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FINAL REPORT

Optimising and Industrialising Black Soldier Fly (BSF)

ENDFOODWASTE COOPERATIVE RESEARCH CENTRE

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Panel Review Statement

End Food Waste CRC recognises the value of knowledge exchange and the importance of objective peer review. It is committed to encouraging and supporting its research teams in this regard. The author(s) confirm(s) that this document has been reviewed and approved by the Project Leader and Industry Partner.

This project has also been evaluated by the End Food Waste CRC publication review panel. These reviewers evaluated its:

- Methodology articulated clearly
- Positioning of findings within the current literature
- Acknowledged compliance with food safety standards
- Conclusions against results
- Relevant human and/or animal ethic approvals obtained

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Industry Partner Foreword

At Goterra, our mission has always been to innovate at the intersection of technology, biology, and sustainability. Our collaboration with the End Food Waste CRC (Formerly Fight Food Waste) and the University of Queensland has been instrumental in furthering this mission, and the outcomes of this partnership have had a profound impact on the trajectory of our business.

The development of a comprehensive literature review on insect protein regulation, has not only deepened our understanding of the existing regulatory landscape but has also enabled us to actively shape the framework around waste processing with insects. In an emerging industry like ours, regulatory clarity is crucial, and this work has provided Goterra with a clear path forward as we continue to scale and innovate. We now stand at the forefront of regulatory developments in the insect protein sector, and this has strengthened our position as industry pioneers.

Another pivotal achievement of this collaboration is the creation of a data set for Near-Infrared (NIR) technology that allows us to profile incoming waste with unparalleled accuracy. This capability has transformed the way we calibrate our machines, optimising them for more efficient consumption and ultimately improving our bottom line. This milestone is only the beginning of a larger vision—comprehensive data collection across our entire supply chain. With this foundation, we can track emissions, predict protein yields, and drive more sustainable practices. This project has set the stage for smarter, data-driven operations that will be essential to our future growth.

Additionally, the research produced by our PhD candidate has had a significant impact on Goterra's reputation within the industry. These academic contributions have elevated our standing as market leaders, underlining the scientific rigor behind our operations and validating our approach to insect-based waste management.

This project has not only accelerated our technological and scientific capabilities but has also reaffirmed Goterra's leadership in the sustainable waste processing space. Our collaboration with the End Food Waste CRC (Formerly Fight Food Waste) and the University of Queensland has been invaluable, and we look forward to building on this success as we continue to innovate, scale, and lead the future of sustainable waste management.

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Olympia Yarger

CEO, GOTERRA

20/09/2024

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Executive Summary

Annually, 1.3 billion tonnes of food waste is generated globally. The reduction of food waste by insect larvae and their use as feedstock for animals has been receiving attention lately. Black soldier flies (*Hermetia illucens*) are recognised as an ideal species for upcycling organic wastes owing to the ability of their larvae to reduce the different types of biological wastes. The nutritional composition and amino acid profile of black soldier fly larvae (BSFL) raised on these organic wastes is similar to several animal feed constituents making them a suitable material for animal feed. However, the commercialisation of BSFL in Australia is limited due to existing legislative regulations and the lack of information regarding their safety as feed. This research project aimed to determine the safety and nutritional composition of BSFL reared from different food waste streams. BSFL harvested from different waste streams in commercial production facilities was examined for different chemicals (mycotoxins, heavy metals) and biological safety issues (detection and enumeration of pathogenic microbes). The initial phase of the project involved rearing BSFL (5^{th} instar) and pre-pupae (6^{th} instar) on homogenous soy waste and customised bread-vegetable waste diet to examine the effect of larval instar in influencing the safety and nutritional quality of the product. The project's second phase involved rearing the BSFL on complex waste streams typically encountered in the industry, such as food waste from supermarkets, and a food waste mixture from supermarkets, retail stores, a childcare centre, and fast-food restaurants. BSFL reared using these food wastes were subjected to two different processing steps, blanching and drying, to investigate the effect of processing on the nutritional and safety aspects of the larvae. The project also explored the potential of using near-infrared spectroscopy (NIRS) in combination with chemometric approaches to quickly monitor the production process, quality, and microbial safety of larvae reared using various food waste streams.

BSFL reared on commercial waste streams had limited presence of several pathogenic microbes, including *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli,* and *Staphylococcus aureus*. The thermal processing methods utilised in this study ensured that the larvae met the microbial safety standards established for animal feeds by regulatory bodies. However, these post-harvest treatments were insufficient to mitigate the risk of spore-forming bacterium *Clostridium perfringens*. The frass resulting from the bioconversion process had microbial counts similar to those of the substrate and fell within the limits recommended for fertilisers by EU Regulation (EC No. 142/2011).

The concentrations of heavy metals in BSFL were found to be below the maximum threshold limits (MTL) established for animal feeds in both Australian and European legislation. Their concentration mainly influenced the distribution of heavy metals in BSFL in the food waste stream. Certain heavy metals were found to be more concentrated in 6^{th} instar BSFL compared to 5^{th} instar BSFL. The processes of blanching and drying, despite showing varied effects with different heavy metals, ensured that the concentration of heavy metals in the larvae remained within the MTL. The residual frass from various food waste streams was also within the MTL established for fertilisers by regulatory bodies. Additionally, mycotoxins such as Aflatoxin B1 (AFB1), Deoxynivalenol (DON), Ochratoxin A (OTA), and Zearalenone (ZEN) were found to be below the limit of quantification in BSFL from the different food waste streams.

The nutritional profile of BSFL was influenced by rearing substrate, larval age, and post-harvest treatments. Of these, the rearing substrate was the most critical factor. Post-harvest treatments in the studies did not significantly alter the nutritional composition, including chemical composition, amino acid, fatty acid, and mineral composition. The larval instar was found to affect the crude fat and fatty acid content of the BSFL, with a partial effect on their amino acid composition. Furthermore, the mineral contents of the BSFL were also influenced by the substrate, while the age of the BSFL had minimal to no effect on them.

The study evaluated the potential of BSFL as a protein and energy source in a balanced commercial poultry diet. After determining the true digestive values of BSFL from three different waste streams: Brisbane (waste from cafes and restaurants mainly consisting of leftover bread, vegetables, egg shells, coffee beans etc.), Hume (all sorts of food waste including waste from retail outlets, and

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supermarkets), and Lendlease (retail and household food waste), diets were formulated for grower and finisher stages with four inclusion levels (0%, 3%, 6%, and 9%) of BSFL. This resulted in 12 experimental diets, all meeting Aviagen nutrient recommendations. As expected, neither the source of the BSFL nor the inclusion levels affected the production parameters (Average Daily gain (ADG), Food Conversion Ratio (FCR)) of the broilers at 42 days of age.

Studies were conducted to investigate using NIR spectroscopy to trace the feed source and distinguish between 5th and 6th instar larvae fed on different food waste streams. The study used NIRS in combination with chemometric techniques such as Partial Least Squares-regression to predict various nutritional properties, including crude protein (CP), crude fat (CF), starches, sugars, neutral detergent, and acid detergent fibre for 5th and 6th instar BSFL, as well as their waste (frass) from soy waste and a custom bread-vegetable diet. The calibration models showed good accuracy for acid detergent fibre (ADF) but moderate accuracy for total carbon, CP, CF, NDF, starch, and sugars.

This project successfully delivered all intended outcomes and has made recommendations for future research to build upon the work in this project. Reports like this can contribute to policy changes, such as the regulations being relaxed appropriately regarding the use of BSFL that will, in turn, continue to drive the growth of the BSFL industry and upcycling of food waste into valuable products.

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1. Introduction

1.1 Previous Research & Literature

1.1.1 General introduction

The rapid population growth accompanied by urbanisation and improved economies in developing nations has increased the consumption and demand for animal-based proteins (Boland et al., 2013; Ismail et al., 2020; Kim et al., 2019a,b). Livestock rearing by conventional agricultural practices is decreasing due to the reduced availability of land, feed, and several other resources caused by climate change and various other socio-environmental factors (Kim et al., 2019). Several studies have revealed that the use of edible insects as feed for livestock can provide a sustainable solution to meet the meat demand of the growing population (Dobermann et al., 2017; Guiné et al., 2021; van Huis & Oonincx, 2017).

Black soldier fly (*Hermetia illucens*) larvae (BSFL) have received interest among several other edible insects in the food and feed sector. BSFL can be reared on different organic waste substrates including household waste, spent grains, human faeces, and animal manure (Banks et al., 2014; Barbi et al., 2020; Nguyen et al., 2013; Nyakeri et al., 2017; Oonincx et al., 2015; Shen *et al*., 2024). Black soldier flies have exhibited the efficiency to bio-convert these organic side streams into value-added products including BSFL and Frass. The nutritional composition, amino acid, and fatty acid profile of BSFL reared from these bio-products is comparable to that of conventional feed ingredients and is strongly influenced by the composition of the waste stream used to feed the larvae. Several studies have also indicated the suitability of BSFL as feed for monogastric animals as well as in aquaculture, and as an alternative source of protein for rearing livestock (Lu et al., 2022; Seyedalmoosavi et al., 2022; Salahuddin et al., 2024; Wang & Shelomi, 2017).

Overall, BSFL fed with different biowaste streams have demonstrated the ability to replace more conventional feed ingredients and offer a circular economy-based solution to the agri-food sector. Despite this, the commercialisation of BSFL as a feed ingredient is not widespread. The absence of specific regulations regarding the use of insects as feed is an important factor influencing their commercialisation (Domingues et al., 2020). Several countries cannot decide which agencies should be involved in regulating insects under the context of food and feed. The prevailing ambiguity within these agencies has delayed the development of concrete legislative frameworks for insects as feed (Domingues et al., 2020; Halloran et al., 2014). Unclear legislation makes it challenging to understand the national and international standards of production, processing, and quality of insects reared for food and feed (van Huis et al., 2013). Insect production facilities are increasing in several countries. However, the trade of edible insects as animal feed remains in its infancy and fails to attract investors due to the lack of understanding of the existing regulations (Halloran et al., 2014). Currently, only a limited number of countries, including the USA, Canada, Australia, and nations belonging to the European Union (EU), have drafted specific regulations for the use of BSFL as feed (Lähteenmäki-Uutela & Grmelová, 2016; Lähteenmäki-Uutela et al., 2017). However, using excreta and processed waste containing animal matter for rearing BSFL for livestock feed is prohibited in these regions. The lack of information about safety issues from rearing BSFL on biowastes is an important factor in influencing the development of the regulatory framework (Imathiu, 2020; van der Fels-Klerx et al., 2018).

The literature review gave an overview of BSF, its life cycle, and nutritional composition. It discussed the current legislative landscape for using organic waste streams as feed for BSFL and their subsequent use as animal feed in different countries worldwide. Finally, the safety issues associated with rearing BSFL from organic side streams was discussed further. The literature reviews have been published, and the complete reports are available (Alagappan et al, 2021, 2022a,b).

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1.1.2 BSF- Life cycle and overview as feed

1.1.2.1 Life cycle of Black soldier fly

Black soldier flies (*Hermetia illucens*; L. 1758; Diptera: Stratiomyidae) are widely distributed in the tropical and subtropical regions and are currently considered to be the major player in the rapidly developing insect feed sector (Kaya et al., 2021). The life cycle of BSFL begins once the adult female flies oviposit and lay around 200-600 eggs (Liew et al., 2022; Purkayastha & Sarkar, 2021). The eggs are laid on dry surfaces and hatched into larvae within 4 days (Hoc et al., 2019). The larvae of BSF go through six larval instars, and these larvae are polyphagous saprophages that can actively forage on a variety of organic products (Barragán-Fonseca et al., 2020). The duration of this larval stage can range between 10-52 days depending upon the type of substrate, temperature, and humidity of the feeding environment (Liew et al., 2022). Larval instars 1-4 are whitish in colour, creamy in appearance, and differ in length and size. The length of the 1st, 2nd, 3rd, and 4th instar larvae are found to vary between 2-5 mm, 6-9 mm, 10-13 mm, and 14-16mm, respectively. The 5th instar larvae are brownish grey in colour and are approximately 17-20mm in length. A prominent dark brownish-black colour characterises the pre-pupae and are around 20-22mm in length (Barros et al., 2019; Kim et al., 2010; Sivanantharaja & Gnaneswaran, 2018).

Upon reaching the pre-pupal stage, BSFL stops feeding and disperse from the feed. The emergence of pupa from the 6th instar BSFL or the pre-pupa takes 14 days and involves 6 stages of molting (Purkayastha & Sarkar, 2021). The pupae develop into adult BSF generally in 10-14 days at suitable environmental conditions (Holmes et al., 2013). The adult BSF are 15-20 mm in length. The adult flies do not have mouth parts, and they rely on their fat stores and liquid supplements for survival and mating (Tomberlin et al., 2009). The mating between the male and female adult flies commences two days after eclosion during flight or in ground under sunlight or similar environmental conditions and, successful mating leads to oviposition in two days (Heussler et al., 2018; Sheppard et al., 2002).

1.1.3 Chemical composition

BSFL are especially known for their protein and fat content. The protein and fat content in BSFL are highly influenced by the substrate used for rearing (Bessa et al., 2020; Franco et al., 2021; Gold et al., 2020). Franco et al. (2021), in their review, reported that the average protein content of BSFL reared on organic food waste streams is observed to be 31.2%. The fat content in these larvae was found to vary between 15-49% (Franco et al., 2021). The fat composition in BSFL is observed to exceed the minimum fat requirements for livestock (Fasakin et al., 2003). The carbohydrate content in the substrate typically influences the lipid content of BSFL, while the protein content is mediated by the type of macronutrients present in the feed (Gold et al., 2020; Oonincx et al., 2015a,b). The use of organic waste streams with a balance in protein and carbohydrate values usually results in BSFL with better protein values (Gold et al., 2020). The protein content in BSFL generally decreases with age (Bessa et al., 2020); this is attributed to the enzymatic catalysis of protein, resulting in the development of chitin (Purkayastha & Sarkar, 2021). The fat content of BSFL reared with different types of manures, including chicken, cattle, and pig manure, ranges between 15-35% (Arango Gutiérrez et al., 2004; Newton et al., 2005). The utilisation of fruit waste, restaurant waste, and similar waste rich in non-fiber carbohydrates typically results in BSFL with a fat content exceeding 35% (Meneguz et al., 2018; Spranghers et al., 2017).

The amino acid composition defines protein quality. The amino acid profile of BSFL consists of a well-balanced mix of essential and non-essential amino acids (Surendra et al., 2020). Lysine is an essential amino acid required in livestock feed and is found at lower concentrations in conventional grain-based ingredients. (Bessa et al., 2020). Lysine in BSFL constitutes ≈5.6% of the total amino acids, making it a good source of this amino acid for livestock (Hopkins et al., 2021). BSFL is also an excellent source of arginine owing to the better bioavailability of this compound in the larvae (Belghit, et al., 2019). Amino acids such as tryptophan, threonine, valine, and cysteine, which are essential in swine and chicken diets, are present in higher proportions in BSFL compared to

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soybean meal and other plant-based feeds (Belghit, et al., 2019). It is important to note that the amino acid profile of BSFL is not significantly affected by the amino acid composition of the substrate fed to BSFL (Ravi et al., 2020).

The fatty acid profile of BSFL is dominated by saturated fatty acids that account for 60-80% of the total fatty acid content (Franco et al., 2021; Surendra et al., 2020). The predominant saturated fatty acid in BSFL is Lauric acid (C12:0) (32-60%) followed by palmitic acid (c16:0) (8-20%) and oleic acid (C18:1 n-9) (5-12%) (Franco et al., 2021; Surendra et al., 2020). The concentration of essential unsaturated fatty acids such as linoleic acid and α-linolenic acid is relatively low in BSFL (Meneguz et al., 2018). The fatty acid content of BSFL is solely dependent on the type of substrate used to rear them (Franco et al., 2021). Therefore, BSFL reared on organic waste streams with low nutrient (lipids) value are expected to have these essential unsaturated fatty acids in lower concentrations (Gold et al., 2020). Also, the concentration of unsaturated fatty acids in BSFL decreases with the increase in the age of the larvae (Liu et al., 2017). However, it is noteworthy that the concentration of these essential fatty acids in BSFL can be enriched by adding these specific constituents to the feed (Liland et al., 2017; Truzzi et al., 2020).

BSFL are known to accumulate minerals and vitamins from the substrate (Wang & Shelomi, 2017). Minerals such as manganese, calcium, iron, phosphorous, zinc, and Vitamin E are accumulated by BSFL (Liu et al., 2017; Spranghers et al., 2017; Surendra et al., 2020). Iron and zinc are micronutrients found at higher concentrations in BSFL (Bessa et al., 2020). The micronutrients are generally available at higher concentrations in 6th instars rather than in the 5th instar larvae (Liu et al., 2017).

1.2 Legislative Landscape

The consumption of insects as food and feed is rapidly increasing in Australia, and it has gained the attention of legislative and regulatory bodies. The Food Safety Australia and New Zealand (FSANZ) has recognised super mealworm (*Zophobas morio*), mealworm beetle (*Tenebrio molitor*), and house crickets (*Acheta domesticus*) as traditional foods (Marone, 2016). Insects are considered as animals and their usage as feed is mediated by the stockfeed laws prevailing in different states across Australia. Each of these states have different regulations pertaining to the feeds for ruminants, pigs, poultry, and aquaculture (AgriFutures Emerging Industries, 2020; IPAA, 2020).

1.3 Gaps in Current Knowledge

The use of BSFL as feed is becoming popular in several regions across the globe. It is approved in the EU, USA, Australia, and Canada under specific legislative policies. The use of biowaste containing meat and other products of animal origin as a substrate for rearing BSFL is banned in the EU. In Australia, different states have specific regulations regarding using bioproducts for rearing insects intended for use as feed. Most Australian states prohibit the use of insects reared from biowaste as feed for ruminants.

A pre-requirement from the livestock industry is that any feedstuff used in animal nutrition should have a known chemical/nutritional value. It is known that the chemical composition of the waste stream influences the composition and safety of the BSFL reared thereupon. Therefore, more research is required to see what the effect of feeding different composite waste streams, as is typically encountered by Goterra, has on the nutritional value and safety of the BSFL. However, the BSFL reared typically undergoes some form of processing before being utilised as animal feed – the effect of this post-harvest processing needs to be quantified to ensure that Goterra produces a product with a known composition.

The following gaps were identified and addressed during the research period:

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- Defining a roadmap to harmonise current BSFL production as practiced by Goterra with existing standards/regulations governing livestock feed and defining research priorities.
- Characterise the chemical composition and biological safety of BSFL reared on different waste streams utilised by Goterra and develop screening methods to discover and control waste streams appropriate for producing animal/chicken feed.
- Evaluate the impact of different processing methods on the suitability and safety of BSFL reared in different waste streams.
- Evaluate the suitability of the frass derived from the different waste streams utilised by Goterra as a soil enhancer.
- Develop a rapid, non-invasive method to determine the nutritional value of BSFL raised on different waste streams.

2. Methodology

Presently, the use of insects in research does not require ethical approval. Nevertheless, due care was taken during the various experiments to ensure the BSFL did not experience any perceived suffering.

Due to COVID-19, we were unable to access Goterra's facility but managed to work with a local BSFL rearing facility where we were able to do some of the initial experiments.

The experiments consisted of several different trials evaluating the effect of different feed combinations, harvesting of larvae at different developmental stages, and conducting different post-harvest processing methodologies (***Figure 1*** Graphic design of the experimental .

The BSFL was subjected to basic proximate analyses, fatty acid analyses, and amino acid analyses. It was also evaluated and tested for various biological and chemical contaminants.

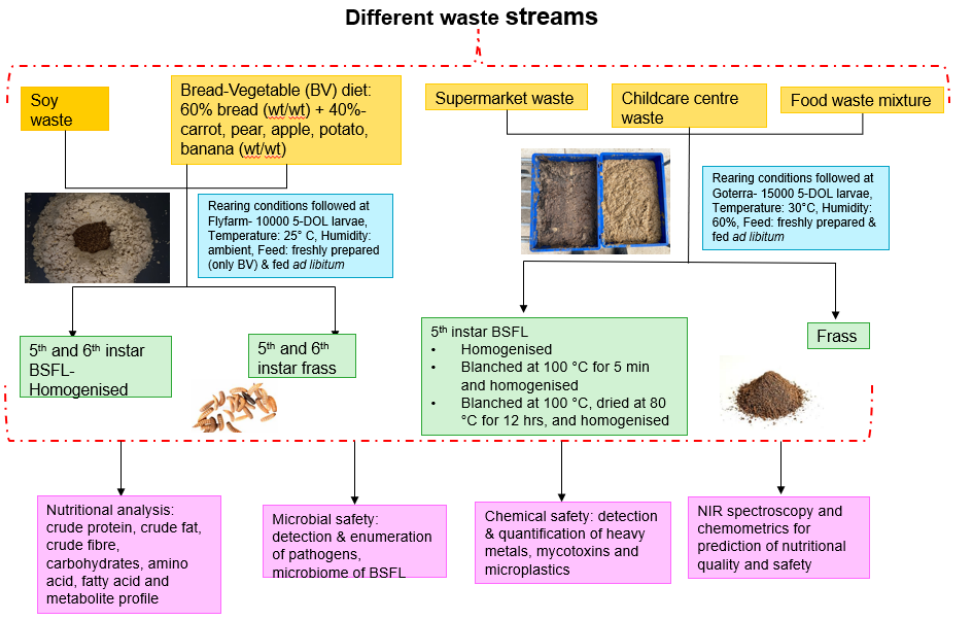

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Figure 1 Graphic design of the experimental layout

2.1 Biological safety of BSFL reared on different food waste streams

2.1.1 Preparation of waste streams

Five different waste streams viz. soy waste (SW), customised bread-vegetable waste (BV), supermarket waste (SM), childcare centre waste (CC) and a food waste mixture (WM) were used for rearing BSFL. The wet, homogenous SW was obtained from a custard manufacturer. BV was prepared by chopping and mixing bread (60% w/w) and the following fruits and vegetables: banana, apples, pears, carrots, and potatoes (8% each w/w) from a local supermarket. The experiments with heterogenous food waste streams were carried out in Goterra's facilities (**Figure *2*** Goterra's insulated production systems (picture courtesy of Goterra)). The SM waste was primarily comprised of bread, meat, salads, pastries, potato chips and dairy products. The CC waste was constituted mainly of salads, bread and other cooked foods. The WM was essentially a mixture of supermarket waste, fast food waste, restaurant waste, childcare waste, and food waste from local retail outlets. The received waste was removed from its packaging material using an industrial de-packager and ground using an industrial grinder before feeding to the larvae (**Figure *3*** Typical waste fed to BSFL within the Goterra production system (picture courtesy of Goterra)). It should be noted that the rearing conditions such as temperature, humidity, and other related processing conditions are not specified due to confidentiality agreements with the commercial production facilities. Nonetheless, the conditions were controlled to ensure maximum growth of the larvae.

Figure 2 Goterra's insulated production systems (picture courtesy of Goterra)

2.1.2 Rearing of BSFL

A detailed description of the rearing environment and procedures is provided in Alagappan et al., (2022) and Alagappan et al., (2023). The 5th instar BSFL were harvested with sterile tweezers from random spots in the tray following the first sighting of 6th instar (pre-pupae) larvae. The 6th instar BSFL was harvested similarly upon sighting of the 1st pupae. The 5th and 6th instar frass were also collected upon harvesting the larvae, suspended in 40% (v/v) glycerol and stored at -80°C for future analysis.

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Figure 3 Typical waste fed to BSFL within the Goterra production system (picture courtesy of Goterra)

2.1.3 Post-harvest treatments of BSFL

Harvested BSFL samples were immediately subjected to the three processing treatments viz. a) homogenisation (unprocessed) - the whole live larvae samples collected from the trays were homogenised with a food processor; b) blanching - the whole live larvae collected were subjected to blanching at 100°C for 5 minutes and homogenised using a food processor; c) drying - the whole live larvae were blanched according to the conditions mentioned above and subjected to drying in a commercial dehydrator at 70°C for 12 hours followed by homogenisation (**Figure *4*** Dried Black Soldier Fly Larvae (picture courtesy of Goterra)).

2.1.4 Detection and enumeration of microorganisms

The Australian Standards for enumerating yeast and mould (Y&M; AS 5013.29 – 2009), *L. monocytogenes* (AS 5013.24.1 – 2009), *S. aureus* (AS 5013.12.3 – 2004), *Salmonella* spp. (AS 5013.10 – 2009), *Bacillus cereus* (AS 5013.2 – 2007), and *E. coli* (AS 5013.15-2006) were followed. The confirmatory tests included in the standards were not conducted, and the colonies observed based on standards were reported as “presumptively positive”.

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Figure 4 Dried Black Soldier Fly Larvae (picture courtesy of Goterra)

2.2 Chemical safety of BSFL reared on food waste streams

2.2.1 General

These studies used five different waste streams: soy waste (SW), customised bread-vegetable diet (BV), supermarket waste (SM), waste mixture (WM), and childcare centre waste (CC), which were sourced and prepared as mentioned in sections 2.1.1 and 2.1.2. The postharvest treatments of BSFL were as described in section 2.1.3.

2.2.2 Heavy metal analysis

The heavy metals analysis was carried out by the SAFS analytical services unit, University of Queensland (Brisbane, QLD, Australia) using standardised techniques (Alagappan, et al., 2024).

2.2.3 Mycotoxin analysis

The Australian Superintendence Company (Brisbane, QLD, Australia) analysed the mycotoxins. BSFL samples from the different trials were subjected to analysis for the following mycotoxins: Aflatoxin B1 (AFB1), Deoxynivalenol (DON), Ochratoxin A (OTA), Zearalenone (ZEN). It is to be noted that mycotoxin analysis was done only on pooled BSFL samples obtained from the different waste streams employed in this study (Alagappan, et al., 2023).

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2.2.4 Data analysis

GraphPad Prism 10.0.2 was used for the univariate data analysis. A one-way analysis of variance (ANOVA) was conducted at a significance level of $p \leq 0.05$ to assess the significant differences in means among the various samples including substrates (waste steams), BSFL and, frass for the different pathogens enumerated. Additionally, Bartlett's test was utilised to evaluate whether the samples exhibited equal variances, which subsequently guided the selection of an appropriate post hoc test. For sample groups with equal variances, Bonferroni's Tukey test was applied, while for those with differing variances, Dunnett's T3 test was conducted at $p \leq 0.05$ to make comparisons.

2.3 Effect of larval Instar, post-harvest treatments, and substrate on the nutritional profile of BSFL

2.3.1 General

The five different waste streams: Soy waste (SW), customised bread-vegetable diet (BV), supermarket waste (SM), waste mixture (WM) and childcare centre waste (CC) were sourced and prepared as mentioned in sections 2.1.1 and 2.1.2 were used in these studies. The Post harvest treatments of BSFL were as described in section 2.1.3.

2.3.2 Chemical composition

Proximate analyses were carried out by the analytical services unit of the School of Agriculture and Food Sustainability, The University of Queensland (St Lucia, Brisbane, QLD, Australia). Association of Official Analytical Collaboration (AOAC) methods 960.39 and 992.15 were used to determine crude fat (CF) and crude protein (CP) (total Nitrogen x 5.62), respectively. AOAC methods 2002.04 were followed to determine neutral detergent fibre (NDF) (AOAC, 2019).

The different food waste streams and the resulting BSFL were subjected to mineral analysis, which was carried out by the SAFS analytical services unit (UQ).

The SAFS analytical services unit (UQ) conducted amino acid and fatty acid analyses on BSFL samples from different waste streams.

The methodologies utilised are reported in detail in Alagappan, et al. (2023, 2024).

2.4 Effect of black soldier fly larvae reared on different waste streams on broiler production performance.

A total of 576 one-day-old Ross 308 male broiler chickens were obtained from a commercial supplier (Aviagen, NSW) and transferred to the Queensland Animal Science Precinct (QASP) facility at the UQ-Gatton Campus. The birds were housed in pens within a controlled environment. Each experimental diet was allocated to six replicate pens, with eight birds per pen, resulting in 48 birds per experimental group. Infrared lamps were used for the first three weeks to maintain a temperature consistent with standard breeding practices. Samples of each feed ingredient were collected and analysed in duplicate for gross energy, dry matter, crude protein, crude fat, and ash content. These nutrient compositions were used to formulate diets for each dietary phase: starter (1 to 10 days), grower (10 to 29 days), and finisher (29 to 42 days).

The experiment focused on the grower and finisher phases (32 days). Four inclusion levels (0%, 3%, 6%, and 9%) of full-fat BSFL, sourced from three different waste streams (Brisbane, Hume, and Lendlease), were used in the grower and finisher diets, resulting in 12 experimental diets and one starter diet (fed from days 1 to 10), all formulated to meet Aviagen nutrient recommendations. All experimental diets included 0.5% titanium dioxide as an indigestible marker to assess nutrient digestibility. Birds were weighed on

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the first day of the trial and again at the end of each growth phase (days 10, 29, and 42) to determine live body weight. Average daily gain and average daily feed intake were recorded individually and on a pen basis at the end of each growth period. The feed conversion ratio was calculated for each phase and for the entire experiment.

2.5 Near-infrared spectroscopy for monitoring the quality of BSFL

2.5.1 Traceability of BSFL

2.5.1.1 Rearing of BSFL on soy waste and spent brewer's grains

The homogenous soy waste and spent brewers' grain were used as the feed for rearing BSFL in these trials at the commercial BSFL production facility "A". A total of 50 5th instar larvae were collected from each tray and randomness was ensured in sample collection by picking larvae from different spots in the trays. The pre-pupae (6th instars) were harvested in a similar manner upon sighting of the first pupae in the trays. Live samples of 5th instar larvae and pre-pupae were then subjected to scanning by NIR spectrometer.

2.5.1.2 Collection of NIR spectra and data Analysis

The FT-NIR spectra of the larvae samples was collected using a Bruker Tango-R spectrophotometer with a gold-coated integrating sphere (diffuse reflection). Samples were placed in a borosilicate-glass cuvette 10 mm diameter. The reflectance spectra were recorded using OPUS software (version 8.5, Bruker Optics GmbH, Ettlingen, Germany) with 64 interferograms at a resolution of 4 cm^{-1} in the wavenumber range of 11,550 to 3950 cm−1.

2.5.1.3 Data analysis

Multivariate analysis was performed using the Unscrambler X software (v11, CAMO ASA, Oslo, Norway). The NIR spectra were smoothened and pre-processed using the Savitzky- Golay differentiation filter as it has been recognised widely for correcting slope and baseline effects of the spectrum (Savitzky & Golay, 1964). The Savitzky and Golay algorithm at the following operating parameters: second-order polynomial and a smoothing window size of 10 points were used for data pre-processing in these studies (Savitzky & Golay, 1964). Principal component analysis (PCA) was performed to visualise the data structure and identify patterns, trends, outliers in the spectra and other dominant features in the samples according to feed and instars (Cozzolino et al., 2021; Cozzolino et al., 2019). Discrimination models between the NIR and reference data (dummy values for each feed) were developed using partial least squares regression (PLS) using cross-validation (Bureau et al., 2019). The optimal number of factors for the calibration model was selected based on the minimal value of the predicted residual sum of squares (PRESS) and the highest correlation coefficient (R^2) between actual and predicted values was used for the selection of optimal number of factors for the calibration model being developed. The PLS models were evaluated in terms of the number of factors, the standard error of cross-validation (SECV), and the correlation coefficient was used to evaluate the PLS models, and the accuracy of the model was determined using the residual predictive value (RPD) (Bureau et al., 2019; Williams et al., 2017).

2.5.2 Prediction of Nutritional Quality

2.5.2.1 Rearing of BSFL on soy waste and customised bread and vegetables (BV) diet

The 5th and 6th instar BSFL along with their respective frass reared on homogenous soy waste and customised bread-vegetable diet were used in these experiments. The harvested larvae that were subjected to homogenisation and stored at -20°C prior to NIR analysis.

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2.5.2.2 NIR spectra collection

Stored samples were thawed at room temperature and scanned as described in section 2.5.1.2.

2.5.2.3 Proximate analysis

Proximate analyses were carried out as described in section 2.3.2.

2.5.2.4 Data analysis

The Vektor Direktor (version 1.0, Kax Group, Scottsdale, Arizona, USA) was used for the multivariate data analysis. NIR spectral data obtained for the larval and frass samples were analysed as described in section 2.5.1.3. The proximate analysis results were reported as mean ± standard deviation. In addition, a *t-test* ($p < 0.05$) was performed on the proximate analyses results for the different nutritional attributes and samples obtained from the two different feed sources.

2.5.3 Prediction of microbial quality

2.5.3.1 Preparation of feed (food waste steams), BSFL and frass

The five different food waste streams: soy waste, customised bread-vegetable diet, waste mixture and supermarket waste prepared in section 3.2.1 were used in these experiments. The different BSFL and frass samples obtained by following the procedures mentioned in section 3.2.2 and 3.2.3 were used in these studies. The various samples collected in this study was stored at -20°C prior to NIR analysis.

2.5.3.2 Detection and enumeration of micro-organisms

The different samples employed in the study were subjected to testing for yeast and mould counts, *L. monocytogenes*, *S. aureus*, *Salmonella* spp., *B. cereus* and *C. perfringens*.

2.5.3.3 NIR spectra collection

Stored samples were thawed at room temperature and analysed using a Fourier Transform-NIR instrument (Tango-R, Bruker Optics GmbH, Ettlingen, Germany). Samples were placed in a glass cuvette (10 mm diameter) where the spectra were collected as the average of 64 interferograms at a resolution of 4 cm^{-1} in the wavenumber range of 11,550 to 3,950 cm^{-1} (OPUS software, version 8.5, Bruker Optics GmbH, Ettlingen, Germany). The cuvettes were cleaned with 70% ethanol and wiped dry using paper towels between samples.

2.5.3.4 Data analysis

The Vektor Direktor (version 1.0, KAX Group, Sydney, NSW, Australia) was used for multivariate data analysis. The NIR spectral data were smoothed and pre-processed using the Savitzky–Golay second derivative (second order polynomial and a smoothing window size of 10 points) before analysis. Trends, patterns, and outliers in the data set were visualised by performing principal component analysis (PCA) (Cozzolino et al., 2019). Partial least squares regression (PLS) was utilised to develop models for the prediction of Y&M counts in the feed, frass and BSFL samples obtained from the different experiments (Cozzolino, 2021). A summary of the different data set used to develop the PLS models is depicted in

Table 1 Summary of different data sets used to develop the partial least square regression models A leave-one-out cross validation was applied during the development of the PCA and PLS models. The cross-validation models were evaluated using the coefficient of determination (R^2_{CV}), the standard error in cross validation (SECV), bias, slope and the residual predictive deviation (RPD) (SD/SECV) (Williams et al., 2017).

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Data set	Details of samples
Feed	Substrate used for rearing BSFL: SW, customised BV, supermarket waste, childcare waste, waste mixture; n=15
Larvae	5th and 6th instar BSFL from SW and BV waste; unprocessed, blanched, and dried larvae from supermarket waste, childcare waste, waste mixture; n=39
frass	5th and 6th instar frass from soy and BV waste trials. 5th instar frass from supermarket waste, childcare waste, waste mixture; n=21
Facility A	Feed, 5th and 6th instar BSFL and frass from soy waste and BV waste; n=30
Facility B	Feed, unprocessed, blanched, dried BSFL, and frass from supermarket waste, childcare waste, waste mixture; n=45
Complete data set	Complete set of samples obtained from both facility; n=75

Table 1 Summary of different data sets used to develop the partial least square regression models.

3. Results and Discussion

3.1 Legislative road map of insect uses as animal feed in Australia.

An updated review of the legislative map of the use of insects, including BSFL was conducted towards the end of the research period of which the findings are summarised in ***Table 2*** **Current Status of insects as feed in Australia**.

Table 2 Current Status of insects as feed in Australia

State	Regulations	Substrate constituents	Status of Insect reared from the specified substrate
Western Australia	Biosecurity and Agriculture Management (Agriculture Standards) Regulations 2013	Animals, matter derived from animals, mammals, mammalian parts/tissue	Restricted for ruminant and Pig feed. Allowed for aquaculture and poultry feed
Victoria	Agricultural and Veterinary Chemicals (Control of Use)(Ruminant Feed) Regulations 2015	Animals, matter derived from animals, mammals, mammalian parts/tissue	Restricted for ruminant and pig feed. Allowed for aquaculture and poultry feed
Tasmania	Animal Health Act 1995	Animals, matter derived from animals, mammals, mammalian parts/tissue	Restricted for ruminant and pig feed. Allowed for aquaculture and poultry feed

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State	Regulations	Substrate constituents	Status of Insect reared from the specified substrate
Northern Territory	Livestock Regulations 2009	Materials derived from mammals, birds, and fishes	Restricted for ruminant and pig feed. Allowed for aquaculture and poultry feed
Queensland	Queensland Biosecurity Regulation 2016	Materials derived from whole or part of vertebrates, mammals, and bird carcasses	Restricted for ruminant, pig, and poultry feed. Allowed for aquaculture
New South Wales	Biosecurity Regulation 2017	Materials derived from whole or parts of vertebrates, and mammals	Restricted for ruminant and pig feed. Allowed in aquaculture and poultry feed
South Australia	South Australian Livestock Regulations 2013	Materials derived from whole or part of vertebrates, mammals, and bird carcass	Restricted for ruminant, pig, and poultry feed. Allowed for aquaculture
Australian Capital Territory	Animal Diseases Act 2005	Material of mammalian and poultry origin, fishmeal, meal from mammalian and poultry tissue	Restricted for ruminant and pig feed. Allowed in aquaculture and poultry feed

Stockfeed standards in Western Australia (WA) are currently regulated by the Biosecurity and Agriculture Management (Agriculture Standards) Regulation 2013. According to this regulation, a material or ingredient intended to be used as feed for animals must not contain antibiotic/hormones, the presence of contaminants should be within the maximum tolerable limits, veterinary chemical and feed additives that are unregistered with Australian Pesticide and Veterinary Medicines Authority (APVMA) should not be present. Feed containing animals or matter derived from animals are considered as Restricted Animal Material (RAM) and is prohibited for use as feed for ruminants in WA (Western Australia Department of Primary Industries and Regional development, 2013). The Agriculture and Veterinary Chemicals (Control of use) (Ruminant Feed) Regulations 2015 and The Animal Health Act 1995 governs the stock feed regulations in Victoria and Tasmania, respectively (Agriculture Victoria, 2015; Department of Primary Industries Parks Water and Environment Tasmania, 1995). The 'RAM' statement definitions in these two states are similar to that of WA and RAM is not allowed to be fed to ruminants. Hence, protein derived from insects is considered as RAM and cannot be fed to ruminants irrespective of the type of substrate used for rearing. Feed containing mammals or tissues/parts of mammalian origin are considered as Prohibited Pig Feeds (PPF) in these three states (Agriculture Victoria, 2015) (Western Australia, Department of Primary Industries and Regional development 2013; Department of Primary Industries Parks Water and Environment Tasmania, 1995). This categorisation prohibits the use of insects reared from substrates containing mammalian products to be used as feed for pigs in the above-mentioned states. According to the 'Livestock Regulations 2009' of the Northern Territory (NT), materials derived from mammals, birds and fishes are defined as RAM and, materials obtained from mammalian and poultry origin are considered as PPF (Northern Territory Government Information and Services, 2015). Hence, insects and insect proteins reared from substrates free from the above stated materials can be used as feed for ruminants, pigs, and poultry in the NT. The stockfeed standards in Queensland, New South Wales and South Australia are regulated by 'Queensland Biosecurity Regulation 2016',

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'Biosecurity Regulation 2017' and 'Livestock Regulation 2013', respectively. Animal feed containing whole, or part of vertebrate material is defined as RAM in these states and, materials of mammalian origin are considered as PPF in NSW. Feeds containing materials of vertebrate origin and bird carcasses are considered as PPF in QLD and SA and, their usage as poultry feed is also restricted (Agriculture and Primary Industries- Queensland Government, 2016; Department of Primary Industries- New South Wales, 2017; Department of Primary Industries and Regions-South Australia, 2013). Insects reared from these substrates are prohibited from use as feed for pigs and ruminants in NSW and, also for poultry in SA and QLD. Fish meal and tissues from mammals and poultry are defined as RAM in the Australian Capital Territory (ACT) under the Animal Diseases Act 2005. The same act describes material of mammalian origin and substances that have been in contact with it as PPF (ACT Legislation Register, 2005). Insects reared from listed RAM and PPF materials are prohibited for use as feed in ACT. Importantly, insects can be used as feed in aquaculture across the nation under existing stock feed regulations, irrespective of the substrate used for rearing.

3.2 Biological safety of BSFL reared on food waste streams

3.2.1 Microbial load in substrate

As expected, the different organic waste streams employed in these trials were presumptively positive for *C. perfringens, B. cereus*, and Y&M, for which the colonies were enumerated (Table 3). Spore-forming bacteria, including *B. cereus* and *C. perfringens*, were found to be distributed in relatively higher concentrations (1.2 log CFU/g - 6.6 log CFU/g) than Y&M (1.3 log CFU/g - 4.4 log CFU/g). *Salmonella*, *E. coli* and *L. monocytogenes* were not found in the waste streams. In the case of *B. cereus*, the highest counts were observed in SW. The heterogeneous waste streams, including SM, CC waste and WM, had similar microbial loads for B. cereus (3.3 log CFU/g – 3.5 log CFU/g) and did not differ ($p > 0.05$) when compared with each other. The *B. cereus* count in BV diets was below the detection limit of 1.2 log CFU/g. This can be attributed to BV diets being formulated with bread and vegetables purchased fresh from a supermarket. The *B. cereus* counts in different food waste streams reported here in this study are similar to that of the levels reported in other agricultural products, including spent grains and cereals (Bessa et al., 2021). The counts of *C. perfringens* in BV diets were similar to that of *B. cereus,* i.e., below the detection limit of 1.2 log CFU/g (***Table 3***). The counts of this organism noted for the other three waste streams (SM, CC, and WM) were found to be relatively close, ranging from 4.8 log CFU/g to 5 log CFU/g with no difference ($p > 0.05$) noted (***Table 3***). It is interesting to observe that the *C. perfringens* counts for these waste streams were found to be lower than other values previously reported for organic waste streams, including food waste (6 log CFU/ml) and blood-bone meal (5.7 log CFU/ml) (Casagrande et al., 2013; Rounsefell et al., 2013).

	***B. cereus* (log CFU/g)**	***C. perfringens* (log CFU/g)**	**Yeast & molds (log CFU/g)**
SW	6.6 ± 0.8^{a}	4.4 ± 0.3^{a}	4.4 ± 0.1^{a}
BV	$<1.2\pm0^{b}$	1.2 ± 0^{b}	1.4 ± 0.2^{b}
WM	3.5 ± 0.1^{a}	5 ± 0^{a}	$<1.3\pm0^{c}$
SM	3.6 ± 0.1^{a}	5 ± 0.1^{a}	4.3 ± 0.1^{a}

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CC	3.3±0[a]	4.8±0[a]	3.2±0.2[b]

Table 3: Microbial load (log CFU/g) of different food waste streams fed to black soldier fly larvae

All values are reported as mean and standard deviation from three replicates. Different superscripts in the same column differ ($p<0.05$). SW- Soy waste, BV- bread vegetable diet, WB- waste mixture, WL- Supermarket waste, CC- childcare centre waste.

Previous studies have reported the presence of pathogenic microbes including *L. monocytogenes*, *S. aureus*, *E. coli* and *Salmonella* spp. in different types of food waste (Govorushko, 2019; Grewal et al., 2007; Sundberg et al., 2011). However, the waste stream samples in our study tested negative for the above stated pathogenic bacteria. This can be attributed to either the absence of these organisms in the food wastes or the suppressive action of the indigenous microbiota present in these waste streams.

The suppression of pathogenic bacteria including *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella enterica* in food waste mixtures due to natural composting has been previously reported. These organisms failed to survive due to the suppressive effect of the natural microbiota present in the food waste (Lemunier et al., 2005; Paniel et al., 2010). The different food waste streams used in our study could potentially have undergone natural composting, prior to homogenisation, thereby enabling the indigenous microbiota to inhibit the survival of these pathogenic bacteria (including *L. monocytogenes*, *S. aureus*, *E. coli* and *Salmonella* spp.). Bacteria including *B. cereus* and *C. perfringens* can exist either in vegetative or spore form. Their thick-walled spores are resistant to heat and are found to be responsible for contamination of different types of processed and cooked foods, thereby aiding in the survival of these microorganisms in food waste matrices (André et al., 2017; Majumdar et al., 2018; Tewari & Abdullah, 2015). It should also be noted that the survival of pathogenic bacteria is further influenced by the bacterial species, availability of specific nutrients required by the different bacteria, and several other intrinsic and extrinsic factors (Lemunier et al., 2005; Sahlström, 2003).

3.2.2 Microbial quality of BSFL

The BSFL samples obtained from SM and SW tested positive for *B. cereus*, *C. perfringens*, and Y&M, but were negative for *L. monocytogenes*, *Salmonella* spp., *E. coli* and *S. aureus*. Y&M counts for BSFL reared on these waste streams ranged from 1.2 log CFU/g to 4.3 log CFU/g. In general, the microbial counts for the pathogens that tested positive were higher for larvae reared with

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simpler homogenous waste streams (SW and BV diets). Y&M counts for BSFL reared on these waste streams ranged from 1.2 log CFU/g to 4.3 log CFU/g. BSFL reared on spent grains, human faecal sludge and sawdust were found to have higher Y&M counts than our findings, ranging from 5.07 log CFU/g to 8 log CFU/g (Campbell et al., 2020; Were et al., 2022). This can be attributed to differences observed in the initial pathogen load and several other underlying factors. Similar observations were made regarding the *B. cereus* counts noted in our experiments. The *C. perfringens* counts in BSFL reared on SW and BV diets ranged from 1.2 log CFU/g to 5.3 log CFU/g, which was found to be higher than the values reported by Van Looveren et al. (2022), who conducted their studies using a simple vegetable-based feed substrate for the rearing of BSFL. However, the initial load of the pathogen in their feed substrate was lower than what we noted in our feed waste substrates. Overall, the extent of contamination in the larva is found to be strongly influenced by the initial pathogen in the waste stream used for rearing (Bessa et al., 2021).

The unprocessed BSFL reared in this study exhibited lower loads of pathogenic microorganisms compared to the waste streams used as substrates for rearing them (Table 4). The *B. cereus* and *C. perfringens* loads in BSFL from the heterogenous food waste streams (SM, CC, and WM) were lower than the SM, CC, and WM waste used for rearing the larvae. Similarly, the *B. cereus* counts in BSFL reared on a SW diet (5.7 log CFU/g) were lower than the SW (6.6 log CFU/g). The *C. perfringens* was found to be distributed at higher counts in BSFL from both the SW (5.3 log CFU/g) and BV diets (1.7 log CFU/g) than the SW (4.4 log CFU/g) and BV (1.2 log CFU/g) diets. In case of Y&M counts, BSFL from different waste streams, except BV and WM diets, had lower counts (2.2 log CFU/g) - 3.9 log CFU/g) than the substrates (3.2 log CFU/g - 4.4 log CFU/g). The reduced pathogen load observed can probably be attributed to the cationic antimicrobial peptides (CAMP) produced by BSFL (Morrill, 2021). The synthesis of these CAMP's is regulated by the body fat content of BSFL. The ability of CAMPs from BSFL to reduce Gram-positive organisms like *S. aureus* and *B. subtilis* has been explored previously, and these molecules are found to form the basis of humoral immunity in several species of edible insects (Morrill, 2021; Park et al., 2014). Various studies have reported on the discovery of different classes of CAMPs extracted from *Hermetia illucens*, including defensins, cercopin, attacin, sacrotoxin, and stomoxyn (Lee et al., 2020; Shin & Park, 2019; Xia et al., 2021). It is however noteworthy that the secretion and action of these antimicrobial peptides by BSFL is regulated by several factors such as the compositional properties of the rearing substrate, larval density, and rearing conditions (Morrill, 2021; Wynants et al., 2019; Xia et al., 2021).

Table 4: Microbial load (log CFU/g) of BSFL of 5th and 6th instar Black soldier fly larvae from homogenous waste streams

	***B. cereus* (log CFU/g)**	***C. perfringens* (log CFU/g)**	**Yeast & moulds (log CFU/g)**
5IS	5.7±0.4^{ab}	5.3±0^{a}	3.9±0.3^{a}
6IS	4.7±0.5^{b}	3.6±0.3^{b}	4.3±0^{a}
5IB	6.1±0^{a}	1.7±0.3^{c}	2.1±0^{b}
6IB	2.4±0.6^{a}	<1.2±0^{c}	2.8±0.2^{c}

All values are reported as mean and standard deviation from three replicates. Different superscripts in the same column differ ($p<0.05$). 5IS and 6IS- the 5th and the 6th instar larvae from soy waste, 5IB and 6IB- the 5th and the 6th instar larvae from bread-vegetable diets

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3.2.3 Effect of Larval Instar

BSFL larvae from SW and BV diets were harvested at different larval stages to explore the effect of larval instar on microbial loads. The counts of the spore-forming Gram-positive bacteria were reduced in the 6th instar compared to the 5th instar, harvested from both the SW and BV diets (Table 4). The *B. cereus* was found to be reduced by 1 log unit and 4 log units in the 6th instar BSFL from SW and BV diets, respectively. Similarly, a 1 log unit reduction in *C. perfringens* was observed for the 6th instar BSFL harvested from SW diets. It is also noteworthy that no significant differences were observed between the 5th and 6th instar BSFL for microbial load of different enumerated organisms, except for *B. cereus* and Y&M counts for larvae reared on BV diets, and *C. perfringens* counts for larvae reared on SW. It is established that BSFL empties their guts upon reaching the 6th instar (pre-pupae) to prepare themselves for pupation (Soetemans et al., 2020). Pre-pupae rely on their fat storage for survival, and they naturally have a higher fat content than the 5th instar larvae (Zhu et al., 2019). Also, the most predominant fatty acid in BSFL is lauric acid (Ewald et al., 2020; Leong & Kutty, 2020; Shumo et al., 2019). Our studies observed that the lauric acid content in the 6th instar BSFL was relatively higher than in the 5th instar BSFL reared from SW and BV diets. Lauric acid's ability to exhibit antimicrobial activity against different bacterial species has been well explored (Abbas et al., 2017; Altieri et al., 2009; Anzaku et al., 2017). Hurtado-Ribeira et al. (2023) and Spranghers et al. (2018) observed that BSFL fat exhibited better antimicrobial suppression against Gram-positive bacteria including *S. aureus*, *L. monocytogenes*, *B. subtilis*, and D-Streptococci caused by the lauric acid content of the larvae. Therefore, this could be a plausible explanation for the 6th instar BSFL having reduced microbial loads compared to the 5th instar BSFL in this study. The microbiota of BSFL is also observed to change with the age of the larvae, along with changes as a result of feed and rearing conditions (Wynants et al., 2019). The shift in microbiota is also speculated to change the antimicrobial activity of the 5th and the 6th instar larvae (Shi et al., 2024). This in turn could have also contributed to the 6th instar larvae potentially exhibiting antimicrobial activity against the pathogenic bacteria.

Interestingly, the Y&M count for the 6th instar BSFL from both BV (2.8 log CFU/g) and SW diets (4.3 log CFU/) were higher than the 5th instar larvae- 2.1 log CFU/g and 3.9 log CFU/g from BV and SW diets, respectively. It is speculated that several intrinsic and extrinsic factors associated with substrate and the rearing conditions could have facilitated the growth of Y&M during the experiment, which could have minimised the antimicrobial effect displayed by BSFL against these organisms. Overall, BSFL's ability to exhibit antimicrobial activity against pathogenic microbes is mediated by several factors involved with the rearing process as stated earlier (Wynants et al., 2019; Xia et al., 2021).

3.2.4 Effect of Thermal Processing

The effect of post-harvest treatments, including blanching and drying, on reducing the microbial load of BSFL reared from the heterogeneous waste streams (SM, WM and CC; Facility B) was evaluated (***Table 5***). The *B. cereus* counts for the blanched and dried BSFL from all three waste streams were reduced to below the detection limit (1.3 log CFU/g). Blanching was observed to reduce the counts of Y&M and *C. perfringens*, compared to that of the unprocessed larvae, but was still found to be above the detection limit. However, drying of blanched samples was found to reduce the *C. perfringens* load below the detection limit (1.2 log CFU/g). It was also observed that there was no significant difference for YM and *C. perfringens* between the blanched and dried BSFL samples from all three waste streams.

Table 5: Effect of thermal processing on microbial load (log CFU/g) of BSFL from heterogeneous waste streams

	***B. cereus* (log CFU/g)**	***C. perfringens* (log CFU/g)**	**Yeast & moulds (log CFU/g)**

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5IWB	2.1±0.2[a]	2.8±0.6[b]	1.6±0.6[bc]
5IWB-B	<1.3±0[a]	1.8±0.6[ab]	1.4±0.1[c]
5IWB-D	<1.3±0[a]	<1.2±0[b]	<1.2±0[c]
5IWL	1.8±0.7[a]	1.7±0.8[ab]	2.2±0.2[bc]
5IWL-B	<1.3±0[a]	1.8±0.6[ab]	<1.2±0[c]
5IWL-D	<1.3±0[a]	<1.2±0 b[ab]	<1.2±0[c]
5ICC	2.8±0.4[a]	3.5±0.3[ab]	2.4±0[ab]
5ICC-B	<1.3±0[a]	2.3±1.3[ab]	1.8±0.4[bc]
5ICC-D	<1.3±0[a]	2±0.8[ab]	<1.2±0[c]

All values are reported as mean and standard deviation from three replicates. Different superscripts in the same column differ ($p<0.05$). 5IWB, 5IWB-B, 5IWB-D- 5th instar larvae from waste mixture subjected to homogenisation, blanching and drying respectively 5IWL, 5IWL-B, 5IWL-D- 5th instar larvae from supermarket waste subjected to homogenisation, blanching and drying respectively, 5ICC- 5th instar larvae from child-care centre waste subjected to homogenisation, blanching and drying respectively.

The Feed Safe Program defines the feed safety standards in Australia, and it does not directly emphasise Maximum Tolerable Limits (MTL) for different pathogenic microbes but directs manufacturers to identify hazards and establish critical control points. The Australian rendering standard for processing animal material-based feed ingredients (BSFL falls under this category) has included MTL for *C. perfringens*, which is 10 CFU/g (Staff, 2000). The counts for *C. perfringens* in thermally processed BSFL samples exceeded this value. The international Good Manufacturing Practise (GMP) guidelines for feed safety suggests that *Salmonella* spp. in feed ingredients should be absent in 25g of samples (International, 2020). Due to limited sample availability, we could not test the samples against these limits, but *Salmonella* spp. were not detected in this study. MTL for Y&M counts by GMP is 6 log CFU/g (International, 2020), and we observed that thermal processing followed in this study ensured that the Y&M counts were well below this limit. Feed safety standards do not report MTL for bacteria, including *L. monocytogenes, S. aureus, E. coli,* and *B. cereus*. However, our work demonstrated that these organisms were found to be below the threshold for the microbiological limits of these microbes for (human) food included in Food Standard Australia New Zealand (FSANZ) code 1.6.1 (Code, 2021).

Pathogens that were detected in both the waste streams and larvae were the only microorganisms detected in the BSFL frass. The microbial load of *B. cereus* (6.2 log CFU/g) and Y&M (4.7 log CFU/g) in the 5th instar frass from SW was found to be similar to that of the counts observed in the SW stream (*B. cereus*- 6.6 log CFU/g and Y&M- 4.4 log CFU/g) used in the rearing of these larvae. The *C. perfringens* counts in the 5th instar frass reared on SW was found to be relatively higher (6.6 log CFU/g) than the load of this microorganism detected in the SW (4.4 log CFU/g). The microbial load of all the above stated microorganism for the 5th instar frass

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from BV diets was found to be relatively higher in counts, when compared to the waste streams. This observed trend in the results could be attributed to the antimicrobial action exhibited by BSFL to suppress the proliferation of microbes, thus resulting in frass with loads similar to that of the initial substrate (Shi et al., 2024; Wynants et al., 2019). The 6th instar frass from both SW and BV diets had lower *C. perfringens* loads, while higher counts were recorded for *B. cereus* and Y&M counts. Interestingly, no significant differences were noted between the 5th and the 6th instar frass for all microorganisms from both SW and BV diets that were evaluated, except for *C. perfringens* in the frass from SW. The conditions prevailing upon bioconversion of food waste streams and gut emptying by BSFL, upon reaching the pre-pupal stage, could have resulted in the increased microbial load observed in the 6th instar frass (Alagappan et al., 2023; Xia et al., 2021).

Frass from the CC waste stream had the highest Y&M counts (5.1 log CFU/g), followed by frass from SM (2.5 log CFU/g) and WM (2.1 log CFU/g), respectively. Interestingly, the frass from the SM and WM waste streams showed no statistical differences for Y&M counts. The Y&M count in frass from SM, WM, and CC trials were found to vary, owing to the differences observed in the microbial load among the three waste streams. Also, it has been reported that the microbiome of BSFL alters the fungal diversity of the food waste stream used for its rearing, thereby influencing the overall microbial load of Y&M in the resulting frass (Kuznetsova et al., 2022). The microbial counts of *C. perfringens* in frass from the three heterogenous waste streams were not significantly different to each other. This can be attributed to the similar pathogen loads in the substrate and the identical rearing conditions followed during the rearing of BSFL on these diets.

The commercial application of BSFL frass as soil fertiliser and conditioner is currently being explored in several regions across the globe (Basri et al., 2022; Klammsteiner et al., 2020). It has been reported that pathogenic bacteria such as *L. monocytogenes* and *Salmonella* spp. could be transferred from the fertiliser products used in the soils, to raw agricultural products (Black et al., 2021; Lemunier et al., 2005). Thus, it is of importance to ensure that the load of pathogenic microbes in frass is safe, as the frass can be characterised with diverse pathogenic microbes, as we observed in our study. Van Looveren, et al., (2022) subjected the frass from their experiments to thermal processing at 70°C for 60 minutes, and observed that total viable counts, along with *Salmonella* spp. and *C. perfringens* counts, were reduced and were within the limits suggested for fertilisers by EU Regulation (EC No. 142/2011). The effect of similar thermal processing steps to reduce the microbial load and the overall quality of BSFL frass as fertiliser must be explored further.

3.3 Chemical safety of BSFL reared on food waste streams

3.3.1 Heavy Metals

The concentrations of all heavy metals analysed (Cu, Mn, Zn, As, Cs, Co, Cr, Ni, and Pb) were found to be distributed at relatively higher concentrations in complex food waste streams rather than in the homogenous SW and BV diets. Only Mn, Co, Cr, and Pb were found to be distributed at significantly higher concentrations ($p \leq 0.05$) in heterogenous waste streams compared to SW and BV diets. Addeo et al. (2024), using different formulated substrates with vegetable and butchery waste, observed similar trends. These authors reported that the concentrations of heavy metals were higher in the substrates with higher proportions of animal matter. Among the heavy metals, Mn was distributed at highest concentration followed by Zn, and Cu. The distribution of As, Cd, Pb, Ni, Co and Cr was found to be at, or slightly above, 0.1 mg/kg of waste. The distribution of heavy metals among the three complex food waste streams (SM, WM and CC) did not differ ($p > 0.05$). This could probably be attributed to the similarity in constituents making up the three complex food waste streams. Interestingly similar trends in results were observed in the homogenous waste streams BV and SW for all heavy metals except Zn In this study, Zn was found to be at a higher concentration ($p < 0.05$) in SW compared to BV diets.

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The concentration of heavy metals observed in the different food waste streams is similar to that reported for conventional feed ingredients including chicken feed, Gainesville diet and other grain-based feed ingredients (Purschke et al., 2017; van der Fels-Klerx et al., 2016; Wu et al., 2020).

The distribution of heavy metals in the BSFL followed similar trends as observed in the substrate. Mn was found to be distributed at highest concentration in unprocessed BSFL followed by Zn and Cu for the larvae reared with SM, WM, and CC waste streams. In case of BSFL from the SW and BV diets, Zn was found to be distributed at higher concentrations ($p \leq 0.05$) than Mn. Similar trends were observed in the substrate as well. The concentration of As, Cd, Co Cr, Ni and Pb ranged between 0.5-1.7 mg/kg of BSFL for unprocessed larvae from heterogenous food waste streams and did not exceed 0.1 mg/kg for BSFL from SW and BV diets. This could be attributed to the fact that the food waste streams employed as feed for BSFL in these experiments were comprised of foodstuff initially classified safe for human consumption. These findings are in accordance with previous reports which suggested that the concentration of heavy metals was mainly influenced by the concentration of these elements in the substrates used for rearing the larvae (Biancarosa et al., 2018; Shumo et al., 2019).

The occurrence of Hg, Pb and Cd in animal feeds are regulated under the Australian legislation. The maximum tolerable limit (MTL) for Cd and Pb in animal feeds and feed ingredients is 2 mg/kg of feed (DM basis) and 5 mg/kg of feed (DM basis), respectively (S Alagappan et al., 2022; New South Wales Department of Primary Industries, 2017; Queensland Government, 2017; Western Australia Department of Primary Industries and Regional development, 2013). The maximum tolerable limits for As in animal feed ingredients set by the European Union is 2mg/kg of the feed ingredients (Commission, 2023). The two different larval instars grown on SW and BV diets are within this limit for these three heavy metals. The concentration of Ni and Co was also found to be below 0.1 mg/kg of larvae. Diener et al. (2015) and Gao et al. (2017) observed that the concentration of minerals in 6^{th} instar larvae was found to be higher compared to that of the 5^{th} instar BSFL. In our studies, the distribution of Cu, Zn, and Mn was higher ($p \leq 0.05$) in the 6^{th} instar larvae. Heavy metals including Zn and Mn are observed to be essential for certain physiological functions and regulating metabolism in BSFL (Addeo et al., 2024; Hanson, 2022). Similarly, Cu accumulating cells are found to be available at the midgut of BSFL to promote acidification of the gut and thereby promote bioconversion of the feed (Bonelli et al., 2019; Seyedalmoosavi et al., 2022). BSFL tends to accumulate more biomass during the pre-pupal stages so as to support the metamorphosis of the adult larvae (Liu et al., 2017). It is therefore speculated that these factors could have resulted in the higher distribution of these heavy metals in the 6^{th} instar larvae.

The concentration of Pb and Cd was below the MTL for unprocessed and processed BSFL from all three waste streams. The As was found to be slightly higher (2.7 mg/kg) in dried and blanched BSFL reared with WM and CC waste streams, respectively. Blanching is normally observed to be associated with leaching of minerals (Inobeme et al., 2020; Manditsera et al., 2019). This trend was observed in our studies for Mn, Zn, Cd, and Cr in blanched BSFL samples from all three heterogenous waste streams. It is noteworthy that the concertation of these heavy metals did not differ ($p > 0.05$) among blanched and unprocessed samples from the three heterogenous waste streams. Blanched BSFL samples from SM had relatively higher levels of Cu, As, Co, Ni and Pb although this was not significant ($p > 0.05$) when compared to the unprocessed BSFL samples. Ssepuuya et al. (2020) observed similar trends after blanching of edible insects. This slight increase in heavy metal content upon blanching can be due to the binding of minerals with chitin and specific protein structures in the larvae (Ssepuuya et al., 2020). This could have led to relatively higher heavy metal content observed only in blanched BSFL from the SM waste stream. Drying is usually associated with the concentration of minerals due to removal of moisture (Zulkifli et al., 2022). This effect was prominent for heavy metals including Cu, Zn, and Mn but was not observed for other toxic heavy metals. This probably due to the low concentration of these heavy metals in the unprocessed BSFL.

Bioaccumulation factor (BAF) is defined as the ratio of heavy metal concentration in the larvae to that of the concentration in the substrate used for rearing the larvae (Biancarosa et al., 2018). BAF values of heavy metals of physiological importance (Cu, Mn,

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and Zn) was found to be higher in BSFL from SW and BV diets than the larvae from heterogenous waste streams (SM, WM, and CC). BSFL has been reported to vary in bioaccumulation patterns for minerals including heavy metals based on the type of substrate and concentration of these minerals in the substrate used for rearing (Addeo et al., 2024; Proc et al., 2020). It is noteworthy that these BAF values reported in our studies for these heavy metals are in accordance with previously published findings (Proc et al., 2020; Purschke et al., 2017; van der Fels-Klerx et al., 2016; Wu et al., 2020).

It was observed that the BAF values were below 1 for most of the toxic heavy metals (Cd, Co, Ni, Cr, and Pb) except Ni (1.5) and Pb (1.3) for 5th and 6th instar BSFL from SW and BV diets. BAF values were below 2 for most of these toxic heavy metals except Pb (dried BSFL from WM), As (unprocessed BSFL from WM) and Ni (blanched and unprocessed BSFL from SM and CC respectively). The low BAF values reported for As and Pb observed in our studies is in accordance with previously reported findings (Diener et al., 2015; Proc et al., 2020; Purschke et al., 2017). However, Cd even when distributed at lower concentrations in the rearing substrate has been reported to accumulate at BAF values ranging between 2.8 to 10.5 (Addeo et al., 2024; Proc et al., 2020). Tschirner and Simon (2015) observed that the type of substrate and its constituents also plays a significant role apart from the concentration of mineral elements in influencing their bioaccumulation patterns. Hence, it can be speculated that this could have affected the bioaccumulation of Cd in our trials.

The fate of the minerals including heavy metals in frass is as follows: reduced concentration in residual frass due to the accumulation in larvae, increased concentration in frass caused by the utilisation of other nutrients available in the substrate by BSFL and, increase in concentration caused by the excrements of the larvae during the bioconversion process (Addeo et al., 2024; Proc et al., 2020).

The concentration of Cu, Mn, and Zn was higher in the frass from SW and BV diet trials when compared to their concentrations in original substrate. The above-mentioned heavy metals were also accumulated in the 5th and 6th instar larvae from these trials. Therefore, it can be inferred that the utilisation of other nutrients (starch, protein, fat, trace elements etc.) available in the waste streams along with the dilution effect brought by varying moisture content in feed and frass samples has concentrated these heavy metals in the resulting residual frass.

3.3.2 Mycotoxins

The maximum tolerable limits for AFB1, DON, OTA, and ZEN in feed ingredients as listed by EU and Australian regulations are 20 PPB, 8 PPM, 250 PPB, and 2 PPM, respectively (Commission, 2023; New South Wales Department of Primary Industries, 2017; Queensland Government, 2017; Western Australia Department of Primary Industries and Regional development, 2013). The concentrations of different mycotoxins analysed in our study are below the detection limits. The detection limits for AFB1 (< 2 PPB), DON (<0.05 PPM), OTA (<1 PPB) and ZEN (< 5PPB) in these studies are found to be well within the MTL established by regulatory bodies. This could probably be attributed to one of the following factors i.e.: 1. the absence of mycotoxins in the different food waste streams employed in the study; 2. excretion of the mycotoxins to the residual frass and; 3. metabolisation of mycotoxins to secondary metabolites by BSFL. The ability of BSFL to excrete and metabolise mycotoxins when reared on conventional feeds spiked with varying concentrations of mycotoxins has been reported (Camenzuli et al., 2018; Gulsunoglu et al., 2019; Purschke et al., 2017; Siddiqui et al., 2023). Overall, it can be suggested that the BSFL samples harvested at two different larval instars, and from different heterogenous waste streams were found to be safe against different mycotoxins that are regulated in stockfeed ingredients.

3.3.3 Microplastics

Several studies have been initiated worldwide to investigate the effects of microplastic on BSFL. Most of these studies investigated the impact of microplastic-contaminated growth substrates on important factors of BSFL development, such as pupation,

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emergence or nutrient composition. For example, Romano and Fischer (2021)] reared BSFL on a substrate spiked with 0.22 % w/w concentration PP microplastics approximately 55±4 µm diameter, approximately 20 million particles. After two weeks of growth, they measured larvae size, weight, pupation %, substrate reduction % and short chain fatty acid (SCFA) profile. They observed a reduction in rates of pupation compared to their control as well as a shift in levels of butyric and propionic acid with the BSFL. They concluded that microplastic exposure can significantly delay development and likely impacted the gut microbiome, resulting in the change of the SCFA profile. However, Planche et al (2024) observed that microplastics did not impact larval growth.

Heussler and Dittmann (2023) conducted a similar exposure study on microplastic spiked substrate. Their study looked at the effects of biodegradable polylactic acid (PLA) and non-biodegradable polyamide (PA – nylon) microplastics spiked at 0.22% w/w of a chicken-feed based diet on growth and development and histology. They observed a decrease in larval weight in the PA treatment compared to their control and PLA treatment. PLA impact on larval weight was negligible. Their study demonstrated that microplastics were only found in the gut and were mostly cleared form the gut after 9 days of feeding at their fifth instar. This study evidenced the ability for BSFL to depurate microplastics, keeping in mind the proviso that the method used would not have detected nanoplastics. On the other hand, Gold et al (2024) noted that the amounts of nanoparticles ingested depended on the size of the particles – 15 µm passed through faster than the 58 µm. Interestingly, after 1080 min of egestion, larvae contained low amounts of 15 µm while no 58 µm were noted. Planch et al (2024) also noted that a 3-day starvation caused at 96% reduction in microplastics present in the larvae, however, this long starvation duration caused a ~30% weight reduction and the authors correctly postulate that this loss would be unfavourable in a commercial scenario.

Cho et al (2020)] investigated the effects of microplastics and salinity on BSFL. They reared BSFL on food waste spiked with microplastics and NaCl adjusted salinity levels. They spiked polyethylene (PE) and (polystyrene (PS) microplastics (400-500µm) at 5,10 and 20% w/w and measured growth rate, survival rate and pupation rate every 2-3 days. Additionally, for the 20% microplastic concentrations they also tested 1, 2 and 3% NaCl treatments. Their results showed negligible inhibition of larval growth, weight and pupation for the microplastic treatments. However, the addition of NaCl to the 20% microplastic treatments resulted in a decrease in larval weight and pupation ratio.

A key study by Lievens et al (2023) to understand important baseline features of microplastic ingestion and excretion by BSFL used blue fluorescent microplastic spheres. They spiked rearing substrates with w% 0, 0.01, 0.1, 1, and 3% spheres and observed accumulation of between 131 particles in the 0.01% treatment and 4866 particles in the 3% treatment; however, the bioaccumulation was negligible as most particles were depurated. Additionally, they used SEM to develop an assessment of mouth size relating to the age of the larvae, providing a fundamental commentary the inextricable relationship between age, mouth opening size and the ability to uptake microplastics. Their assessment based on SEM measured mouth opening size ranging from 20-110 µm at 5 DAH and 17 DAH, respectively. Experimentally, they observed microplastic uptake at 10 DAH where the mouth size was approximately 65 µm. This study highlighted the biological restriction for particle size ingestion by BSFL. This is an important feature when identifying the fate of plastics during BSFL valorisation of food waste. While the study clearly demonstrated the high levels of depuration with spherical virgin plastics, the author noted that further work is required. The nature of the BSFL's grinding mouth parts is such that weathered or irregular shaped microplastics, which would be more common, could be degraded into smaller plastics. Likewise, the depuration and excretion of the plastic particles may be different when the particles are not perfect spheres and composed of a uniform virgin polymer.

Generally, studies that have spiked the growth substrate of BSFL with plastics (ranging from 0.22 -20% w/w) have seen negligible effects on larval growth parameters such as the weight, survival and pupation of larvae. When synthesising the findings of such studies several important aspects should be considered to assess the observed effects:

- Were the microplastics the right size to be ingested by the larvae?

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- What is the chemical composition of the plastics (to understand the effects of chemicals associated with plastics).
- Are they representative of plastic materials found in food wastes?

As previously stated microplastics tend to be efficiently excreted by BSFL when they are present in rearing substrate (Lievens et al., 2022, 2023) suggesting that microplastics are ultimately ending up in frass. As so much of the substrate mass is valorised by BSFL in the process, the concentration of microplastics in frass is likely to be higher than that of the substrate the larvae were reared on. While there has been a limited investigation into concentration factor that may occur, the potential problem of microplastic and persistent chemical accumulation is synonymous with biosolid production. While both biosolid and frass are conceptually an ideal and circular soil fertiliser option, the heterogeneity of the feedstock poses a problem – each batch of feedstock and therefore finished product may be differentially contaminated.

For plastics, the mass conversion occurring to the substrate and likely subsequent magnification effect occurring highlights the need for rapid and robust decision-making tools regarding the contamination load, or quality, of the frass fertiliser.

The existing literature on microplastics in BSFL has focused on the larvae as a food or feed product and the resulting frass as a fertiliser and soil amendment. Studies have typically fed microplastics to laboratory raised BSFL larvae to understand whether accumulation is occurring, however typical of such studies, many variables that exist in industrial settings are not well represented including the variability of food waste feedstock, growing conditions and plastic type, content, shape and size.

Overall, the literature identifies BSFL as resilient food waste stream recyclers. Generally, their effectiveness as waste valorisers is not reduced by microplastics (up to 20% w/w of feed has been trialled). Their ability to depurate microplastics after feeding suggests that plastic does not accumulate in the larvae, however, there is no study effectively analysing for nano plastics and further work is needed for more appropriate risk management.

3.4 Effect of larval Instar, post-harvest treatments, and substrate on the nutritional profile of BSFL

The chemical composition of the waste streams is reported in **Figure *4***. The chemical composition of the heterogeneous substrates (SM, CC, and WM) were similar, with no significant differences ($p>0.05$) for crude protein (CP), crude fat (CF), and neutral detergent fibre (NDF). However, the soya diet had the lowest ($p<0.05$) starch whilst the bread-vegetable (B) had the highest starch concentration ($p<0.05$). The latter (B) also had a significantly ($p<0.05$) higher NDF than the other samples, which did not differ from each other ($p>0.05$) The CP and CF content of the heterogeneous waste streams were comparable to those reported by others (Barbi et al., 2020; Shumo et al., 2019; Spranghers et al., 2017). The chemical composition of CP and CF was lower ($p<0.05$) in B streams while Starch and NDF was higher ($p<0.05$) compared to SW.

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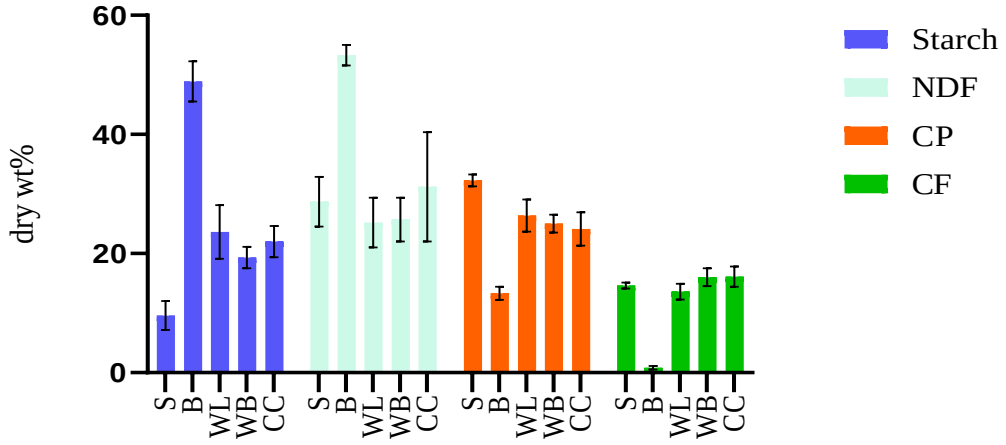

Figure 5 Chemical composition of food waste streams used for rearing BSFL
All values are reported as mean and standard deviation from three replicates. S- Soy waste, B- bread vegetable diet, WB- waste mixture, WL- Supermarket waste, CC- childcare centre waste. NDF- neutral detergent fibre, Cp – crude protein, CF – crude fat

Pertaining to the mineral composition of the diets fed to the BSFL, Calcium (Ca) was the dominant element followed by potassium (K), sodium (Na), phosphorus (P), sulphur (S), and magnesium (Mg). The concentration of the above stated elements along with boron (B) and barium (Ba) (except Na) was found to be higher ($p<0.05$) in the SW compared to BV diets. No statistical differences were observed for selenium (Se) within BV and SW waste stream. The mineral concentration of the BV diet was similar to those reported by other researchers (Fischer & Romano, 2021; Hashmi et al., 2021). However, the mineral content of the SW waste stream was found to be relatively low compared to other reported studies (Li et al., 2013; Rahman et al., 2021). No significant differences ($p>0.05$) were found for Ca, K, B, and Se in samples from the heterogenous waste streams (SM, WM, and CC) despite the variation observed in the values. The concentration of S was lower ($p<0.05$), and Ba was higher ($p<0.05$) in CC waste on comparison with SM and WM waste streams. Differences in these elements can be explained by the type of ingredient in the waste. The CC included salads, bread, and other cooked foods while, both WM and SM mainly consisted of meat, leftover foods, pastries, and other dairy products. Mg content also differed ($p<0.05$) between CC and SM waste streams. Similar trends were observed between CC and WM waste streams for Na and P. Overall, the mineral concentration in all the waste streams align with those reported by other authors for similar food waste streams (Fitriana et al., 2022; Shumo et al., 2019)

The chemical composition of the BSFL samples obtained from the different waste streams and diets is reported in ***Figure 5*****7** .NDF content despite being higher in 6th instar BSFL from both SW and BV diets, was found to be significant ($p<0.05$) only for the 5th and 6th instar BSFL from BV diets. The age of the BSFL did not influence the CP in the samples reared on SW. However, differences ($p<0.05$) were observed for CF content in 5th and 6th instar BSFL raised on SW (**Figure *6***). The CP for the 5th and 6th instar BSFL was higher while CF was found to be significantly reduced. Previous research has shown that different agro-industries by-products with composition similar to that of SW has resulted in BSFL with reduced CP and higher CF contents (Gold et al., 2020; Gold et al., 2018; Siddiqui et al., 2022). It has also been reported that the CP of BSFL upon reaching a certain threshold does not differ, even with changes in the CP content of the substrate (Barragán-Fonseca et al., 2018; Barragan-Fonseca et al., 2019; Eggink et al., 2023). The 6th instar BSFL have a higher chitin content in their exoskeleton, resulting in a higher protein estimation (Rampure et al., 2023). However, CP values for 6th instar BSFL from SW were lower than for the 5th instar. Although the CF was lower, samples of 6th instar BSFL sourced from SW had higher CF content than 6th instar sources from the other waste streams. However, it has been reported that fat levels in the 6th instar seem to decrease during pupation. The plausible reason for this discrepancy observed with

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CF and CP is that the larvae in this study from SW could have been harvested slightly before their actual intended life stage. Liu et al. (2017) reported that the CP and CF content of BSFL increased during the early stages of larval development.

In the BSFL samples from the BV diets, the CP of the 6th instar BSFL was higher ($p<0.05$) compared to 5th instar larvae. The increase in CP content observed for the 6th instar BSFL from BV diets could be attributed by the increased distribution of chitin in the exoskeleton of BSFL pre-pupae (Rampure et al., 2023). The CF content, as stated earlier was observed to decrease due to the utilisation of fat stores by the pre-pupae.

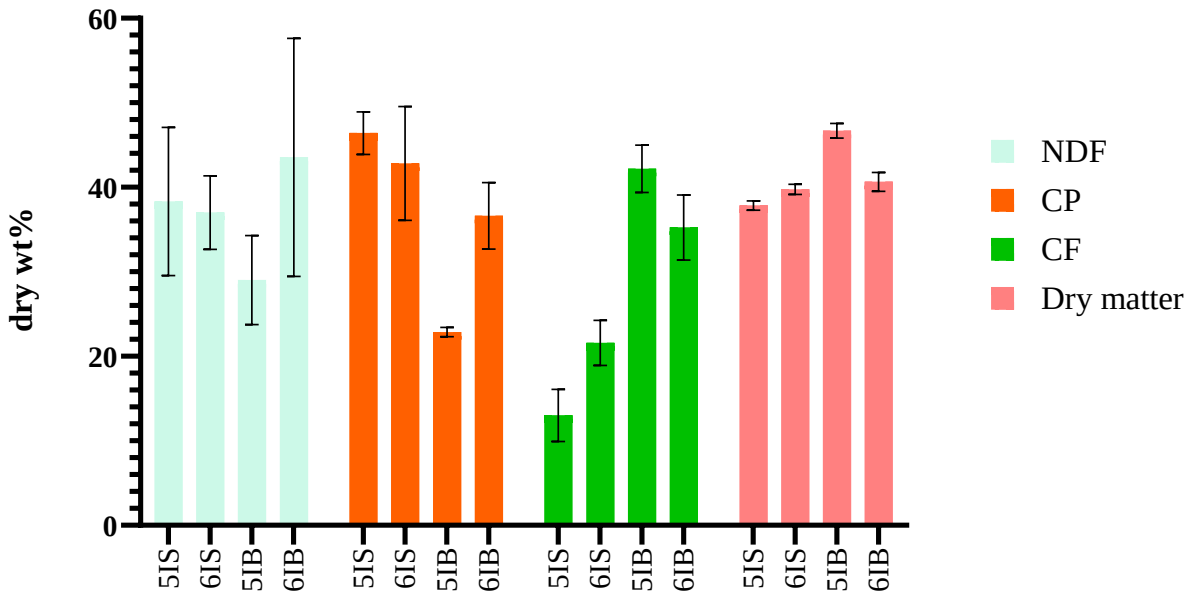

Figure 6 Effect of larval instar on chemical composition of BSFL from homogenous waste streams

All values are reported as mean and standard deviation from three replicates. 5IS and 6IS- 5th and 6th instar larvae from soy waste, 5IB and 6IB- 5th and 6th instar larvae from bread-vegetable diets. NDF- neutral detergent fibre, Cp – crude protein, CF – crude fat

The mineral concentration of BSFL reared from homogenous and heterogeneous wastes was similar as reported by others (Romano et al., 2023; Scieuzo et al., 2023; Shumo et al., 2019). The 5th and 6th instar BSFL reared on BV diets did not differ in Ca and K ($p>0.05$). No differences ($p>0.05$) in the P and S content of the 5th and 6th instar BSFL reared on SW were observed. Smets et al. (2020), reported that the concentration of Ca, K, Mg, P and Na can differ between the larval and prepupal stages. Similar trends were observed in this study, however, only for B and Mg. Minerals such as Na, P, S, Ba, and Se did not differ ($p>0.05$) despite the variations observed in the 5th and 6th instar BSFL grown on SW. Similarly, minerals including K and Ca did not differ ($p<0.05$) for 5th and 6th instar BSFL from the BV diets. Notably, Ca was found to be distributed at higher concentrations in the 6th instar BSFL from both SW and BV diets. This can be explained by the deposition of Ca in the cuticle at the later stage in the life cycle of the larvae (Do et al., 2021). Spranghers et al. (2017) also reported that the concentration of minerals in 6th instar BSFL reared with chicken feed, vegetable and restaurant waste remained unaltered irrespective of the substrate used. However, in this study, the concentration of most of the minerals evaluated varied in the 6th instar BSFL reared from the two waste streams. These

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results indicated that the waste substrate plays a greater role in in determining the variation in mineral concentration than the age of the larvae.

In case of the amino acids (AA), the concentration of Lys was low while Met was high in all the samples (**Figure 7**). This discrepancy in the content of these AA with those reported by other authors could be attributed to the differences in the geographical strain of BSFL (Makkar et al., 2014). Similar trends were reported for Cys, Met, and Thr, while the overall AA profile in the different BSFL samples was comparable to that of the conventional SM and fish meal used in animal feeds (Siddiqui et al., 2022).

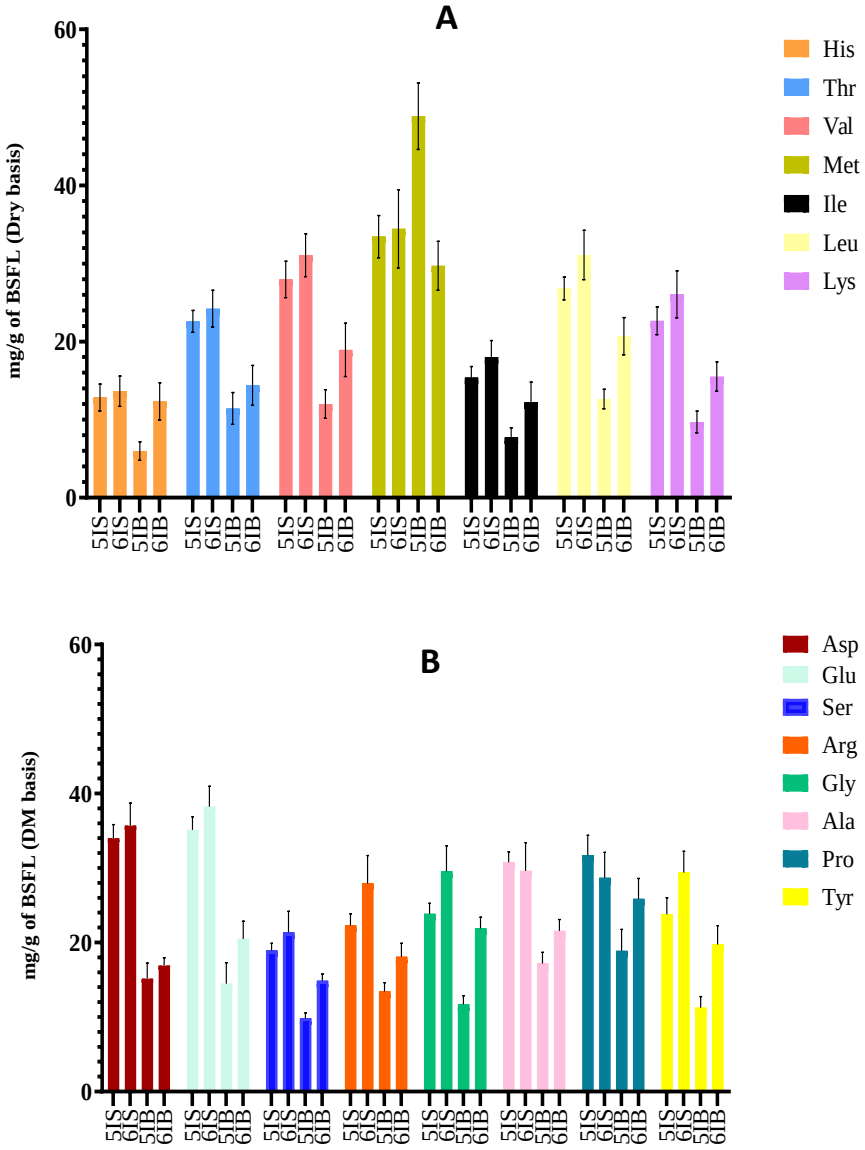

Figure 7 Effect of larval instars on amino acid composition (mg/g of DM BSFL) of BSFL from homogenous waste streams

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A. Concentration of limiting amino acids in 5th and 6th instar BSFL; B. Concentration of non-limiting amino acids in 5th and 6th instar BSFL. All values are reported as mean and standard deviation from three replicates. 5IS and 6IS- 5th and 6th instar larvae from soy waste, 5IB and 6IB- 5th and 6th instar larvae from bread-vegetable diets

The AA composition of BSFL samples from the BV diets were found to be relatively lower compared to that of samples from SW. It is established that the AA profile of BSFL varies based on the substrate composition hence, this could be attributed to the difference in protein quality brought by the substrates (Lalander et al., 2019; Ottoboni et al., 2018). The distribution of limiting AA (LAA) was higher ($p<0.05$) for most of the AA in the 6th instar BSFL compared to 5th instar larvae from BV diets except for Thr (no difference) and Met (lower in 6th instar). In the SW trials, Leu and Lys were the only LAA found to be distributed at higher ($p<0.05$) concentrations in the 6th instar BSFL. The distribution of non-limiting AA (NLAA) including Asp, Glu ,Ser, Pro, and Ala did not differ ($p>0.05$) among the two larval instars from the SW diets. In case of the BV diets, the concentration of all NLAA except Asp was found to be significantly higher ($p<0.05$) in the 6th instar BSFL. Overall, it was observed that the AA content (except Met) in pre-pupae was found to be relatively higher than of the larvae. Smets et al. (2020) in their studies reported that the concentration of certain AA's increased in pre-pupae which is in accordance with our findings. Overall, these results suggest that the amino acid content of the BSFL is party influenced by the larval instar and partly by the substrate used for rearing.

The relative distribution of fatty acids (FA) in the BSFL samples is reported in **Figure *8***. Saturated FA (SFA) were found to be predominant in BSFL samples reared on different agro-industrial by-products. This was followed by poly-unsaturated (PUFA) and mono-unsaturated FA (MUFA). Among the SFA's, lauric acid was found to be predominant (26.2% - 71.3%) followed by palmitic acid (4.9% - 16.9%) and myristic acid (0.8% - 4.1%). This trend was similar to previous findings reported (Ewald et al., 2020; Liland et al., 2017; Meneguz et al., 2018). BSFL is reported to synthesise lauric acid and other saturated fatty acids from carbohydrates present in the substrate (Liu et al., 2017; Spranghers et al., 2017). The same trend was observed in our trials as we observed relatively very small difference for the distribution of other saturated fatty acids for BSFL from different substrates. The distribution of PUFA was found to vary among the homogenised 5th instar BSFL from different trials. This is because BSFL like many other species of insects, does not have the enzyme precursor needed for *de novo* synthesis of the unsaturated fatty acids (UFA) and therefore its UFA composition is highly influenced by the compositional properties of the substrate fed (Ewald et al., 2020). It is also noteworthy that the distribution of UFA in unprocessed BSFL from SM and WM trials did not vary owing to the similarity in the constituents of the waste stream.

It was observed that the distribution of MUFA such as lauric acid, myristic acid, linoleic acid and palmitoleic did not differ ($p>0.05$) among the instars but varied ($p<0.05$) between SV and BV diets; relatively higher in BSFL from BV diets compared to others. Ewald et al. (2020) observed that BSFL fed with bread waste had higher lauric acid compared to the other BSFL samples. This is attributed to the larvae's ability to synthesise lauric acid from the carbohydrate content present in the substrate (bread). Fatty acids including myristic acid, stearic acid, oleic acid, gamma linoleic acid and nervonic acid did not differ ($p>0.05$) between the 5th and 6th instar BSFL grown on both SW and BV diets.

The distribution of palmitic acid was higher ($p<0.05$) in the 6th instar BSFL but only for larvae reared on SW. Overall, the fatty acid content of BSFL was found to be distributed at relatively higher concentrations in the 6th instar BSFL compared to the 5th instar larvae. This is due to the fact that BSF in their adult stages rely on their larval fat reserves for survival and other metabolic functions (Liu et al., 2017; Siddiqui et al., 2022).

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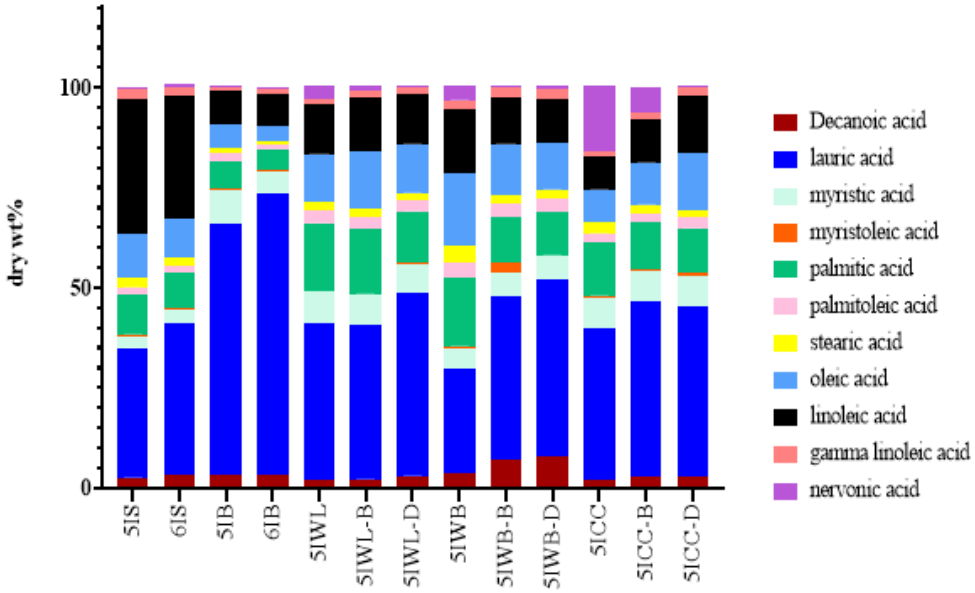

Figure 8 Relative distribution of fatty acids in BSFL reared with different food waste streams

All values are reported as mean and standard deviation from three replicates. 5IS and 6IS- 5th and 6th instar larvae from soy waste, 5IB and 6IB- 5th and 6th instar frass bread-vegetable diets. 5IWB, 5IWB-B, 5IWB-D- 5th instar larvae from waste mixture subjected to homogenisation, blanching, and drying respectively 5IWL, 5IWL-B, 5IWL-D- 5th instar larvae from supermarket waste subjected to homogenisation, blanching and drying respectively, 5ICC- 5th instar larvae from child-care centre waste subjected to homogenisation, blanching and drying respectively.

Ravi et al. (2020) observed that blanching and drying modified the CP content in BSFL. However, in this study, the CP content of the BSFL samples reared on three different heterogenous waste streams did not differ ($p>0.05$) with each other after the post-harvest treatments (**Figure *9***). This could be attributed to the drying conditions used. Zulkifli et al. (2022) dried BSFL samples with a conventional drying oven at different temperatures and time which influenced the CP, CF, and crude fibre content of the BSFL samples. The CP content reported for the BSFL in these heterogenous trials (Figure 9) is in accordance with other previously reported values. The CF content was found to be significantly lower ($p<0.05$) for blanched samples on comparison with dried and unprocessed BSFL from WM. The CF content of these samples ranged between 14.7 to 39.4 dry wt% with the lowest value observed for blanched BSFL samples from the WM waste stream. Unlike CP, CF content in BSFL samples varied greatly with changes in the carbohydrate content. Studies have revealed the linear relationship between the CF content of BSFL and the carbohydrate content of the rearing substrate (Barragán-Fonseca et al., 2018; Barragán-Fonseca et al., 2019; Eggink et al., 2023). Consequently, this observed discrepancy could be attributed to the variation observed in the CP to carbohydrate ratio of substrates used in our studies. The trends in results for NDF content were similar to that of crude protein content. The NDF values reported for BSFL in these trials are comparable to that of BSFL samples reared on similar diets (Shumo et al., 2019). Overall, the carbohydrate content of BSFL from these trials are comparable to values reported in previous studies (Lu et al., 2022). Post harvest treatments such as blanching and drying conducted in these studies did not significantly affect the CP, CF, and fibre contents of BSFL.

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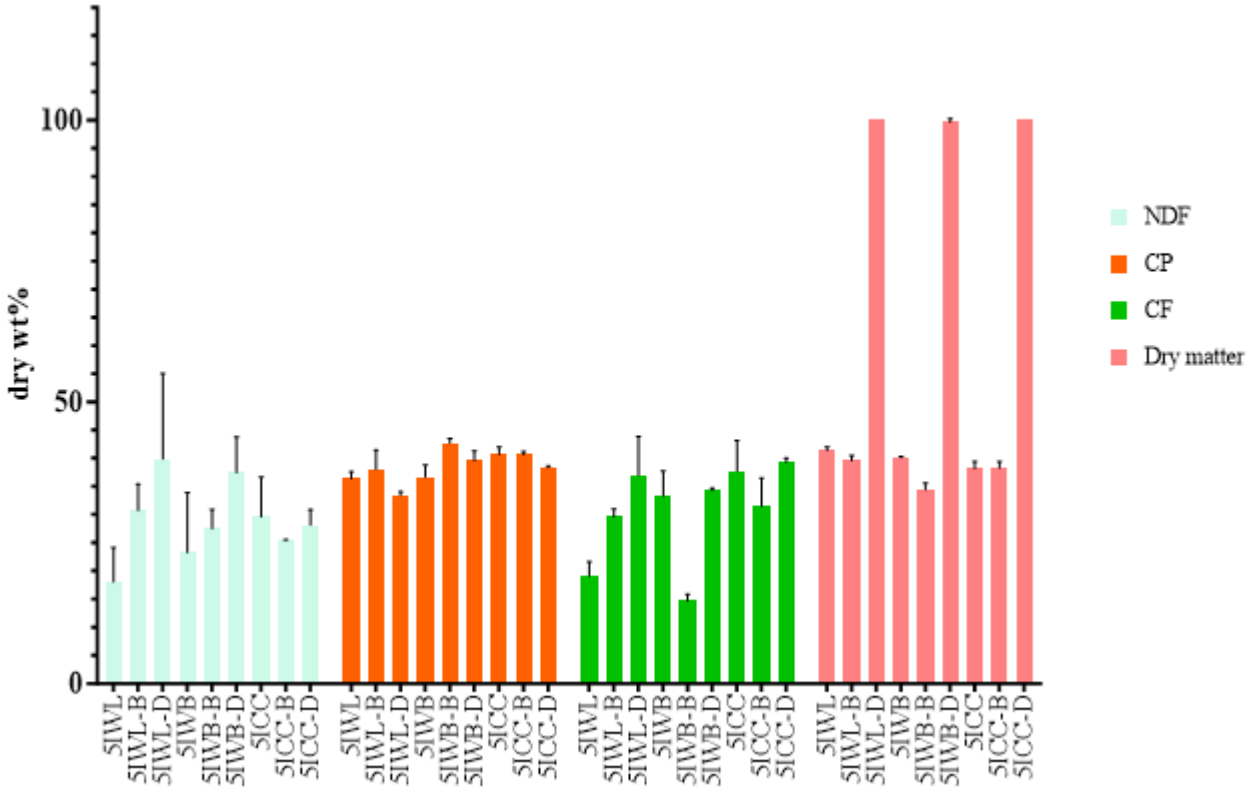

Figure 9 Effect of post-harvest treatments on the chemical composition of BSFL from heterogeneous waste streams

All values are reported as mean and standard deviation from three replicates. 5IWB, 5IWB-B, 5IWB-D- 5th instar larvae from waste mixture subjected to homogenisation, blanching, and drying respectively 5IWL, 5IWL-B, 5IWL-D- 5th instar larvae from supermarket waste subjected to homogenisation, blanching and drying respectively, 5ICC- 5th instar larvae from child-care centre waste subjected to homogenisation, blanching and drying respectively.

3.5 Effect of black soldier fly larvae reared on different waste streams on broiler production performance

The production performance of the broilers fed balanced commercial diets containing various levels (0, 3, 6, 9%) of BSFL reared on three waste streams is depicted in **Table *3***. For robustness, three control diets (without and BSFL) were included in the experimental design. Except for average daily feed intake (ADFI), neither the BSFL source nor their inclusion rates differed for the other production parameters. The statistical differences in ADFI were only between one of the control diets fed (Brisbane control: 106.99 g/day) and the diet containing the BSFL reared on the Brisbane waste at a 9% inclusion rate (99.96 g/day); the latter did not differ from the other two control diets.

The diets were expected to have the same production parameters for the broilers, as they all had been formulated to have the same level of digestible ingredients (amino acids and energy). Similar results were noted in an earlier study (Pieterse et al., 2019)), which also found that the chicken breasts tasted the same when evaluated by a trained sensory panel. Adegbenro et al. (2024) noted that when the fish meal was replaced with various levels of BSFL, the food conversion ratio, final body weight, and dressed yield did not differ between treatments. The BSFL diets were found to be more economical as the level of BSFL increased due to the lower costs of the BSFL compared to the fish meal.

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Table 3 Production performance of broilers fed commercial diets containing BSFL (at 0, 3, 6, 9% levels) reared on three commercial waste streams.

Interactions		Performance parameters (42-day trial)			
BSFL Stream	**Inclusion rate**	**FBW, g**	**ADG, g/day**	**ADFI, g/day**	**FCR**
Brisbane	0	3128.33	73.5665	106.99[a]	1.46
	3	2993.45	68.9038	100.29[ab]	1.46
	6	3071.83	72.2272	103.6[ab]	1.43
	9	2982	70.0794	99.96[b]	1.43
Hume	0	3071.21	72.1994	103.6[ab]	1.44
	3	3013.57	69.4008	100.97[ab]	1.46
	6	3058.02	71.8953	102.03[ab]	1.42
	9	3051.96	71.7421	103.4[ab]	1.44
Lendlease	0	2947.71	69.2688	100.08[ab]	1.45
	3	3035.96	71.3839	103.19[ab]	1.45
	6	3050.46	71.7222	103.41[ab]	1.44
	9	3018.17	70.9196	104.03[ab]	1.47
SEM		90.86	2.342	2.372	0.019
p-value		0.52	0.43	0.01	0.67
Main effect					
BSFL Stream	Brisbane	3043.9	71.294	102.71	1.44
	Hume	3048.69	71.309	102.5	1.44
	Landlease	3013.07	70.823	102.68	1.45
SEM		75.7	1.932	1.994	0.013
p-value		0.64	0.89	0.97	0.51
Main effects					
Inclusion rate	0	3049.08	71.678	103.56	1.45
	3	3014.33	69.896	101.49	1.45
	6	3060.1	71.948	103.02	1.43
	9	3017.37	70.913	102.46	1.45
SEM		77.53	1.981	2.039	0.014
p-value		0.71	0.36	0.37	0.4

Where FBW- final body weight, Adg – average daily gain, ADFI – average daily feed intake, FCR – food conversion ratio

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3.6 Near infrared spectroscopy for monitoring the quality of BSFL

NIR was able to distinguish between the larvae sourced from two different feeds (**Figure *10***). Similarly, the 5th instar larvae and the 6th instar pre-pupae were clearly separated from one another (**Figure *11***). This suggests that the information collected in the NIR spectra is capable of differentiating the 5th instar BSFL larvae from the 6th instar pre-pupae irrespective of the feed source used to rear them. NIR spectroscopy has demonstrated its ability to identify the age of several insect species, including *Musca domestica* (common house flies), *M. autumnalis* (flies), *Stomoxys calcitrans* (flies), *Culicoides sonorensis* (midges), *Anopheles gambiae* (mosquito), and *Anopheles arabiensis* (mosquito)) (Johnson & Naiker, 2020; Mayagaya et al., 2009; Perez-Mendoza et al., 2002; Sikulu et al., 2010). However, no reports were found on the use of NIR spectroscopy to distinguish BSFL samples based on their larval morphology. However, some 5th instar larvae were noted to overlap with the 6th instar pre-pupae samples - a plausible explanation is that some of the harvested 5th instar samples might be approaching the next morphological stage, which is the pre-pupae or the 6th instar.

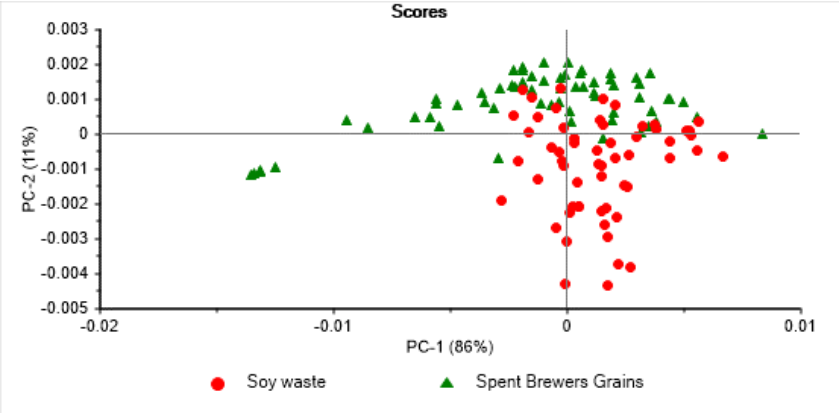

Figure 10 PCA plot for 5th instar larvae reared on two feeds

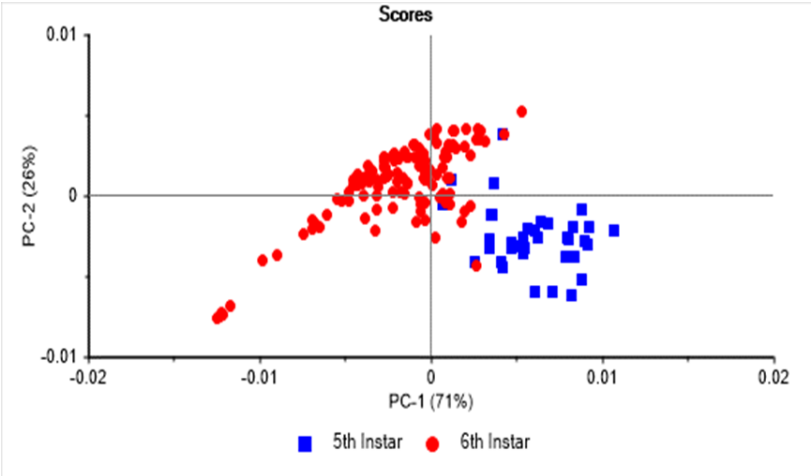

Figure 11 PCA plot for 5th instar larvae and 6th instar (pre-pupae)

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The cross-validation statistics for the prediction of proximate analysis using the second derivative NIR spectra obtained from BSFL and frass samples from two different diets gave variable results. The ratio of performance to deviation (RPD) value defined as the ratio of standard deviation to standard error of cross validation (SD/SECV) is used as a dimensionless statistic to assess the predictive ability of a calibration model. It has been reported that an RPD value higher than 4.0 can be considered excellent for routine analysis, while values between 3-4 are considered good for process or quality control applications and RPD values below two are deemed to be fit only for screening purposes (Fearn, 2002a; Williams, 2014). In this study, good cross-validation statistics (RPD value: 3.6) were obtained for the prediction of ADF in both larvae and frass samples. A moderate cross-validation (RPD values around 2.1) was obtained for total Carbon (TC) content suggesting that the model could be used for rough screening purposes. However, moderate to low RPD values were obtained for the prediction of CP (1.4), CF (1.6), starch (1.4), sugar (1.4) and NDF (1.6). These calibrations can be considered adequate as qualitative model (e.g. low, medium and high content). The poor cross-validation statistics obtained for some of the proximate analysis parameters can be explained by the narrow range in composition of the samples analysed (Fernández-Ahumada et al., 2006).

Additionally, the poor cross-validation statistics obtained for the prediction of CP could be attributed by the narrow range in protein (92.6-200.1 fresh weight g/kg). It is important to note that insects have high concentration of chitin as one of the main constituents of their exoskeleton. The concentration of chitin has been observed to be higher in the 6th instar larvae as well as in frass due to the shedding of their exoskeletons as the larvae reaches the adult stages (Soetemans et al., 2020). The analytical method employed to determine CP, quantifies the total nitrogen content of the sample, where CP is obtained by multiplying N by the conversion factor of 5.62. As BSFL and frass samples contain both protein and chitin, it is suggested that the accurate prediction of CP by NIRS-PLS models will be influenced by the chitin distributed in the samples. Kröncke and Benning (2022) and Cruz-Tirado et al. (2023) observed similar results in their studies for predicting CP in single live mealworm larvae and in BSFL using either NIR or NIR-Hyperspectral imaging, respectively.

4. Other outputs

4.1 Scientific papers:

A number of scientific papers emanated from this work:

Alagappan, S., Rowland, D., Barwell, R., Mantilla, S. M. O., Mikkelsen, D., James, P., Yarger,O, & Hoffman, L. C. (2022). Legislative landscape of black soldier fly (*Hermetia illucens*) as feed. *Journal of Insects as Food and Feed*, *8*(4), 343-355.

Alagappan, S., Rowland, D., Barwell, R., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2022). Organic side streams (bioproducts) as substrate for black soldier fly (*Hermetia illucens*) intended as animal feed: Chemical safety issues. Animal Production Science, 62(17), 1639-1651.

Alagappan, S., Hoffman, L. C., Mantilla, S. M. O., Mikkelsen, D., James, P., Yarger, O., & Cozzolino, D. (2022). Near Infrared Spectroscopy as a Traceability Tool to Monitor Black Soldier Fly Larvae (*Hermetia illucens*) Intended as Animal Feed. Applied Sciences, 12(16), 8168.

Alagappan, S., Dong, A., Mikkelsen, D., Hoffman, L. C., Mantilla, S. M. O., James, P., Yarger, O., & Cozzolino, D. (2023). Near infrared spectroscopy for prediction of yeast and mould counts in black soldier fly larvae, feed and frass: a proof of concept. Sensors, 23(15), 6946.

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Alagappan, S**.,** Hoffman, L., Mikkelsen, D., Mantilla, S. O., James, P., Yarger, O., & Cozzolino, D. (2024). Near-infrared spectroscopy (NIRS) for monitoring the nutritional composition of black soldier fly larvae (BSFL) and frass. *Journal of the Science of Food and Agriculture*, *104*(3), 1487-1496

Alagappan, S., Mallard, S., Cozzolino, D., Mikkelsen, D, James, P., Olarte Mantilla, S.M., Yarger, O., Hoffman, L.C. (2024). Effect of larval instar and post-harvest treatments on heavy metals in BSFL and frass reared on commercial food waste streams. *International Journal of Food Science & Technology,* https://doi.org/10.1111/ijfs.17511.

Alagappan, S., Kolobaric, A., Hoffman, L.C., Cozzolino, D. (2024). Current and potential applications of vibrational spectroscopy as tool in the black fly soldier production and circular economy. *Applied Sciences*, https://doi.org/10.3390/app14167318

Alagappan, S., Hong, H., Mikkelsen, D., Mantilla, S. O., James, P., Yarger, O., Hoffman, L., & Cozzolino, D. Investigating the Effect of Larval Instar, Post-Harvest Treatments, and Substrate on the Nutritional Profile of Black Soldier Fly Larvae (*Hermetia illucens*). Submitted to *Journal of Animal Production Science* in March 2024

Alagappan, S., Hoffman, L.C., Mikkelsen, D, Olarte Mantilla, S.M., James, P., Yarger, O., Cozzolino, D.. (2024). Near Infrared Spectroscopy (NIRS) for monitoring the nutritional composition of black soldier fly larvae (BSFL) and frass. Journal of the Science of Food and Agriculture, 104, 1487-1496. https://doi.org/10.1002/jsfa.13044

Cozzolino, D., Alagappan, S., Ochoa, M., Zhang, S., Yarger, O., Hoffman, L.C., Mikkelsen, D. (2124). Monitoring compositional changes in black soldier fly larvae after processing (drying and blanching) using near infrared spectroscopy. Infrared Physics & Technology, 138, 105212, 1-7. https://doi.org/10.1016/j.infrared.2024.105212

Mendez, C., Alagappan, S., Hoffman, L.C., Yarger, O., Cozzolino, D. (2024). Effect of Sample Presentation on the Classification of Black Soldier Fly Larvae using Near Infrared Spectroscopy. Applied Sciences, 14, 3841. https://doi.org/10.3390/app14093841

4.2 Higher degree by research students

A PhD student, Shanmugam Alagappan, has completed his PhD “Optimising and industrialising black soldier fly (BSF) production - redirecting food waste to livestock feed production using insects”.

Five Master students were also involved in this project, **Table *4* Masters students involved in this investigation**lists their subjects and status.

Table 4 Masters students involved in this investigation

Student Name	Degree	Project	Objectives	Publications	Completion Status
Monica Cely Ochoa	Masters in Food Science	Impact of post-harvest treatments on microbial quality	- BSFL samples grown on food waste were subjected to different blanching & drying treatments - Microbial quality of samples was investigated from a feed-safe perspective	NA	Completed-Dec 2022

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Shuxin Zhang	Master's in food science	Use of rapid analytical methods to monitor composition in black soldier fly larvae	- Investigate the use of NIR and MIR technologies for monitoring the compositional changes in BSFL grown on different food waste streams - Investigate the use of NIRS for monitoring the compositional changes in BSFL subjected to different post-harvest treatments	https://www.mdpi.com/1420-3049/27/21/7500 https://doi.org/10.1016/j.infrared.2024.105212	Completed-Jun 2023
Carmen Mendez Sanchez	Visiting Student-PhD	Traceability of BSFL with portable NIR spectroscopy	- Classify BSFL grown on different waste streams based using portable NIR - Investigate the effect of sample size and presentation on influencing the classification of BSFL with portable NIR	https://www.mdpi.com/2076-3417/14/9/3841	Completed- Sep 2023
Abdullah Hasib	Master's in food science	Invitro digestibility of BSFL grown on food waste streams	- BSFL samples grown on different food waste were subjected to invitro digestion - The digestibility of BSFL was investigated	NA	Ongoing-Expected June 2024
Xing Liu	Master's in food science	Extraction and characterisation of chitin from BSFL	- Effect of food waste on chitin content and quality was investigated - Effect of larval instar on chitin content and quality will be investigated - Effect of post-harvest techniques(Blanching & Drying) on chitin content and quality will be investigated	NA	Ongoing-Expected Dec 2024

4.3 Conference abstracts and presentations

Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L (2021). Optimising and industrialising black soldier fly (BSF) production -redirecting food waste to livestock feed production using insects. Annual Fight Food Waste Conference 2021. Brisbane, Australia, 17-18 November 2021 **(Oral Presentation)**

Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2022) Near Infrared Spectroscopy as a tool to trace the organic waste used to grow black soldier fly larvae destined for animal feed (2021). Australasian Milling conference: Insect Program, Gold Coast, Australia, 15-17 May 2022 **(Oral Presentation)**

Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2022). Near Infrared (NIR) Spectroscopy for the Prediction of Nutritional Composition of Black Soldier Fly Larvae. International conference for Insects to feed the world, Quebec, Canada, 12-16 June 2022 **(Virtual-Oral Presentation)**

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Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2022). Predicting the Nutritional Profile of Black soldier fly larvae (Hermetia illucens) using Near Infrared Spectroscopy. Australian Near Infrared Spectroscopy Group conference, 12-14 September 2022 **(Oral Presentation)**

Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2022). Mighty Maggots: Transforming food waste to feedstock. National Australian Food waste Summit Brisbane, Australia, 23-24 November 2021 **(Poster Presentation)**

Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2023). Microbial safety of black soldier fly larvae. Emerging Researchers Session at AIFST convention, Melbourne, Australia 24-25 July 2023 **(Oral Presentation)**

Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2023). Near Infrared Spectroscopy (NIRS) to assess the quality of black soldier fly larvae (BSFL). 21st International conference on Near Infrared spectroscopy, 20-24 August 2023 **(Oral Presentation)**

Alagappan, S., Cozzolino, D., Mikkelsen, D., Mantilla, S. M. O., James, P., Yarger,O, & Hoffman, L. (2023). Limitations and Implications for Optimising and industrialising black soldier fly (BSF) production intended for animal feed. Annual Food waste Convention 2023, Sydney, Australia, 22-23 November 2023. **(Oral Presentation)**

5. Conclusions & Recommendations

5.1 Conclusions

BSFL foraging upon the food waste delivers a product rich in various nutrients, making it a suitable feed ingredient in livestock farming. Despite being beneficial in food waste management and livestock farming, BSFL is not widely commercialised as a feed ingredient in Australia. The lack of knowledge about safety and the effect of processing treatments on the safety and quality of BSFL are a key factor influencing its commercialisation. This research explored various microbial and chemical safety attributes and several nutritional parameters of BSFL reared on food waste streams in commercial manufacturing facilities. The effect of larval instars and processing treatments on the safety z

nd nutritional quality of BSFL were also studied.

The results of this study indicated that BSFL were safe with respect to chemical safety parameters, including heavy metals and mycotoxins. The survival rate, growth rate, and biomass gain of BSFL in the presence of mycotoxins and heavy metals, were found to be largely unaffected. BSFL are prone to accumulate some heavy metals, including cadmium, arsenic and lead, which could potentially cause deleterious effects to animals fed BSFL or lead to residues in secondary animal products. The extent of risk from these safety hazards in BSFL appears to be influenced by the nature of the organic stream used for rearing. Rather than accumulating pesticides and mycotoxins, BSFL either excrete them or reduces the concentrations of these contaminants in the residual frass. Information regarding the chemical safety of the larvae when exposed to microplastics, dioxins, and other organic pollutants commonly found in the environment is limited and requires further research. Current studies have revealed the chemical safety of larvae when reared on conventional stockfeed and other homogenous substrates. Further research is also needed to investigate the cumulative effect caused by various contaminants that might occur together in organic side streams. The findings from future research will aid us in identifying different safety hazards associated with BSFL reared on different side streams, which

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in turn will aid in the determination of management approaches to mitigate specific risks and the development of a regulatory framework to promote the commercialization of BSFL in the animal feed sector.

The post-harvest treatments used, did not affect its nutritional profile. Overall, the post-harvest processing treatments carried out in this study appear to ensure the safety of BSFL, as microbial loads for the foodborne pathogens, except for *C. perfringens*, were below the detection limits. However, it must be noted that the extent of reduction in microbial counts will vary based on the type of organism and the initial load of the organism in the samples.

BSFL larvae grown on different food waste streams, feeds, and frass were tested for potential pathogenic microbes. This research demonstrated that the BSFL reared on diverse food waste streams seems relatively safe, where microbial counts for a broad set of pathogenic bacteria to animals and humans were within acceptable limits or, at times, below the detectable limits. However, if mixed food waste (including meat) were to be used as insect feed and BSFL only heat treated as outlined in this study it would not render the BSFL safe for animal feed from a biosecurity perspective. There needs to be caution set here. Currently, such material could only be rendered (AS rendering standard) before being fed to pigs and poultry (but would remain prohibited for ruminants). Safety also needs to consider animal bacterial and viral pathogens not investigated here.

The larvae's microbial load was primarily influenced by the feed source, the initial microbial load in the substrate, and several other associated intrinsic factors. In this study, *Salmonella, E. coli* and *L. monocytogenes* were not detected, and it is not clear whether the BSFL would accumulate these pathogens if present in the feed and/or whether the post-harvest treatments would negate their presence in the larvae, if present. The 6th instar larvae were lower in microbial counts than the 5th instar BSFL commonly used in feeding applications. Nonetheless, the thermal processing treatments in this study ensured that the 5th instar BSFL met the microbiological safety criteria for animal feeds. However, blanching and drying treatments were inadequate to reduce the spore-forming bacterium *C. perfringens* risk. The frass resulting from the bioconversion process had microbial counts similar to that of the diet feeds, potentially owing to the antimicrobial action exhibited by BSFL.

BSFL reared on different food waste streams in a commercial production facility was tested for the prevalence of chemical contaminants, including heavy metals and mycotoxins. The effect of larval instars and commonly practiced post-harvest treatments, including blanching and drying, on the chemical safety of the larvae was also evaluated. The concentration of heavy metals in BSFL was found to be primarily influenced by the substrate and its constituents. The findings of these studies suggested that the concentration of certain heavy metals was higher in 6th instar compared to that of the 5th instar BSFL. The effect of blanching and drying on influencing the concentration of heavy metals in BSFL was found to vary with different heavy metals. The concentration of heavy metals in the resulting residual frass was also found to be within the maximum tolerable limits suggested for soil fertilizers. The concentration of different mycotoxins analysed was below quantification limits in BSFL samples obtained from the different trials.

The nutritional profile of BSFL is influenced by several factors including the rearing substrate, larval age and post-harvest treatments, with substrate being the most influential factor. The study investigated the effect of larval instar and post-harvest treatments on the nutritional profile of BSFL reared on different food waste streams in two different commercial production facilities. The post-harvest treatments carried out in these studies did not significantly alter the nutritional composition of the different nutritional parameters analysed. The larval instar was found to influence the crude fat and fatty acid content of the BSFL but only partially affected their amino acid composition. The mineral contents of the BSFL were also influenced by the substrate, with little to no effect established by the age of the BSFL. The outcomes of this study will assist the commercial partner in selecting substrate(s) with desired nutritional properties to obtain BSFL with a desired nutritional quality. It will also assist Goterra in tailoring their post-harvest treatments to obtain BSFL suitable to meet varying market requirements.

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Feeding BSFL to broilers at different concentrations did not affect production parameters given the correct nutritional composition of the BSFL was used in the diets. Similarly, the source of the waste fed to the larvae had no impact on the broiler performance when nutritionally balanced diets were formulated and fed. BSFL is a suitable ingredient for use in commercial broiler diets. As broiler diets are formulated on a least-cost basis, the challenge for the BSFL industry is to produce sufficient quantities of BSFL at a competitive price.

NIR spectroscopy could be used as a non-invasive rapid tool for the real-time traceability of BSFL. A traceability system for BSFL will aid in risk evaluation and the identification of hazards associated with it, thereby assisting in improving the safety and quality of BSFL intended to be used by the animal feed industry. Also, the potential for utilising NIR spectroscopy to monitor the nutritional quality of BSFL and frass was explored. The NIR calibration models showed good to moderate prediction accuracy for ADF and TC content prediction for two different BSFL instars and frass reared on two different diets. However, calibration models developed for predicting CP, CF, starch, sugars, and NDF resulted in models with limited prediction accuracy. The poor prediction models for these nutritional attributes are thought to be due to the narrow range of data or non-uniform distribution of data over the ranges analysed. The lower accuracy of the calibration model for CP is likely due to the analytical errors involved in CP quantification brought about by the distribution of chitin in the samples. The reference data imposed certain restraints that influenced the accuracy of the PLS model developed for the larvae samples. NIR spectroscopy, in combination with chemometrics, can be used as an instantaneous tool to monitor the microbial quality of the feed samples intended to be used as the substrate for rearing BSFL. This will assist the commercial rearing facilities in selecting suitable feed sources for the larvae. The utilisation of this technique can also facilitate the selection of suitable processing methods either for the feed or for the larvae being reared based on the extent of contamination observed in the substrate.

5.2 Recommendations for future research

BSFL has been reported to exhibit antimicrobial action against a diverse set of pathogens. The effect of different food waste streams on influencing the antimicrobial activity of BSFL needs to be further explored. This will provide valuable information that can assist in improving the utilization of BSFL and its role as a feed ingredient. The effect of thermal processing treatments on different food waste streams and their subsequent impact on influencing the microbial and nutritional quality of the larvae also needs to be explored. For example, a quick blanching at 100°C was found to be efficient in killing various microbes but resulted in some of the fat/oil in the larvae leaching out; a lower temperature, more extended period might be more suitable; this needs to be researched further.

It was observed that frass's microbial contamination was higher than that of BSFL. The residual frass obtained from the bioconversion process with BSFL is considered a good organic fertilizer. The transfer of pathogenic microbes from soil fertilisers to agricultural products has been reported in the past; therefore, ensuring a safe pathogenic load of microbes in frass is essential. Hence, further research is needed to explore the effect of thermal treatments on the microbial load in frass and the suitability of frass as an organic fertilizer.

BSFL was observed to be safe against the different chemical contaminants tested. However, further research should be conducted to understand the functional pathways or mechanisms in BSFL that might be responsible for metabolising these chemical contaminants. Cytotoxic and genotoxic effects caused by the exposure of BSFL to chemical contaminants should be investigated as they might influence the growth and reproduction of BSFL. Understanding these degradation mechanisms and cytotoxic profiles would be beneficial to help maximise the productivity of BSFL in commercial rearing practices and to develop practices to reduce undesirable effects such as stunted growth, reduced weight gain, and other quality aspects of the larvae.

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NIR spectroscopy combined with chemometric techniques appeared to be a rapid non-destructive tool to monitor the different quality aspects of the BSFL production chain. The reference data used for predicting certain nutritional parameters had some limitations, which may have affected the calibration models' prediction accuracy. The likelihood of overcoming these limitations by the inclusion of samples from diverse waste streams with varied nutritional and microbial properties needs to be explored. The feasibility of using NIR spectroscopy to predict different quality aspects of BSFL has been shown, however, further research is required to validate the existing classification models to facilitate the use of this technology for real time applications in commercial rearing facilities.

Linked to the use of NIR, more research is required to streamline the production process, enabling real-time monitoring of the environment within the production facility. This will result in a more accurate prediction of the production volumes and the safety and nutritional value of the BSFL produced.

Presently, the BSFL are being dried and sold as animal feed. Evaluation of the actual nutritional digestive values of BSFL reared on different commercial waste streams in the poultry broiler and egg-laying industry will ensure a more focused market based on scientific evidence. This is also applicable to the use of BSFL as an ingredient in other species' feed such as fish, pigs, pets. At the same time, further processing of the BSFL into oil, proteins and chitin might yield alternative, more valuable products.

Although the Goterra system of valorisation of waste is seen as a positive contributor to decreasing waste's environmental impact, a full Life Cycle Analysis of Goterra's systems would greatly add value to their product. Finally, more focus should be placed on selling this "great recycling story."

6. Impact and Ongoing Monitoring

Industry profitability gained: Goterra has signed contracts that will ramp to a total contract value of +$10m AUD per annum due to the outcomes of the NIR and trial work for protein.

During the project's duration, six additional jobs were created through Goterra's growth.

Table 5 indicates the continuous (monthly) amount of food waste valorised and the Greenhouse gas emissions prevented.

Table 5 The amount of organic waste valorised through the utilisation BSFL (data supplied by Goterra)

	June 2024	July2024	Difference	2023 Totals	2024 Totals	Difference to date
Waste processed	249,955kgs	260,887kgs	10,932	1,051,615 kg	1,843,963 kgs	792,348kgs
CO2e mitigated	~474,914	~495,685	20,771	~1,998,069 kgs	~3,503,530 kgs	~1,505,461kgs

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