogas production	80 °C and 90 °C for 1 hours	Not reported	Biogas production: +factor 20 at 90°C, solubilization protein and carbohydrates: +10%	(Appels et al., 2010)
Agricultural crops and residues	Wheat straw, barley straw, rice straw, maize stalks	Biogas production	90°C for 0.5 hours	Not reported	Methane yield: wheat straw: +62%, barley straw: +40%, rice straw & maize stalks: no sig. effect	(Menardo et al., 2012)
Agricultural crops and residues	Buckwheat hull	Biogas production	70°C for 24 hours	Cellulose: no sig. effect, Hemicell.: +24.8%, Lignin: no sig effect	Methane yield: no sig. effect	(Mirko et al., 2021)
Agricultural crops and residues	Walnut shells	Biogas production	50°C for 2 hours	Not reported	Methane yield: +20%	(Şenol, 2021)
	Coffee pulp	Biogas production	50 °C, 70 °C, 90°C for 1 hours	Not reported	Biogas production: +14, 23 and 40%	(Nava-Valente et al., 2021)
Fruit waste	Banana peels	BSFL	120 °C for 1 hours at 2 bar	Not reported	Larval weight gain: -15%, Bioconversion rate: -19%	(Isibika et al., 2019)

Table S-7.3. Effect of alkaline pretreatment on lignocellulosic biomass.

Substrate classification	Substrate	Bioconversion technology	Dose	Time	Temp.	Lignocellulose Composition affected	Effect	Reference
				NaOH				
Animal manure	Cattle manure	Biogas	4 and 8% (w/w)	24 to 48 hours	20 °C	Not reported	Methane production: 13 to 23%	(Angelidaki and Ahring, 2000)
Crop and harvesting residues	Sugarcane bagasse	Compost	1%	Not reported	Not reported	Cellulose: +25%, Hemicell.: -19%, Lignin: -22%	Water retention capacity: +13%	(Ansari et al., 2021)
Crop and harvesting residues	Wheat plant	Biogas	8% (w/v)	1 hours	25 °C	Not reported	Methane yield: +47.5%	(Taherdanak and Zilouei, 2014)
Crop and harvesting residues	Rice straw	Biogas	6%	504 hours	20 °C	Cellulose: -16%, Hemicell.: -37%, Lignin: -28%	Biogas production: +27 to 65%	(He et al., 2008)
	Barley straw	Biogas	30% (w/w)	12 hours	25 °C	Not reported	Methane production: +89%	(Neves et al., 2006)
	Sorghum forage, wheat straw	Biogas	1% and 10%	24 hours	40 °C	No significant reduction at 1% dose, At 10% Cellulose: -13 to 31%, Hemicell.: -45 to 66%, Lignin: -3 to 44%	Methane production: *Dose 1%*: +12 to 14%, *Dose 10%*: +29 to 43%	(Sambusiti et al., 2013)
	Rice straw	BSFL	NaOH + H_2O_2	6 hours	30 °C	After pretreatment: Cellulose: -50%, Hemicell.: -22%, Lignin: -68%	Larval mass +32%	(Liu et al., 2021)

				$Ca(OH)_2$ Lime				
Animal manure	Cattle manure	Biogas	Ca(OH) 2	2, 12 hours	20 °C	Lignin: -23%	Methane yield: No sig. effect	(Niasar et al., 2011)
Municipal solid waste	Mainly kitchen waste	Biogas	Ca(OH) 2	1 to 6 hours	Room temp, under anoxic condito n	Not reported	Methane yield: up to +173%	(López Torres and Espinosa Lloréns, 2008)
Crop and harvesting residues	Rice straw	Biogas	Ca(OH) 2	120 hours	20 °C	Cellulose: -3%, Hemicell.: +3%, Lignin: +1%	Did not compare methane yield to a control	(Liang et al., 2014)
				Urea and ammonia (NH3)				
Fruit waste	Banana peels	BSFL	0.8% and 1% dose (w/w) 24.5% Aq. Ammoni a concen.	168 hours	28 °C	Crude fiber: Dose 0.8%: -11%, Dose 1%: +5%	Larval mass: +31 to 32%, Bioconversio n rate: -1.4 to +33%	(Isibika et al., 2019)
Animal manure	Digested swine manure fibers	Biogas	3% dose (w/w) 5-32% Aq. ammoni a concen.	24, 72 and 120 hours	22 °C	Not reported	Methane yield: +76-104% with 3-days pretreatment	(Mirtsou-Xanthopoulo u et al., 2014)
Animal manure	Digested manure fibers	Biogas	3% dose (w/w) 32% Aq. ammoni a concen.	24, 72, and 120 hours	22°C	Klason lignin: -35%	Methane yield: +17 to 80%	(Jurado et al., 2013)
Crop and harvesting residues	Almond hulls	BSFL	Urea, C/N ratios: 16, 32, 49	0 hours	28°C	Not reported	Waste reduction: 23 to 31%	(Palma et al., 2019)

Crop and harvesting residues	Wheat straw	Biogas	3% dose (w/w) 32% Aq. ammonia concen.	72 hours	22 °C	Not reported	Methane yield: +37%	(Jurado et al., 2013)
Crop and harvesting residues	Sunflower straw,grass	Biogas	3% dose (w/w) 32% Aq. ammonia concen.	72 hours	22 °C	Sunflower straw: Cellulose: -10%, Hemicell.: -26%, Lignin: -11%, grass: Cellulose: -22%, Hemicell.: -30%, Lignin: -7%	Methane yield: sunflower straw: +38%, grass:+26%	(Antonopoulou and Gavala, 2015)
Crop and harvesting residues	Wheat straw	Biogas	1, 3 and 5% Urea	144 hours	20°C	Cellulose: -27 to 47%, Hemicell.: -23 to 34%, Lignin: - 23 to 54%	Methane production: Dose 1%: +45%	(Yao et al., 2018)

Table S-7.4. Effect of fungal or bacterial pretreatment on lignocellulosic biomass. WR for waste reduction.

Substrate Classification	Substrate	Bioconversion Technology	Species	Time	Temperature	Composition affected	Effect	Reference
				Bacterial				
Fruit waste	Banana peels	BSFL	BSFL gut bacteria	7, 14, and 21 days	28°C	Crude fiber: 7 days: -1%, 14 days: -13%	Larval mass: -13% to +45%, 14 days and 21 days led to decrease in larval mass Bioconversion rate: +14% to 101%	(Isibika et al., 2019)
Agricultural crops and residue	Corn stover	Biogas	Microbial Consortium (BYND-9)	6 days	Not reported	Untreated: Cellulose: -26%, Hemicell.: -25%, Lignin: -6% Pretreated: Cellulose: -36%, Hemicell.: -44%, Lignin: -14%, During stable AD period	Biogas production: +25%	(Zhao et al., 2019)

Agricultural crops and residue	Corn straw	Biogas	*Yeast and microbial mixture: Yeast (Saccharomyces cerevisiae., Coccidioides immitis*., and *Hansenula anomala*), Cellulolytic bacteria (*Bacillus licheniformis*., *Pseudomonas, Bacillus subtilis*., and *Pleurotus florida*.), Lactic acid bacteria *Lactobacillus deiliehii*	*5, 10, 15, and 20 days*	20 °C	Cellulose: -2.5 to 17% Hemicell.: -7 to 29% Lignin: -15 to 53%	Biogas production: +11 to 42%	(Zhong et al., 2011)
			Fungal					
Grass	Yard trimming	Biogas	White-rot fungi, *C. subvermispora*	30 days	28 °C	Cellulose: -7% Hemicell.: -28% Lignin: -21%	Methane yield: + 84 to 154%	(Zhao et al., 2014)
Fruit waste	Banana peels	BSFL	*Trichoderma reesi* *Rhizopus oligosporus*	7, 14, 21 days	28 °C	Crude fiber: *T. Reesei* 7 days: -53% 14 days:-40% *Rhizopus oligosporus* 7 days: -10% 21 days:-10%	*T. Reesei* Bioconversion rate: +14 to 61%, Larval mass: +26 to 70% *Rhizopus oligosporus* Bioconversion rate: -7.5 to +108%, Larval mass:	(Isibika et al., 2019)

							-11 to +64%	
Agricultural crops and residues	Rice straw	Biogas	*Trichoderma Reesei* White rot fungi: *Pleurotus ostreatus*	20 days	28 °C, moisture content of rice straw 75%	*T. Reesei:* Cellulose: -4% Hemicell.: -16% Lignin:-23% *P. ostreatus:* Cellulose: -4% Hemicell.: -15% Lignin:-33%	Methane yield: *T. Reesei:* +78%, *P. ostreatus:* +120%	(Mustafa et al., 2016)
Agricultural crops and residues	Corn stover silage	Biogas	White rot fungi: *Phanerochaete chrysosporium*	30 days	28 °C	Cellulose: -20% Hemicell.: -32% Lignin: -23%	Methane yield: +6 to 23%	Liu et al 2014
	Willow sawdust	Biogas	White rot fungi *Leiotrametes menziesii* and *Abortiporus biennis*	14 days	27 °C	*L. menziesii* Cellulose: -11% Hemicell.: -23% Lignin: -16% *Abortiporus biennis* Cellulose: -7% Hemicell.: -18% Lignin: -1%	Methane yield: *L. menziesii:* -9%, *Abortiporus biennis:* +31%	(Alexandropoulou et al., 2017)
Bacterial Co-treatment								

Agricultural crops and residues	Soybean curd residue	BSFL	*Lactobacillus buchneri*	Direct	28 °C	Not reported	Bioconversion rate: +38%, Larval mass: +39% WR: +14%	(Somroo et al., 2019)
Animal manure	Chicken manure	BSFL	*Bacillus subtilis, Kocuria marina (FE01), Mircrococcus luteus *FE02) Enteroccoccus faecalis (FE03) Lysinibacillus boronitolerans (FE04) Gordonia sihwensis (FE06) Proteus mirabilis (FE08)*	Direct	28 °C	Not reported	*K. marina:* Larval mass: +15%, WR: *+3%* *Micrococcus luteus:* Larval mass: -0.4%, WR:*-4.5%* *Enteroccoccus faecalis:* Larval mass: +1.2%, WR:*-1.2%* *L. boronitolerans:* Larval mass: +0.3%, WR:*+3%* *Gordonia sihwensis:* Larval mass: -1%, WR:*-5%* *P. mirabilis:* Larval mass: +19%, WR:-2% *B. subtilis:* Larval mass: +18%,	(Mazza et al., 2020)

WR:+9%

Chapter 3:

Figure S-7.2. Ammonia pretreatment conducted in glass containers with a lid to avoid ammonia loss. To identify the dose and treatment time, pretreatments were conducted in triplicate.

Table S-7.5. Overview of ammonia dose and pretreatment times assessed for each substrate, to evaluate dose and treatment time combination leading to greatest fiber degradation (n=3).

	Treatment	**Treatment time (days)**
Four substrates	Raw (no added ammonia)	0
	Control (no added ammonia)	3
		7
	1% ammonia (25% concentration)	3
		7
	5 % ammonia (25% concentration)	3
		7

Equations S1-3 were used to calculate the amount of 25% aqueous ammonia solution needed for the pretreatments. Equation 1 was adapted from (Li and Kim, 2011).

$$Ammonia\ (g) = \ dose\ \left(\frac{1\ or\ 5\ (g)}{100\ (g)}\right)\ x\ substrate\ dry\ mass\ (g) \qquad (1)$$

$$25\%\ ammonia\ solution\ (g) = \frac{ammonia\ (g)}{ammonia\ concentration\left(\ 25\%=\frac{25g}{100g}\ \right)} \quad (2)$$

$$25\%\ ammonia\ solution\ (mL) = \frac{25\%\ ammonia\ solution\ (g)}{ammonia\ density\left(0.91\ \frac{g}{mL}\right)} \quad (3)$$

Table S-7.6. Values used to calculate the dose of aqueous ammonia that was used in previous publications based on the equations from above.

Substrate	Ammonia density (g/ml)	Ammonia solution reagent concentration	Dry mass (g)	Amount of ammonia solution (ml)	Ammonia (g)	Calculated Dose (%)	Reference
sunflower straw, grass, poplar straw	0.91	32%	1	10	2.912	291.2	Antonopoulou, G., & Gavala, H. N. (2015). The Effect of Aqueous Ammonia Soaking Pretreatment on Methane Generation Using Different Lignocellulosic Biomasses. Waste and Biomass Valorization, 6, 281–291. https://doi.org/10.1007/s12649-015-9352-9
Manure fibers		5%	1	10	0.455	45.5	Mirtsou-Xanthopoulou, C., Jurado, E., Skiadas, I. V, & Gavala, H. N. (2014). Effect of Aqueous Ammonia Soaking on the Methane Yield and Composition of Digested Manure Fibers Applying Different Ammonia Concentrations and Treatment Durations. Energies, 4157–4168. https://doi.org/10.3390/en7074157
Chicken feed		25%	84	3.69	0.84	1.0	This study
Chicken feed		25%	84	11.1	2.53	3.0	This study
Chicken feed		25%	84	18.46	4.20	5.0	This study
Barley straw	0.88	28%	40	5	1.23	3.1	Hartley, R. D., & Jones, E. C. (1978). Effect of aqueous ammonia and other alkalis on the in-vitro digestibility of barley straw. Journal of the Science of Food and Agriculture, 29(2), 92–98. https://doi.org/10.1002/jsfa.2740290204

Figure S-7.3. Set up of thermal pretreatment using a waterbath.

Substrate	**Untreated**	**2mm**	**1mm**	**0.5 mm**
Spent grain				
Grass clippings				

Figure S-7.4. Images of the different treatment conditions for spent grain and grass clippings (L. Velasquez, 2023).

Table S-7.7. BSFL survival rate, final larval weight and larval protein mass on mechanically pretreated spent grain and grass clippings. Data displayed are mean ± standard deviation (n=4).

Substrate	Treatment condition	Survival rate (%)	Final larval weight (mg DM/individual larva)	Larval protein (% DM)
Spent grain	Untreated	97.0 ± 1.7	27.4 ± 2.1	37.9 ± 1.1
	2.0 mm	96.4 ± 2.6	32.5 ± 1.9	39.4 ± 0.6
	1.0 mm	93.6 ± 3.1	27.6 ± 2.2	38.5 ± 0.6
	0.5 mm	99.1 ± 0.7	39.8 ± 3.1	34.6 ± 1.6
Grass clippings	Untreated	98.6 ± 0.5	27.3 ± 0.9	38.4 ± 0.7
	2.0 mm	99.8 ± 0.5	31.4 ± 1.8	35.9 ± 1.1
	1.0 mm	98.9 ± 1.7	31.5 ± 1.2	35.1 ± 0.7
	0.5 mm	99.5 ± 0.4	38.5 ± 0.5	34. 3 ± 0.6

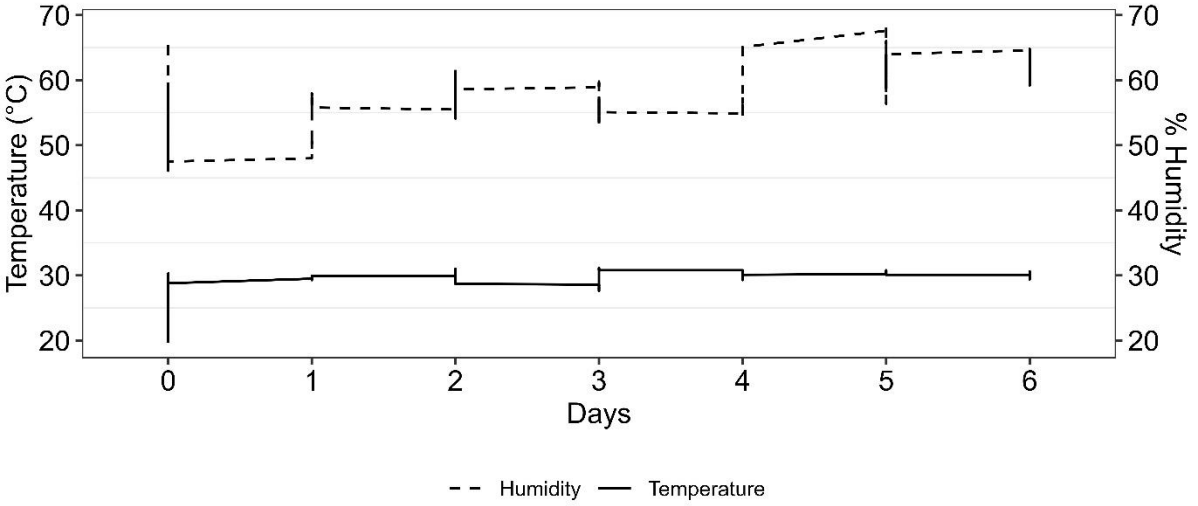


Figure S-7.5. Ambient temperature and humidity in the larval rearing container.

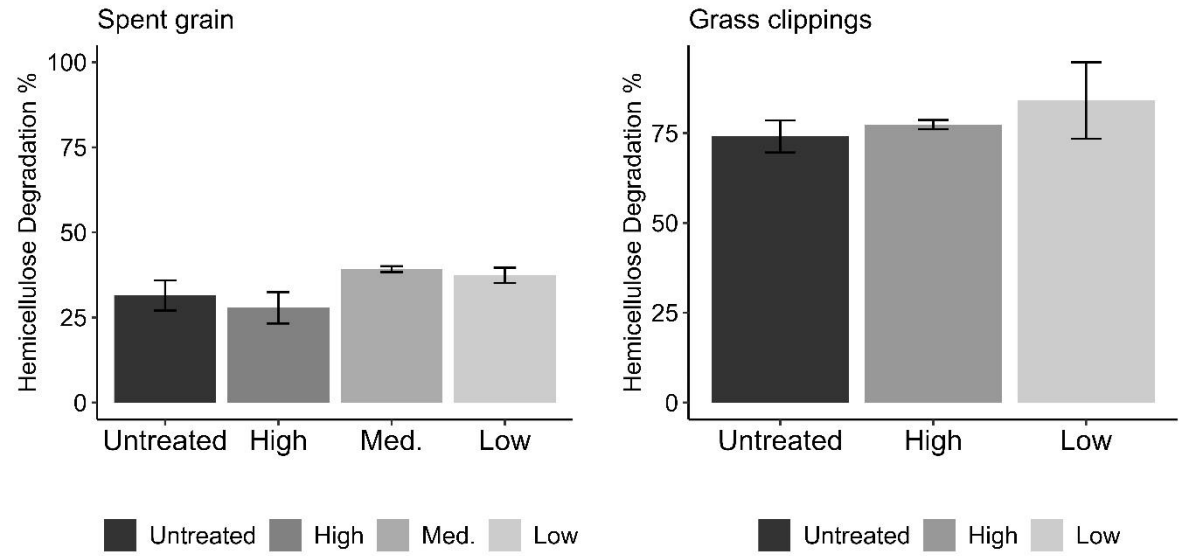


Figure S-7.6. Reduction of hemicellulose was higher on grass clippings than on spent grain. Data displayed are mean with error bars as standard deviation (n=3).

Table S-7.8. Second mechanical pretreatment larval feeding experiment for spent grain and grass clippings untreated and low particle size. Data displayed are mean ± standard deviation (n=3).

Substrate	Treatment condition	Final larval weight (mg DM/individual larva)	Bioconversion rate (% DM)	Waste reduction (% DM)
Spent grain	Untreated	24. ± 1.9.1	10.5 ± 0.6	47.1 ± 1.2
	Low	26.3 ± 0.6	10.9 ± 0.7	37.3 ± 0.8
Grass clippings	Untreated	9.0 ± 0.3	5.6 ± 0.3	17.0 ± 3.2
	Low	11.1 ± 0.6	7. 0 ± 0.4	36.6 ± 1.3

Table S-7.9. Total viable counts in the substrates and treatments (untreated and low) on day 0, 3 and 5 in the second mechanical pretreatment larval feeding experiment. Data displayed are mean ± standard deviation (n=3). *Only one replicate could be analyzed for spent grain low on day 0.

Substrate	**Treatment**	**Log_{10} cfu/g**		
		0	3	6
Spent grain	Untreated	8.4 ± 1.2	9.0 ± 0.6	9.3 ± 0.2
	Low	7.9*	8.7 ± 1.0	8.9 ±0.2
Grass clippings	Untreated	8.6 ± 0.1	9.1 ± 1.2	9.9 ± 0.1
	Low	8.1 ± 0.2	9.4 ±0.2	9.2 ±0.2

Equations used for calculating the LEEB depth-dose distribution of the dried insects.

$$Correction\ factor = \frac{density\ of\ B3\ film\ \left(\frac{g}{cm^3}\right)}{density\ of\ insect\ \left(\frac{g}{cm^3}\right)} \qquad \textit{equation (1)}$$

$$50\%\ LEEB\ depth - dose\ distribution\ of\ insects\ (\mu m)\ = 289\ um\ x\ correction\ factor \qquad \textit{equation (2)}$$

Table S-7.10. Composition of agar media used for aerobic microbial counts.

Aerobic total viable count and bacterial spore count agar	**LB agar (*E. coli*)**	**Yeast and moulds agar**
• 15 g/L Agar; (VWR International, Belgium) • 8 g/L Nutrient Broth, (Difco, Switzerland)	• 15 g/L Agar; (VWR International, Belgium) • 10 g/L Trypton (Sigma-Aldrich Switzerland) • 10 g/L NaCl (Sigma-Aldrich Switzerland) • 5 g/L Yeast extract (Sigma-Aldrich Switzerland)	• 5 g/L Agar; (VWR International, Belgium) • 39 g/L potato dextrose agar (Sigma-Aldrich Switzerland) • 1 ml 10% lactic acid (Sigma-Aldrich Switzerland)

(a) Microwave dried BSFL

(b) Microwave dried mealworm

(c) Oven dried BSFL

(d) Oven dried mealworm

Figure S-7.7. Surface view of the whole dried insect products used for the LEEB trials.

Table S-7.11. Penetration depth at 50% of LEEB dose of BSFL and mealworm and the physical properties. Data displayed are mean values and standard deviation. Thickness and length sample size n=10 and density n = 2−3.

	BSFL				Mealworm			
	Density (g/cm^3)	**Thickness (mm)**	**Length (mm)**	**Depth (µm)**	**Density (g/cm^3)**	**Thickness (mm)**	**Length (mm)**	**Depth (µm)**
Microwave	0.317 ± 0.046	3.6 ± 0.5	2.0 ± 0.2	1021	0.413 ± 0.122	2.1 ± 0.4	2.2 ± 0.3	783
Oven	0.795 ± 0.023	2.3 ± 0.4	1.9 ± 0.3	407	0.604 ± 0.198	2.9 ± 0.3	2.2 ± 0.4	536

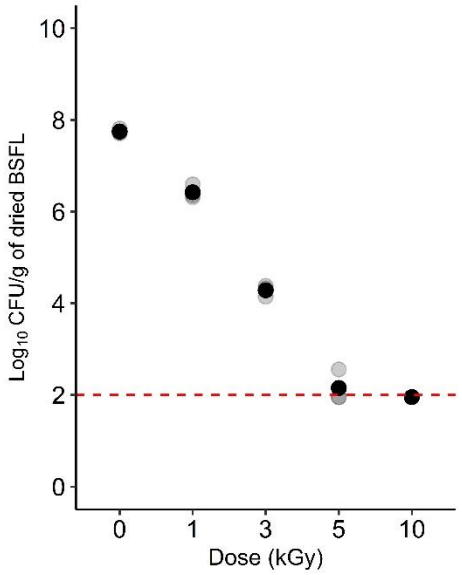


Figure S-7.8. *E. coli* K-12 inactivation using HEEB at 10 MeV with the following doses of: 0, 1, 3, and 5 kGy and 10 kGy. The red line indicates the detection limit of 2-$\log_{10}$ cfu/g. As a conservative approach, samples where colonies were not observed are graphed at the detection limit. Mean is shown in bold with replicates shown in grey (n=3).

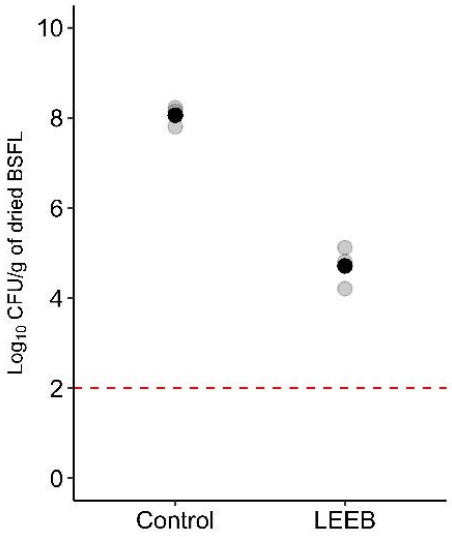


Figure S-7.9. *E. coli* K-12 numbers in the control or LEEB-treated BSFL (250 keV, 12.3 ± 2 kGy). Mean is shown in bold with replicates shown in grey (n=3). The detection limit shown in red is 2-$\log_{10}$ cfu/g dried BSFL.

Table S-7.12. Moisture content (%) and water activity a_w (-) of microwave dried whole BSFL and mealworm. Data displayed are mean values and range (n=2).

Insect	**Treatment**	**Physicochemical parameters**	
		Moisture Content (%)	Water activity a_w
Microwave dried BSFL	Control	6.3 ± 0.05	0.40 ± 0.02
	LEEB-treated	7.2 ± 0.04	0.44 ± 0.01
Microwave dried mealworm	Control	6.1 ± 0.02	0.47 ± 0.00
	LEEB-treated	6.0 ± 0.04	0.46 ± 0.01

Table S-7.13. Moisture content (%) and water activity a_w (-) of BSFL during six-month shelf life test. Data are displayed with mean values and standard deviation (n=3).

Treatment	**Parameter**	**Storage time (months)**						
BSFL		0	1	2	3	4	5	6
Control	Water activity	0.33 ± 0.05	0.37 ± 0.02	0.42 ± 0.01	0.41 ± 0.00	0.48 ± 0.00	0.48 ± 0.01	0.45 ± 0.01
LEEB	Water activity	0.30 ± 0.01	0.38 ± 0.01	0.41 ± 0.01	0.41 ± 0.01	0.47 ± 0.01	0.48 ± 0.01	0.44 ± 0.00
Control	Moisture content	2.33 ± 0.01	3.0 ± 0.2	4.2 ± 0.2	4.0 ± 0.1	5.2 ± 0.1	5.5 ± 0.1	4.9 ± 0.0
LEEB	Moisture content	2.4 ± 0.1	3.1 ± 0.2	4.4 ± 0.1	4.3 ± 0.1	5.0 ± 0.1	5.6 ± 0.1	4.9 ± 0.1

Table S-7.14. Moisture content (%) and water activity (-) of mealworm during six-month shelf-life test. Data displayed are mean values and standard deviation (n=3).

Treatment	Parameter	Storage time (months)						
Mealworm		0	1	2	3	4	5	6
Control	Water activity	0.42 ± 0.01	0.46 ± 0.004	0.47 ± 0.02	0.44 ± 0.04	0.53 ± 0.01	0.53 ± 0.03	0.51 ± 0.01
LEEB	Water activity	0.41 ± 0.01	0.44 ± 0.01	0.42 ± 0.02	0.42 ± 0.04	0.49 ± 0.02	0.49 ± 0.03	0.49 ± 0.01
Control	Moisture content	6.53 ±	6.75 ± 0.11	6.72 ± 0.06	6.04 ± 0.12	7.94 ± 0.51	8.16 ± 0.12	7.09 ± 0.12
LEEB	Moisture content	6.24 ± 0.1	7.00 ± 0.13	6.73 ± 0.07	6.57 ± 0.05	8.32 ± 0.62	8.54 ± 0.05	7.29 ± 0.06

Table S-7.15. Primary (peroxide value) and secondary (p-anisidine value) lipid oxidation were monitored during the oven-dried mealworm shelf-life at four different time points. ND: non-detect. Data displayed is of one replicate (n=1). Except for month 0 where data displayed are mean values and standard deviation (n=3).

Storage Time (months)	Treatment	Peroxide value (meq./kg fat)	p-Anisidine value (-)
0	Control	1.97 ± 0.32	N.D.
	LEEB	3.0 ± 0.1	N.D.
1	Control	1.7	0.6
	LEEB	2.8	0.6
3	Control	2.6	0.6
	LEEB	3.2	1.8
6	Control	2.3	1.8
	LEEB	3.0	1.0

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Zhu, N., Gao, J., Liang, D., Zhu, Y., Li, B., Jin, H., 2021. Thermal pretreatment enhances the degradation and humification of lignocellulose by stimulating thermophilic bacteria during dairy manure composting. Bioresour. Technol. 319. https://doi.org/https://doi.org/10.1016/j.biortech.2020.124149

Curriculum vitae

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EDUCATION

Doctoral Candidate, Sustainable Food Processing October 2023
ETH Zurich, Zurich, Switzerland
Thesis: Pretreatments to improve black soldier fly larvae performance on fibrous biowastes sand safeguarding insect-based food and feed

***Master of Science,* Civil and Environmental Engineering,** December 2018
University California Davis, Davis, CA
Thesis: Evaluating the Microbial Safety of Fecal Sludge Derived Products: Heat Treatment of Fecal Sludge for Black Soldier Fly Larvae Production in South Africa

***Bachelor of Science,* Civil Engineering** May 2013
Specialization: Environmental
Florida State University, Tallahassee, FL

WORK EXPERIENCE

Environmental Engineer and Project Manager May 2014- July 2018
PSI, Miami, FL

- Perform oversight and management of various types of projects, including but not limited to:
  - Phase I Environmental Site Assessments (ESAs)
  - Phase II ESAs
  - Supplemental Site Assessment
  - Monitoring Plans
  - Source Removals
  - Tank Closure Assessments

- Develop scope of work and draft reports under guidance of Professional Engineer.
- Establish and maintain client relations.
- Conduct all relevant field work in compliance with Florida Department of Environmental Protection (FDEP) standard operating procedures.
- Provide presentation to senior management that includes status of project, client relations and budgetary considerations.

Environmental Consultant August 2013-March 2014

African Lion Agriculture Limited, Sierra Leone, Africa

- Report to the CEO to plan, coordinate and implement following certifications:
    - Global G.A.P.
    - Roundtable Sustainable Palm Oil (RSPO)
    - Rainforest Alliance

AWARDS AND RECOGNITION

- J. Williams Fulbright Scholarship July 2018-May 2019
- George and Rosemary Tchobanoglous Graduate Fellowship September 2017-March 2018
- UC Davis Blum Center Poverty Alleviation through Sustainable Solutions July 2017

SKILLS

- Excellent interpersonal and communication skills (written and verbal);
- Multicultural communication skills and bilingual language ability (English/Spanish);
- Proficient user of Microsoft Office products and R software;

PUBLICATIONS

Peguero, D.A., Gold, M., Velasquez, L., Niu, M., Zurbrügg, C. and Mathys, A., (under review). Physical pretreatment of three biowastes to improve black soldier fly larvae bioconversion efficiency. Waste Management Elsevier Ltd.

Peguero, D. A., Gold, M., Duewell, T., Waser, A., Dubovcova, B., Vandeweyer, D., Zurbrügg, C., & Mathys, A. (2023). Low energy electron beam to support product safety of dried insect products. Journal of Insects as Food and Feed.

Peguero, D.A., Gold, M., Endara, A., Niu, M., Zurbrügg, C. and Mathys, A., (2023). Evaluation of ammonia pretreatment of four fibrous biowastes and its effect on black soldier fly larvae rearing performance. Waste Management Elsevier Ltd, 160: 123–134.

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Spykman, R., Hossaini, S. M., **Peguero, D. A.,** Green, A., Heinz, V., & Smetana, S. (2021). A modular environmental and economic assessment applied to the production of Hermetia illucens larvae as a protein source for food and feed. The International Journal of Life Cycle Assessment, 26(10), 1959-1976.

Peguero, D. A., Mutsakatira, E. T., Buckley, C. A., Foutch, G. L., & Bischel, H. N. (2021). Evaluating the microbial safety of heat-treated fecal sludge for black soldier fly larvae production in South Africa. Environmental engineering science,38(5), 331-339.

Peguero, D., Foutch, G., Smay, J., Sahondo, T. M. C., Xaba, L. P., Hayangah, T. P. A., Sindall, R.C., Buckley,

C.A., Bischel, H. N. (2018). Microbial evaluation of the viscous heater for commercial applications in faecal sludge treatment. In WEDC (pp. 1–6).

CONFERENCE CONTRIBUTIONS

Peguero, D.A., Gold, M., Endara, A., Zurbrügg, C., Mathys, A. Ammonia pretreatment of agri-food wastes to enhance black soldier fly larvae bioprocessing performance (2022). *Insects to Feed the World*, Quebec City, Canada, Presentation.

Peguero, D.A., Gold, M., Duewell,T., Dubovcova, B., Waser, A., Zurbrügg, C. and Mathys, A. Assessment of low-energy electron beam (LEEB) treatment of two whole dried insect products (2022). *Insects to Feed the World*, Quebec City, Canada, Presentation.

Peguero, D.A., Gold, M., Endara, A., Zurbrügg, C., Mathys, A. Pretreatment methods to improve black soldier fly larvae bioconversion of agri-food wastes and byproduct (2021). *World Food System Center Research Symposium*, Zürich, Switzerland, Poster.

Peguero, D.A., Green, A., Smetana, S., Mathys, A. Inventory and state of the art for sustainable insect production (2021). 72nd *Annual Meeting of the European Federation of Animal Science*, Poster.

Peguero, D.A., Gold, M., Zurbrügg, C., Mathys, A. Pretreatment strategies to improve insect processing for use as protein rich animal feed ingredient (2021). 72nd *Annual Meeting of the European Federation of Animal Science*, Presentation.

Peguero, D.A., Mutsakatira, E.T., Tikilili, P.Z., Lewis, M., Richards, C.S., Buckley, C.A., Mathys, A. Fecal sludge management using black soldier fly larvae and product safety (2020). *Insects to feed the world*, Virtual, Presentation.
