Opportunities for Organics Valorisation in a Circular Economy: Substrate Sensitivity of Black Soldier Fly (Diptera: Stratiomyidae) Larvae Production

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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Statement of Originality

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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June 2023

Authorship Attribution Statement

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The author of this thesis, Zachary Cohn, designed and performed the experiments, generated and analysed the data and wrote the manuscripts. This would not have been possible without the intellectual support and guidance of Dr Gustavo Fibres Weihs, Prof. Tanya Latty and Prof. Ali Abbas.

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As supervisor of the candidature upon which this thesis is based, I, Prof. Ali Abbas, can confirm that the authorship attribution statements above are correct.

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June 2023

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Abstract

Black Soldier Fly Larvae (*Hermetia illucens*, BSFL) are promising tools for organic waste valorisation in the circular economy, capable of upcycling a wide variety of organic materials into sustainable bio-based products. However, the quality and process performance of BSFL production are highly dependent on the substrate used. This poses challenges for the use of waste-based substrates, which are often heterogenous, poorly defined, and inconsistently available. This thesis investigates the influence of substrate properties on BSFL production. It combines a comprehensive literature review, controlled growth trials, and mixed-integer linear programming to provide a holistic understanding of the influence of substrate properties on BSFL quality and process performance. The research explores various substrate properties, including carbohydrate and fibre profiles, protein and carbohydrate contents, and contamination with perfluorooctanoic acid (PFOA).

Experimental studies reveal that substrate carbohydrate profiles significantly influence BSFL growth, nutritional composition and substrate conversion efficiency. For example, the presence of hemicellulose constituents like galactose and arabinose in the substrate can negatively impact larval process performance, with bioconversion reductions of -14.7 ± 6.8% and -63.0 ± 2.6% relative to the control, respectively. Additionally, larvae crude lipid contents are significantly increased by wheat starch (12.6 ± 3.0%) and decreased by galactose (-15.0 ± 1.4%) and xylan additives (-27.5 ± 3.4%), however fatty acid profiles are largely unaffected and remain dominated by lauric acid. There are minimal significant effects from mono- and di-saccharides or starch additives.

The concentration and source of fibre components influence BSFL growth, nutritional composition and fibre digestibility. Substrates with pure cellulose as an additive are found to enhance larval growth by 22.6 ± 2.2% through both protein and lipid gains. In contrast, the addition of apple fibre containing a complex of cellulose, hemicellulose and lignin, decreases

overall growth by -29.7 ± 3.2% through reductions in lipid and chitin contents, without affecting protein gain. Apple pectin does not alter larval growth at 17% inclusion in the substrate, but additional pectin reduces larval lipid accumulation, decreasing growth by -21.6 ± 8.1%. These effects appear to be due to substrate texture modifications caused by the fibre additives. Lignin and hemicellulose resist digestion, whereas cellulose is reduced from 13 to 23% across treatments. Similarly, soluble fibre resists digestion when present in chicken feed and apple fibre, however the pectin treatment shows reduction of 42.2 ± 5.1%.

Regarding substrate contamination, larvae survival and growth show resilience to PFOA exposure, a contaminant found in agricultural residues and water sources. Quantities bioaccumulated were minimal with bioaccumulation factors between 0.63 and 1.64, indicating the potential for valorisation of PFOA contaminated wastes via BSFL substrates. Preliminary findings show minimal effects on larval fatty acid profiles, however larvae exposed to high levels of PFOA for 11 days contained reduced linoleic acid and total fatty acid contents by -35.3 ± 5.8% and -8.2 ± 1.9%, respectively.

The developed mixed-integer linear programming model, demonstrated through a case study of New South Wales, Australia, highlights the implications of substrate sensitivity for the logistics of large-scale BSFL production. The model identifies optimal locations for BSFL facilities in light of available waste sources of varying protein and carbohydrate contents and demonstrates the potential for substrate diversification to improve profit from BSFL production by 14% when using a subsample of 50 waste generators from the developed NSW dataset. Product prices and maximum facility capacities yielded the most significant variations in the model output. The model provides a practical decision-making tool for policymakers and industry stakeholders to optimise BSFL production using wastes.

This thesis enhances our understanding of how substrate properties influence BSFL quality and production performance. This understanding is valuable for the formulation of

waste-based substrates, enabling BSFL production to align with the principles of the circular economy. Future research should identify methods to mitigate the anti-nutritional effects of poor carbohydrate profiles, exploit dietary fibre dependence of larval composition, and confirm bioaccumulation levels of novel per and poly-fluoroalkyl substances (PFAS) and contaminants. Future logistics optimisation research that considers substrate formulation from diverse wastes and accounts for further substrate properties beyond protein and carbohydrate would be beneficial for industry stakeholders.

Nomenclature

Term	Description
ACN	Acetonitrile
ADF	Acid Detergent Fibre
AMP	Anti-Microbial Peptide
ANF	Anti-Nutritional Factor
ANOVA	Analysis Of Variance
AOAC	Association of Official Analytical Chemists
API	Application Programming Interface
AS	Australian Standard
ATP	Adenosine Triphosphate
AUD	Australian Dollar
BAF	Bioaccumulation Factor
BER	Bioconversion Efficiency corrected for Residue
BSFL	Black Soldier Fly Larvae
BSFLM	Black Soldier Fly Larvae Meal
C	Carbohydrate
C18	Octadecyl silica sorbent
CE	Circular Economy
CEL	High Cellulose treatment
CTL	Control
DCM	Dichloromethane
DF	Dietary Fibre
DM	Dry Matter
EAFC	Equivalent Annual Fixed Cost
ECD	Efficiency of Conversion of Digested food
ECI	Efficiency of Conversion of Ingested food
EI	Electron Impact
EPA	Environmental Protection Agency
EPL	EPA license
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FCR	Feed Conversion Ratio
FM	Fishmeal
GC	Gas Chromatography
HPLC	High-Performance Liquid Chromatography
HSO	Very High Soluble Fibre treatment
HVAC	Heating, Ventilation and Air Conditioning
ID	Internal Diameter
IDF	Insoluble Dietary Fibre
INS	High Insoluble Fibre treatment
IS	Internal Standard
Kp	Nitrogen to Protein Conversion Factor
LC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
LGA	Local Government Area
MILP	Mixed Integer Linear Programming
MS	Mass Spectrometry
NDF	Neutral Detergent Fibre
NFWB	National Food Waste Baseline
NP	Nondeterministic Polynomial time
NPI	National Pollutant Inventory
NSP	Non-Starch Polysaccharides
NSW	New South Whales
P	Protein
PET	Polyethylene Terephthalate
PFAS	Per and Poly-Fluoroalkyl Substances
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
POEO	Protection of the Environment Operations license
PSA	Primary and Secondary Amine sorbent
QUECHERS	Quick Easy Cheap Effective Rugged and Safe
RAM	Restricted Animal Material
RF	Retention Factor
RO	Reverse Osmosis

RRF	Relative Retention Factor
SBM	Soybean Meal
SCFA	Short Chain Fatty Acid
SDF	Soluble Dietary Fibre
SOL	High Soluble Fibre treatment
TDF	Total Dietary Fibre
USD	United States Dollar
VS	Volatile Solids

Nomenclature used in the model formulation of Chapter 6.

Term	Description
$i \in I$	Index and set for candidate facilities
$j \in J$	Index and set for substrates
$k \in K$	Index and set for waste generators
P_L	Value per unit mass of BSFL lipid produced
P_M	Value per unit mass of BSFL meal produced
P_k	Price per unit mass of waste from generator k
$D_{i,k}$	Distance between generator k and facility i
P_t	Cost per kilometre per tonne for transport
P_{EAFC}	Equivalent annual fixed cost per facility
P_v	Operating cost per unit mass of waste processed
G_k	Quantity of waste generated by generator k
F	Minimum fraction of facility capacity used
Q	Maximum capacity of each facility
U	Minimum fraction of total waste to be processed
M	Large constant
τ	Tortuosity factor
C_j^M	Protein conversion factor for substrate j
C_j^L	Lipid conversion factor for substrate j
S_k^P	Protein content of waste from generator k on a wet mass basis
S_k^C	Carbohydrate content of waste from generator k on a wet mass basis
S_j^P	Protein content of substrate j on a wet mass basis
S_j^C	Carbohydrate content of substrate j on a wet mass basis
$y_{i,k,j}^P$	Protein content of substrate j at facility i
$y_{i,k,j}^C$	Carbohydrate content of substrate j at facility i
$b_{i,j}$	Binary variable indicating if substrate j is used at facility i
$w_{i,k,j}$	Quantity of waste from generator k used in substrate j at facility i
e_i	Binary variable denoting the existence of a facility at location i

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Chapter 1. Introduction

1.1. Background

The circular economy is a concept gaining traction worldwide in which resources are continually cycled through the economy rather than disposed of as wastes at the end of their life (EMF, 2013). In this context, the valorisation of organic waste through the production of Black Soldier Fly Larvae (BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), has emerged as a promising strategy (Barry, 2004; Craig Sheppard et al., 1994; Diener et al., 2009; Girotto and Cossu, 2019; Nguyen et al., 2015; Salomone et al., 2017). Black Soldier Fly Larvae can upcycle a wide variety of organic materials - such as manures, food by-products and agricultural residues - into sustainable bio-based products (Diener et al., 2011), preventing these materials from becoming 'wastes' by bringing them back into economic circulation, as per the circular economy ideal (Kirchherr et al., 2017).

However, the quality and process performance of BSFL production are highly sensitive to the substrate used. In terms of BSFL quality, the nutritional composition and safety of larval products are especially sensitive to alterations in the substrate, as are process performance measures such as larval growth rates and substrate conversion efficiency (Fels-Klerx et al., 2018; Gold et al., 2020; Tschirner and Simon, 2015). This sensitivity poses challenges for waste-based substrates, which are often heterogenous, poorly defined and inconsistently available. Substrate sensitivity is especially problematic for industrial-scale operations, where the largest circular economy benefits can be realised, and a consistent supply of reliable substrate is crucial (Joly and Nikiema, 2019; Surendra et al., 2020). For this reason, the use of non-waste materials in BSFL substrates is prevalent, such as those used in traditional animal feeds, sacrificing the fundamental circular economy promise of BSFL valorisation.

Therefore, understanding which substrate properties influence BSFL production – and how – is paramount in developing strategies for managing substrate sensitivity and thereby ensuring the circular economy value of BSFL is realised. This thesis aims to contribute to this understanding.

1.2. Objective

The primary objective of this thesis is to develop our understanding of how substrate properties influence BSFL quality and process performance such that BSFL producers may optimise production using waste-based substrates. The substrate properties and aspects of BSFL quality and production performance explored are detailed in Section 1.3.

1.3. Research Approach and Thesis Structure

The research approach is outlined in Figure 1.1. Chapter 2 comprehensively reviews the literature on the potential of BSFL as a source of circular bioproducts, as well as the production process, identifying gaps in understanding the influence of substrate properties. This chapter presents the benefits and pitfalls of typical approaches to BSFL production as a form of traditional agriculture or composting when investigating substrate properties. Among the identified gaps, the most impactful and feasible to address in the context of this thesis are selected for exploration through experimental studies. These studies are documented in the following three chapters.

Chapter 3 experimentally explores the influence of dietary non-fibre carbohydrates on BSFL production performance and the quality of produced larvae in terms of fatty acid contents and compositions. Chapter 4 expands on the second, investigating the involvement of substrate dietary fibres in BSFL production and their influence on larval performance and proximate composition. Chapter 5 examines the safety aspect of BSFL quality by studying the bioaccumulation of perfluorooctanoic acid (PFOA), a prevalent contaminant across agricultural residues and water sources. Together, these chapters generate new insights into the

dominant components of BSFL substrates and provide a much-needed confirmation of the larvae's safety when exposed to realistic levels of PFOA.

Chapter 6 adopts a mathematical modelling approach involving the development of a mixed-integer linear programming optimisation model to explore the consequences of substrate properties on the logistics of BSFL production at scale. The developed model is demonstrated for several scenarios using New South Wales, Australia, as a case study. This chapter presents the need for further exploration into substrate properties and provides a valuable decision-making tool for policymakers and industry stakeholders in optimising BSFL production or other circular economy technologies.

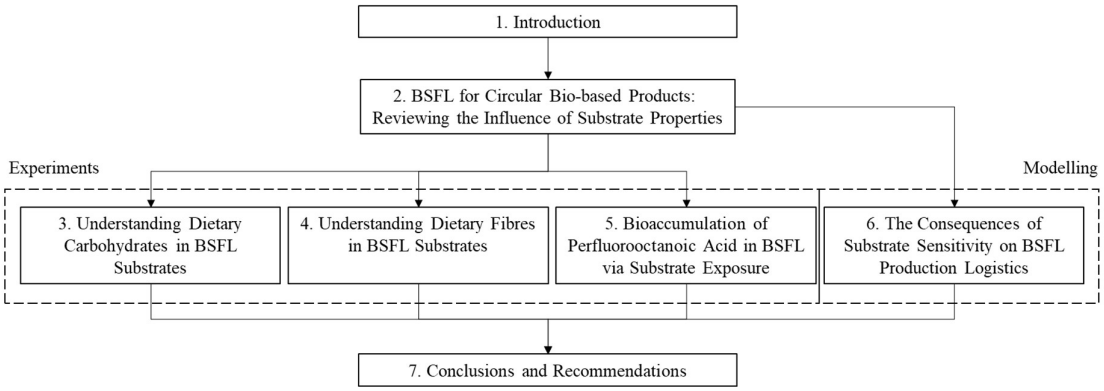

Figure 1.1 Overview of the research approach and thesis structure

Each chapter contributes to the overall objective of the thesis, which is to enhance our understanding of how substrate properties influence BSFL quality and production performance, thereby assisting in the valorisation of organic wastes and facilitating BSFL's contribution to a circular economy. Capping off the thesis are the conclusions and recommendations, along with appendices.

Chapter 2. Black Soldier Fly Larvae for Circular Bio-based Products: A Comprehensive Review of Substrate Properties and Future Research Directions in Larval Production

2.1. The Circular Economy Shift

The linear economic system is inherently unsustainable, relying on the false basis of limitless environmental resources for societal growth (Bonciu, 2014). Although this model has enabled tremendous global development, this has come at the cost of fuelling some of humanity's greatest challenges: climate change and widespread inequality (Bonciu, 2014; Leamer et al., 1999; Stern, 2011). There is now urgency for the adoption of a circular economic system, one more appropriate for a world approaching 10 billion people in the coming decades (Godfray, 2014; Wiebe et al., 2019). The circular economy provides a systems framework for addressing the root of these global challenges by enabling sustainable and resilient development while regenerating the environment (EMF, 2013; Morseletto, 2020; Stahel, 2016).

Definitions of a Circular Economy (CE) differ across sources; however, they often share the same core vision for an economy where resource value is maximised and maintained through material cycling, minimising energy use and emissions (Kirchherr et al., 2017; Reike et al., 2018). These translate into direct environmental and economic benefits plus the creation of new jobs (EMF, 2013; Pauli, 2010). The circular economy requires redesign and integration at multiple levels, from government and industry to local communities and products. Innovative and practical business strategies are often used to achieve this.

It is important to emphasise that the circular economy is distinct from a waste management strategy, such as recycling. Instead of looking at today's 'end of pipe' wastes and working out what to do with them, the circular economy fundamentally calls for a paradigm

shift in product design, whereby products are created with their purpose and complete lifecycle in mind, including waste generation (EMF, 2013). This review is therefore intentionally structured with a focus on products first and wastes second.

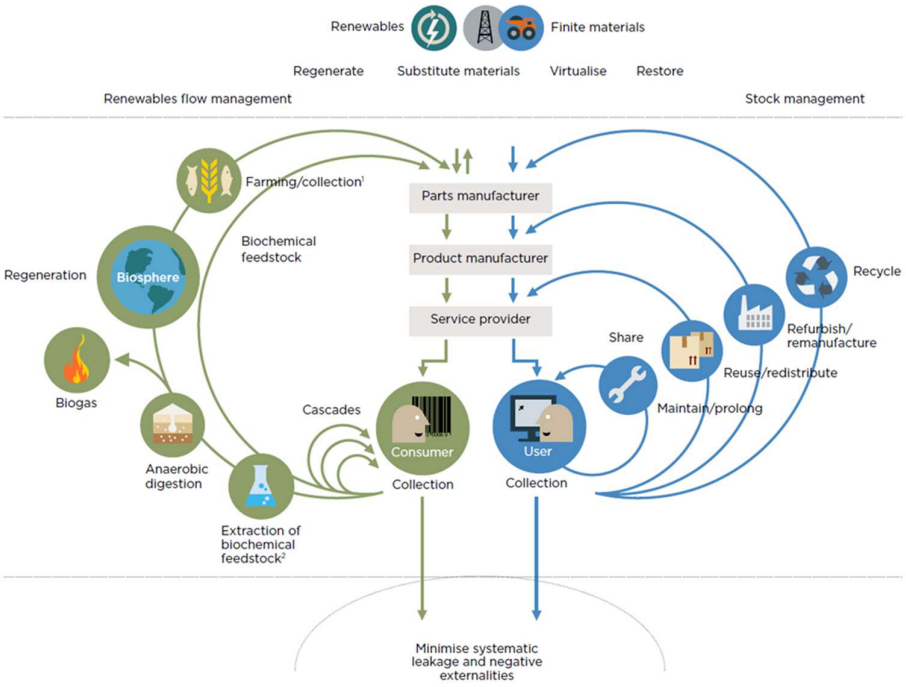

Figure 2.1 The butterfly diagram: a commonly accepted depiction of the Circular Economy (EMF et al., 2019).

The CE concept is commonly visualised by the Ellen Macarthur Foundations' butterfly diagram (Figure 2.1), distinguishing technical materials and biological nutrients, with strategies for value retention (Bianchini et al., 2019; EMF et al., 2019; Velenturf et al., 2019; Velenturf et al., 2020). While conceptually useful, this is regrettably limited in its presentation of biological and technical cycles as distinct, despite many bio-products (e.g., wooden furniture, cotton textiles) benefitting from the same value retention strategies such as sharing, reuse and refurbishment. Furthermore, the biological 'wing' is significantly underdeveloped, with only a general indication of cascades as means for maximising value retention of biological nutrients, presenting anaerobic digestion as a 'one size fits all' processing step for by-products of biochemical feedstock extraction. This oversimplification is understandable given the complexity and range of value retention strategies available for bio-products (Carus

and Dammer, 2018). However, biological nutrients are such immense and fundamentally necessary components of the economy that they require more explicit coverage in the circular economy framework.

2.1.1. Bioeconomy: Designing Products from Organic Resources

The concept of the 'Circular Bioeconomy' addresses the shortcomings of the Ellen Macarthur Foundation's depiction of a circular economy, providing value retention options that are overall complementary to the discourse around circular economy. The Bioeconomy is defined by Carus and Dammer (2018) as "encompassing the production of renewable biological resources and the conversion of these resources and waste streams into value added products, such as food, feed, biobased products and bioenergy". Bioeconomy policies built on similar definitions have been promoted across the world especially since the early 2000's (Birner, 2018). While the Bioeconomy is inherently circular since bio-based products return to the environment through biogeochemical cycling, the 'Circular Bioeconomy' encompasses relevant circular economy ideals (Carus and Dammer, 2018), including:

- Emphasis on bio-based products,
- Minimisation of greenhouse gas emissions,
- Reduced reliance on virgin materials and fossil fuels,
- Sharing, reuse, remanufacture and recycling of bio-based materials through cascading use,
- Use and valorisation of unavoidable organic wastes and side streams,
- Return of biodegradable products to nutrient cycles.

This emphasis on bio-based products (termed 'bioproducts') will be invaluable for our transition to a circular economy (Jain et al., 2022). In particular, by substituting biomaterials into traditionally fossil-based products and thereby enabling their recycling through biochemical means (e.g., substituting polypropylene and polyethylene toothbrushes for renewably sourced polylactic acid or wood composites). Biorefineries are the processing plants

of the bioeconomy, facilitating the conversion of biomass into bioproducts using processes such as fermentation, pyrolysis and gasification (Dahiya et al., 2018; Satchatippavarn et al., 2015; Wang et al., 2017). Through integration of biorefineries with industries such as agriculture, forestry and manufacturing, industrial symbioses may be established, and the ideals of a circular economy realised (Kundu et al., 2021; Tumilar et al., 2021).

To facilitate greater adoption of high value bioproducts, more emphasis on specialised product design is needed in which the ultimate end users are considered. This must happen before process design (Moggridge and Cussler, 2001). The chemicals industry is one example having begun its transition to more specialised, designed products, where value is created from microstructures (e.g. in paints and cosmetics) rather than from molecular structures (as in bulk commodity chemicals like petroleum) (Saraiva and Costa, 2004). Animal feeds are another example, being increasingly designed as formulations of ingredients to improve end product qualities, such as fortifying omega 3 content in chicken eggs (Alagawany et al., 2019), colouring in salmon fillets (Nogueira et al., 2017) and low emissions beef (Kinley et al., 2020).

As with the circular economy, confusion can arise in justifying the circular bioeconomy's value over the traditional waste management hierarchy of value retention, outlined in Figure 2.2.

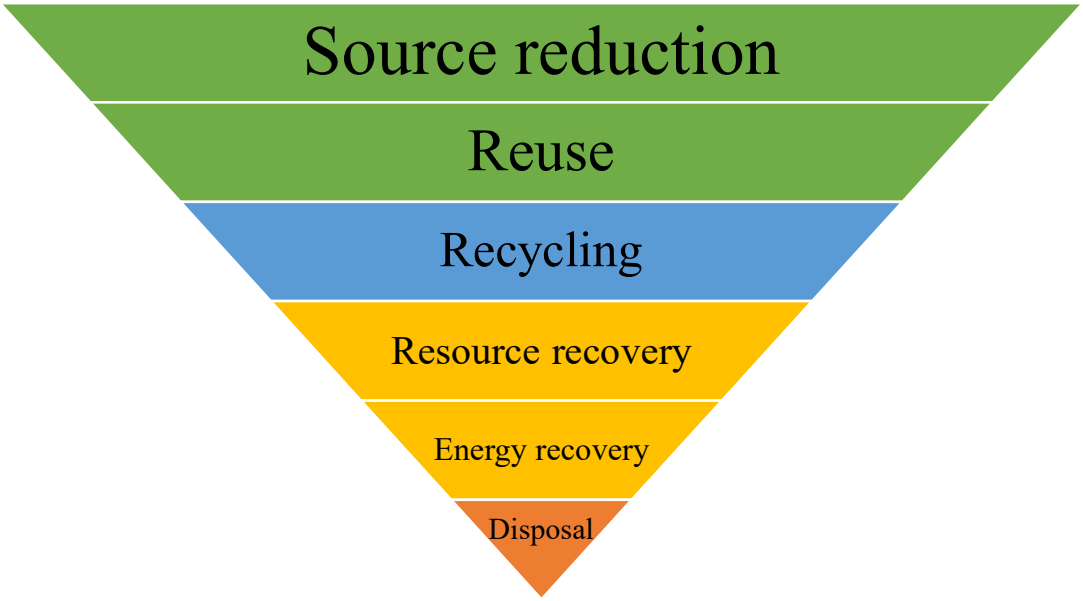

Figure 2.2 The traditional Waste Management Hierarchy (Jibril et al., 2012).

The insufficiency of the traditional waste management hierarchy alone is that it is typically only applied once a resource has been labelled as waste (Korhonen et al., 2018). As such, currently a large proportion of intentionally generated biomass is used foremostly for energy generation, removing any opportunity for reuse as a material (excluding direct carbon capture and use), as would be dictated by the waste management hierarchy for 'waste' biomass (Carus and Dammer, 2018). This effectively wastes much of the embodied value input to the biomass' production and collection. To avoid this inefficiency, the Circular Bioeconomy calls for cascading uses to be pre-planned into the lifecycle of all biomass, as is requested for all materials in a circular economy.

2.2. Black Soldier Fly Larvae as a Source of Circular Bioproducts

Insects such as fly larvae, crickets and cockroaches show significant promise as components of designed bioproducts, including feeds, foods, cosmetics, pharmaceuticals and fuels (Cappellozza et al., 2019; Cickova et al., 2015; Gold et al., 2018; Madau et al., 2020; Makkar et al., 2014; Müller et al., 2017; Nguyen et al., 2015; Ravi et al., 2020; Wang and Shelomi, 2017). Most promising of these are the black soldier fly larvae (BSFL) (*Hermetia*

illucens. Diptera: Stratiomydae). BSFL are uniquely positioned at the intersection between waste and product streams, as they are capable of efficiently transforming various natural organic wastes, such as agricultural by-products, food wastes and animal manures, into protein and lipid-rich biomass (Craig Sheppard et al., 1994; Diener et al., 2011a; Diener et al., 2011b). A notable advantage to BSFL is the potential to modify their body composition using select substrate formulations and process conditions to more closely match desired quality specifications of downstream industries (Barragán-Fonseca, 2018; Ewald et al., 2020a; Siddiqui et al., 2022). They possess an immense growth rate, able to increase in mass by 4000-fold from egg to mature larvae (Liu et al., 2017). These facets create significant opportunity for BSFL as a source of circular bioproducts and has prompted significant growing interest in their production across academia and industry (Gold et al., 2018; Ravi et al., 2020).

Additional advantages to the production of BSFL for bioproducts include:

- Suitability for low-tech production, thereby reducing costs and hazards associated with high temperatures and pressures, as well as machinery used in alternative manufacturing facilities (Dortmans B.M.A. et al., 2017). This increases the technology's accessibility, particularly to low-income areas where organic wastes may otherwise be openly burned or dumped rather than upcycled.
- The species' cosmopolitan distribution, high fecundity and fast growth rates facilitate rapid startup and large-scale, automatable production (Joly and Nikiema, 2019; Makkar et al., 2014).
- Diversity of products and corresponding downstream applications mitigate risk associated with disruptions in any one market and may improve brand strength (Stanley and Bunnag, 2001).
- Extensive substrate reduction (reported up to 80% on a wet basis) and lower environmental costs in terms of energy, water, land, and material use compared to alternative waste management options (Dortmans B.M.A. et al., 2017; Huis and Oonincx, 2017).

When produced in an engineered bioconversion system, thousands of freshly hatched BSFL have been found to consume between 25 and 500 mg of fresh substrate per larvae per day (dependent on rearing substrate and culture conditions) alongside commensurate microorganisms, accumulating substantial proteins, lipids and other biomolecules as they develop (Makkar et al., 2014). Typically after 10-14 days, larvae are separated from the remaining substrate residue having reached a reportedly maximum size of 27 mm long and 220 mg in fresh weight, ready for downstream use (Joly and Nikiema, 2019; Makkar et al., 2014). Some larvae are allowed to pupate into adult flies to supply the next generation of eggs. An approximate mass balance schematic is shown in Figure 2.3, presenting the production steps in producing 125 t of fresh larvae from 1,000 t of fruit and vegetable waste-based substrate.

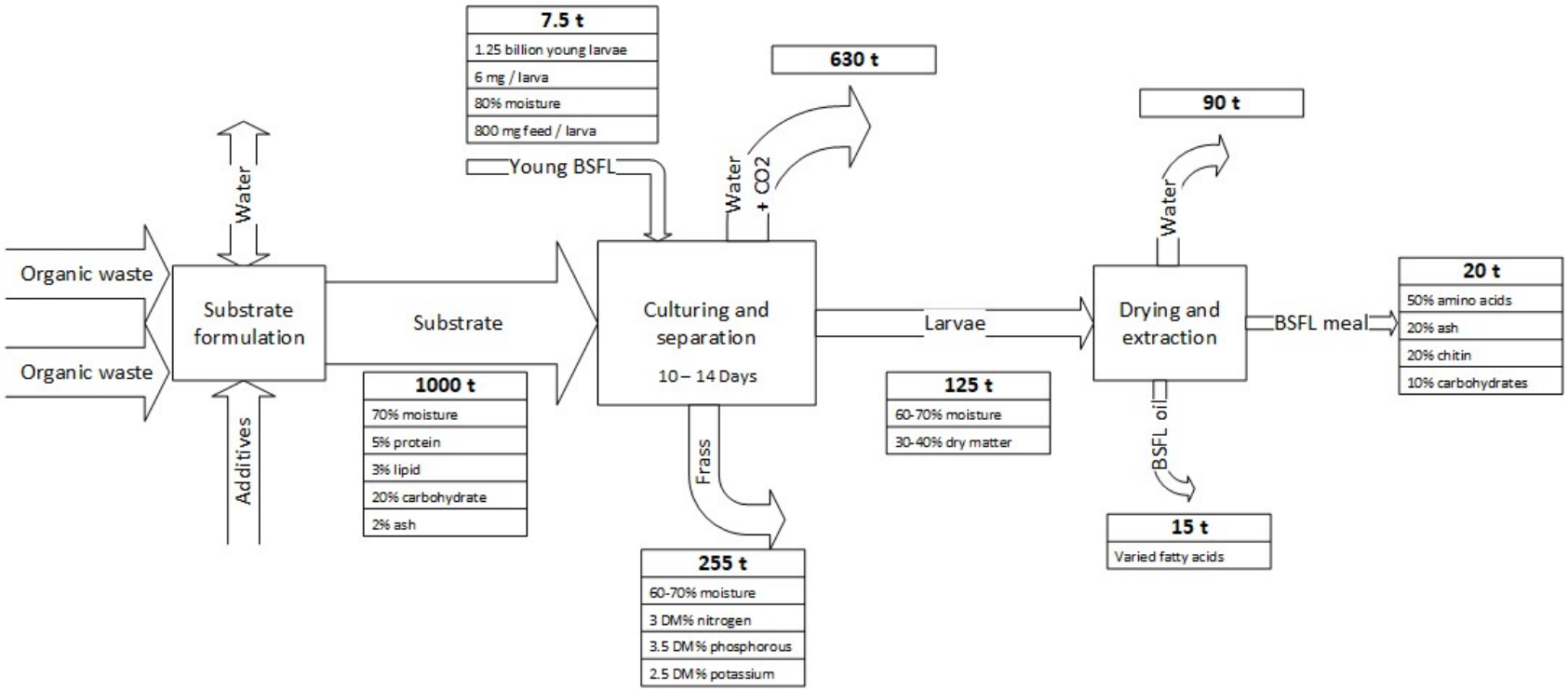

Figure 2.3 An example mass balance on black soldier fly treatment of organic wastes calculated using values from literature (Dortmans B.M.A. et al., 2017; Joly and Nikiema, 2019; Makkar et al., 2014; Ravi et al., 2020). Substrate proximate analysis is based on exemplar values for fruit and vegetable waste.

2.2.1. Products and Relevant Quality Factors

As shown in Figure 2.3, the bulk outputs from BSFL production can be separated into water, larval meal, larval fat and feed-residue fractions. Quality of these fractions is defined by the requirements of the downstream product and are influenced by the substrate and process settings (Bosch et al., 2020; Gold et al., 2020b). BSFL production facilities produce similar types of products, primarily feed ingredients and fertiliser, with larger facilities producing higher quality products using refining equipment (Joly and Nikiema, 2019). These have been identified in the literature and commercially as each being suitable for different downstream use cases, as will be reviewed in this section.

2.2.1.1. Whole Larvae

While live or whole dehydrated larvae are typically valued much more highly than the processed fractions (~$100/kg, ~$80/kg respectively in AUD), they are usually sold at a significantly smaller scales and pose sanitation concerns (Klunder et al., 2012; Meat & Livestock Australia and All Energy PL, 2017). Live larvae additionally pose logistical constraints in terms of storage and transport (Meat & Livestock Australia and All Energy PL, 2017).

2.2.1.2. Proximate Composition

The water, protein, lipid, chitin and ash content of BSFL compose the proximate composition and reflect the larvae's suitability as raw material in animal feed or biochemical productions. In particular, excessive chitin, lipids and ash contents can be detrimental in animal feed formulations, hence quantities of these can be used as measures of whole BSFL quality in animal feed markets (Lalander et al., 2019). The ratio of these components is dependent on the substrate and life stage of the larvae at harvest as shown in Figure 2.4 and Figure 2.5 (Beniers and Graham, 2019; Liu et al., 2017).

Typically, larvae accumulate more lipids as they mature until the prepupal stage, at which point feeding ceases and stored lipids are used for energy (Beniers and Graham, 2019; Tomberlin et al., 2002). Growth trials tend to cease upon the emergence of prepupae for this reason, often between 9 and 14 days from introduction to the bulk substrate (Bosch et al., 2019; Joly and Nikiema, 2019). Numerous studies have shown mature larval water and crude protein content is minimally influenced by substrate, typically 65-75% and 35-43% dry matter in mature larvae, respectively (Cappellozza et al., 2019; Meneguz et al., 2018c; Oonincx et al., 2015). Contrarily, crude lipid content varies widely as a function of substrate (14-49% dry matter in mature larvae) in reflection of substrate lipid and non-fibre carbohydrate contents (Barry, 2004; Joly and Nikiema, 2019; St-Hilaire et al., 2007a). This relation between carbohydrate content in the substrate and fat content in insects is well documented in literature (Barragán-Fonseca, 2018; Makkar et al., 2014; Stanley-Samuelson et al., 1988).

Chitin content increases as the larvae mature, with maximum levels at the pupal stage (Caligiani et al., 2018). For this reason, some studies choose to harvest earlier 5^{th} instar larvae whose chitin content typically ranges from 4-8% dry mass (Gold et al., 2020b; Joly and Nikiema, 2019). While younger larvae at earlier instars contain less lipid and chitin and therefore are more protein rich, the small size of younger larvae poses significant harvesting challenges (Wang and Shelomi, 2017). Additionally, much concern regarding chitin as a detrimental component of animal feed stems from studies on its use in aquafeed in which chitin is inferred to reduce performance without conclusive evidence, hence avoiding higher chitin contents by harvesting early instar larvae may not be worthwhile (Henry et al., 2015). Ash contents vary with values found between 3-25% dry matter, loosely correlated with substrate ash contents (Joly and Nikiema, 2019; Spranghers et al., 2017).

Wet chemistry methods are used to determine proximate composition, typically developed from the Association of Official Analytical Chemists (AOAC) standards (Caligiani

et al., 2018; Ewald et al., 2020b). Differences across studies in analytical methods, alongside rearing conditions and fly genetics, result in immense variations in reported proximate compositions in the literature (Bosch et al., 2020). This is most apparent when attempting to quantitatively identify the effect of substrate on proximate composition from literature data, as may be done in a meta-analysis. The recent systematic review by Hopkins et al. (2021) demonstrates this, finding excessive ranges in literature reported protein and lipid contents of 12.9 – 78.8% and 2.2 – 57.8%, respectively. Omissions in reporting key process conditions makes it challenging to normalise data across studies, highlighting the need for adoption of standardised operating procedures. This lack of standardisation is a significant missed opportunity for furthering understanding of BSFL production, given the large and growing body of studies exploring larvae production on varied substrates (Bosch et al., 2020; Hopkins et al., 2021).

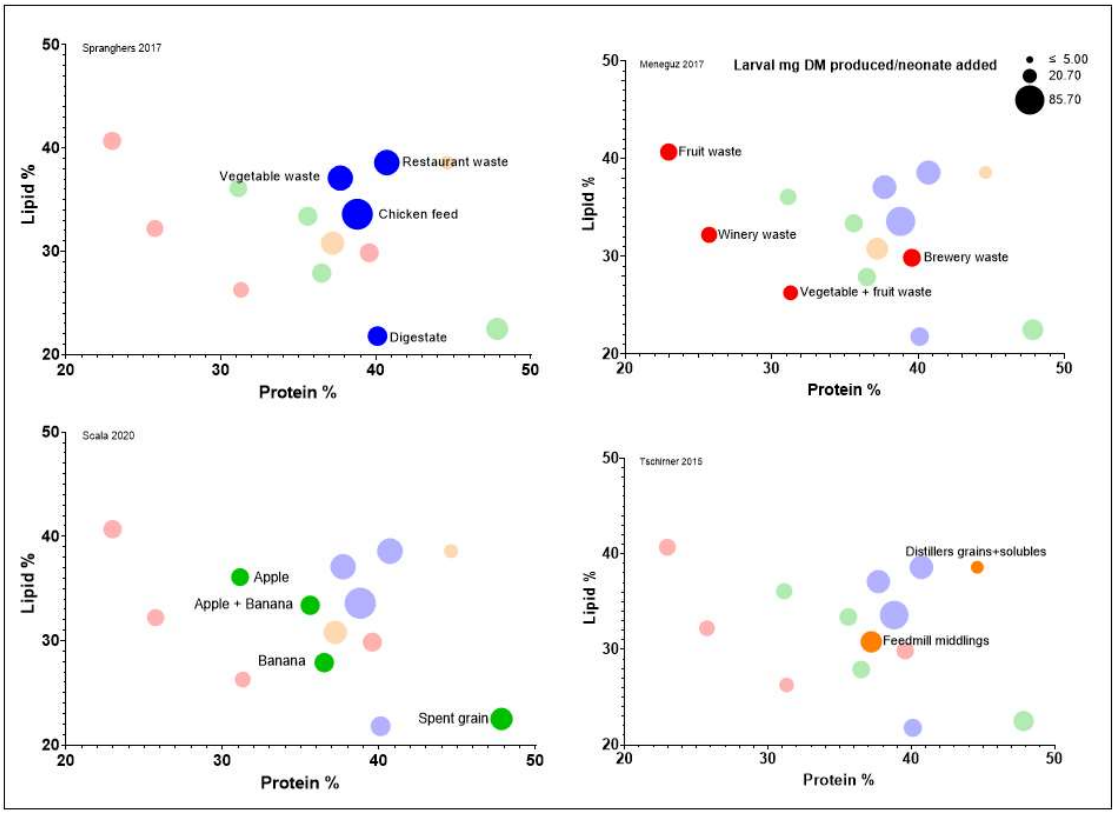

Figure 2.4 Variations in BSFL protein and lipid compositions as produced using varied substrates across several studies. Relative sizes of markers correspond to the larval dry mass produced normalised by the number of neonates added.

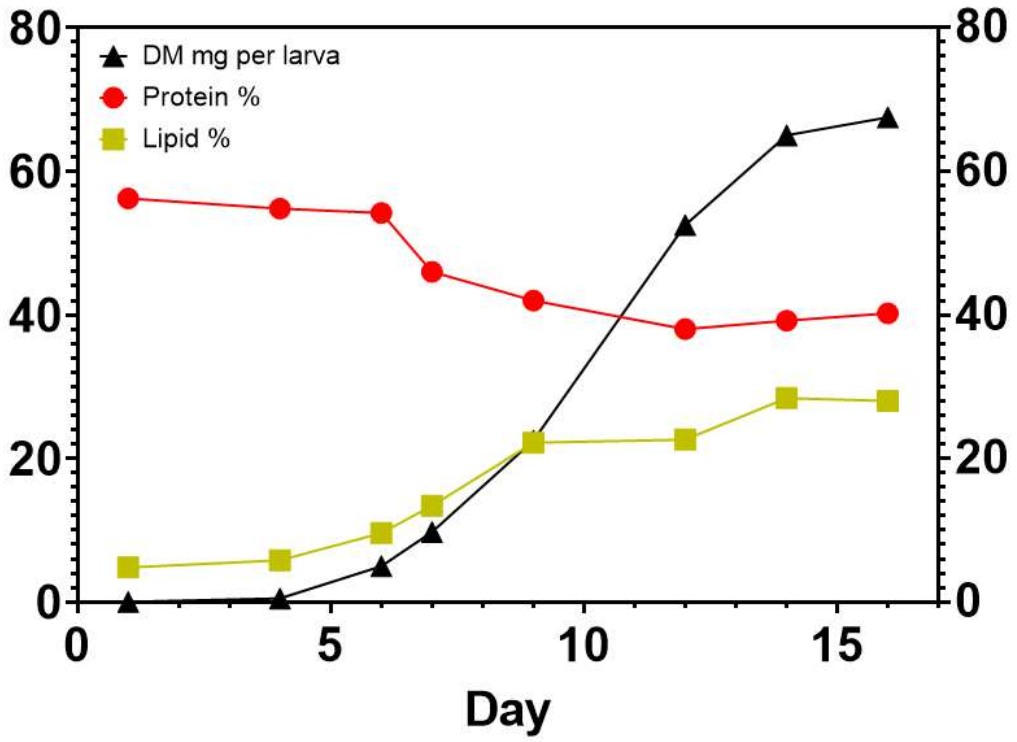

Figure 2.5 Variations in BSFL individual dry mass (mg), protein and lipid contents (%) as a function of age when supplied with excess chicken feed. Adapted from Liu et al. (2017).

2.2.1.3. BSFL Meal

The most dominantly purported use case for black soldier fly larvae is as animal feed (Tomberlin et al., 2015). Primarily, the larvae meal fraction that remains after defatting is fed either directly to the animal or as part of formulated feeds in place of alternate protein components such as fish and soybean meals (Jayanegara et al., 2017; Leiber et al., 2017; St-Hilaire et al., 2007b). Larvae are defatted by either a wet or dry fractionation process, with each process influencing the nutritional profile of the resulting BSFL meal – an area of active research (Ravi et al., 2020). Wholesale prices of larval meal vary widely, ranging from 200-2000 USD per metric tonne, dependent on the target market and product quality (Joly and Nikiema, 2019). For comparison, the soybean price between 2018 and 2023 ranged between 339 and 737 USD per metric tonne, hence the economics of substituting soybean meal with BSFL meal is highly context dependent (World Bank, 2023). The successful market

penetration of BSFL meal into animal feeds is noted in literature as crucial to the future of BSFL production as an industry (Surendra et al., 2020).

Regulations as to which animals may be fed BSFL products vary greatly by jurisdiction, usually depending on the presence of animal products in the larval substrate and pathogen levels (Insect Protein Association of Australia, personal communication) (Alagappan et al., 2022b). High income countries pose more restrictions, however Joly and Nikiema (2019) note the increased approval of companies BSFL based products and that regulations may become less restrictive in future. Typically, suitable animals include non-ruminants such as fish, poultry, pigs, and pets such as cats and dogs (Alagappan et al., 2022b; DiGiacomo et al., 2019). BSFL meal has also been proposed as an additive in human foods, however, there remain unknowns regarding consumer acceptance and techno-functional properties for food processing (Bessa et al., 2020; Higa et al., 2021).

Alongside the contents of protein, chitin and ash as described in section 2.2.1.2, quality factors integral to the use of BSFL meal for animal feed include the amino acids profile, anti-nutritional factors, safety and vitamin and mineral contents. For human food formulations, techno-functional properties such as water and oil holding capacity, solubility, emulsification, foaming and gel formation are also important, with studies emerging into processing techniques for manipulating them (Mishyna et al., 2021; Mshayisa et al., 2022; Villaseñor et al., 2022).

2.2.1.4. Safety

Prior to widespread use of BSFL developed on organic waste substrates, confirmation of its safety for intended products is required. This need has been increasingly recognised as evidenced by the magnitude of studies exploring safety related aspects of BSFL production (Alagappan et al., 2022a; Alagappan et al., 2022b; Belghit et al., 2021; Fels-Klerx et al., 2020; Lievens et al., 2021; Schmitt et al., 2019; Vandeweyer et al., 2021). These safety aspects can be grouped as biological (including bacterial, fungal, viral contaminants and associated toxins)

or inorganic (heavy metals, pesticides, pharmaceuticals and others) in nature and are directly influenced by the substrate. Whether produced BSFL meets safety requirements determines suitable downstream markets.

Of particular concern are viral and prion diseases with significant potential for devastating livestock industries, such as foot and mouth disease (*Aphthovirus*), swine fever (*Asfivirus*) and mad cow disease (Bovine Spongiform Encephalopathy) (Ahmed et al., 2021; Alexandersen et al., 2003; Olesen et al., 2023). These may be transferred from animal products to ruminants and pigs via feed, hence prompting regulations restricting the use of non-hygienically processed animal products (referred to as Restricted Animal Material, aka RAM, in Australia) in feed formulations across jurisdictions (Alexandersen et al., 2003; Gogin et al., 2013; Wells et al., 1991). This raises the question of whether BSFL, when cultivated on animal-based substrates, can be safely fed to livestock, or if they would be considered RAM. An assessment of regulations in Australia alongside personal communication with the Insect Protein Association of Australia resulted in recommendations presented in Figure 2.6. In Europe as of 2022, RAM is explicitly restricted from BSFL substrates altogether, regardless of intended downstream use (Mancini et al., 2022). These restrictions are likely to remain in place unless studies can demonstrate an acceptably low risk of disease transmission from animal-based substrates to livestock through BSFL – an area with limited research to date (Olesen et al., 2023; Pienaar et al., 2022). Despite these concerns, ongoing research is evaluating the potential of BSFL as a nutrient source for ruminants and pigs (Ahmed et al., 2021; Jayanegara et al., 2017; Mulianda et al., 2021; Renna et al., 2022), including trials that utilize RAM substrates (El-Dakar et al., 2021a; Lalander et al., 2019; Parodi et al., 2021).

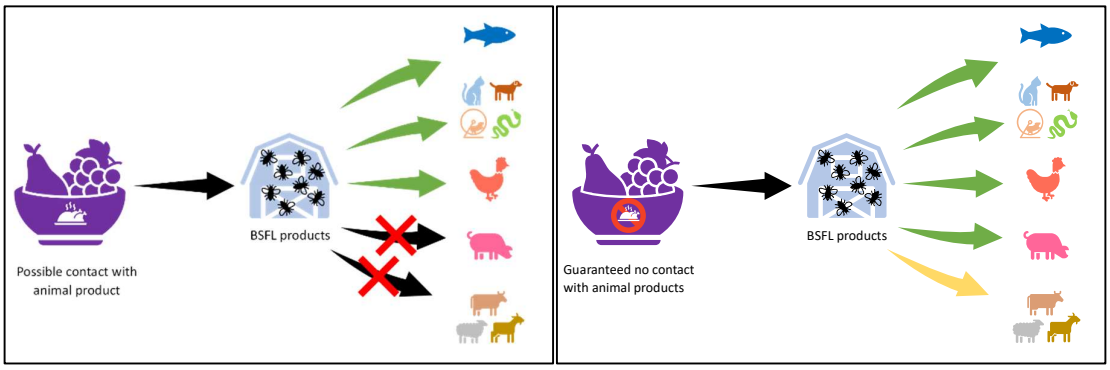

Figure 2.6 Overview of recommendations from an assessment of Australian regulations for feeding BSFL products to livestock as a function of substrate. Animals from top to bottom: fish, pets, poultry, pigs and ruminants.

Pathogenic bacteria and fungi are additional concerns. Deactivation of *Salmonella* and *Escherichia coli* by BSFL have been observed, however, the presence of pathogenic species of *Bacillus*, *Staphylococcus* among others within BSFL require decontamination prior to use in animal feeds, as is the case with other feed ingredients (Jiang et al., 2020; Lalander et al., 2015; Wynants et al., 2019). Various mycotoxins have been assessed for their accumulation potential including aflatoxins B1, B2 and G2, deoxynialenol, ochratoxin A and zearalenone, finding larvae either excrete or metabolise them, allowing maximum safe limits for these chemicals to potentially be greater than in traditional animal feeds (Camenzuli et al., 2018; Purschke et al., 2017).

Regarding inorganic safety, bioaccumulation of toxic metals has been noted as a concern. Bioaccumulation is dependent on the metal and typically not observed to the extent of exceeding legal limits in Europe (Fels-Klerx et al., 2020). Metals Cd, Cu, Hg, Mn, K and Cr have been found to notably bioaccumulate, whereas Se and Zn have not (Attiogbe et al., 2019; El-Dakar et al., 2021b; Elechi et al., 2021; Purschke et al., 2017; Schmitt et al., 2019; Wu et al., 2020). While Zn has not been found to bioaccumulate, Diener et al. (2011b) note its toxic effects regarding larval survival. Variation in bioaccumulation of the same metals across studies indicates there may be an interactive effect with other substrate components (Elechi et al., 2021; Truzzi et al., 2019). Interestingly, Wu et al. (2021) found an increased substrate

copper contents in turn increased antibiotic resistance, providing an additional concern regarding metals influence on BSFL quality. Hence, further clarification of metal bioaccumulation factors is merited prior to the use of substrates containing notable metal contents.

Numerous studies have assessed the safety of black soldier fly larvae meal with regards to pesticides and insecticides, such as azoxystrobin, propiconazole, chlorpyrifos, and pirimiphos. While these chemicals have been shown to have negative effects on survival, no bioaccumulation has been observed (Lalander et al., 2016; Meijer et al., 2021; Purschke et al., 2017). Similarly, pharmaceuticals (carbamazepine, roxithromycin, trimethoprim) and antibiotics (tetracyclines, sulfonamide) have not shown bioaccumulation and gut microbiota have been found to degrade tetracyclines (Cai et al., 2018; Gao et al., 2019a; Lalander et al., 2016; Liu et al., 2021b). Some preliminary investigations have been performed into microplastics (polypropylene, polyethylene, polystyrene), discovering no impacts on larval growth or survival, yet reductions in pupation occurred (Cho et al., 2020; Romano and Fischer, 2021). However, microplastic accumulation in BSFL was not explored in these studies. Some bioaccumulation of dioxins, polychlorinated biphenyls and polyaromatic hydrocarbons occurs, yet levels have not been found to exceed limits outlined in Europe and by the World Health Organisation (Charlton et al., 2015; Fels-Klerx et al., 2018).

2.2.1.5. Amino Acid Profiles

BSFL is typically deemed a source of high-quality protein due to its similar amino acid profiles to traditional protein sources such as soybean and fishmeal (Belghit et al., 2019), as often quantified through high-performance liquid chromatography (HPLC). In particular, BSFL contains high levels of essential amino acids for various animals (Makkar et al., 2014). Existing studies show the amino acid profile varies minimally in relation to the substrate fed, typically only by 1 – 2% (Table 2.1) (Lalander et al., 2019; Spranghers et al., 2017). Variations

still appear across studies, likely as a function of differences in quantification methods, larval development stage or genetics. A representative profile of amino acids found in BSFL compared with those of soybean meal (SBM) and fishmeal (FM) is presented in Table 2.1, showing the relative similarities between the profiles.

Table 2.1 Amino acid profiles of BSFL protein as produced from varied substrates across several studies. Representative profiles of Fishmeal and Soybean meal are included showing the similarities in profiles. Essential amino acids for fish nutrition – a commonly proposed market for BSFL - are in italics (Li et al., 2009).

Amino acid	**Liland et al. (2017)**		**Lalander et al. (2019)**		**Spranghers et al. (2017)**		**Fishmeal (FM) (Makkar et al., 2014)**	**BSFL mean - FM**	**Soybean meal (SBM) (Makkar et al., 2014)**	**BSFL mean - SBM**
(g/100g protein)	Substrate: Wheat	Substrate: Brown algae	Substrate: Poultry feed	Substrate: Food waste	Substrate: Chicken feed	Substrate: Restaurant waste	-	-	-	-
Alanine	6.2	6.4	5.9	5.9	6.5	6.8	6.3	0.0	4.5	1.7
Arginine	4.6	6.5	4.8	4.9	5.2	4.9	6.2	-1.0	7.6	-2.5
Aspartic acid	9.4	8.3	8.4	9.1	9.7	9.1	9.1	-0.1	14.1	-5.1
Cysteine	-	-	0.6	0.5	0.6	0.5	0.8	-0.2	1.4	-0.8
Glutamic acid	10.3	11.9	9.5	9.8	10.8	11.3	12.6	-2.0	19.9	-9.3
Glycine	4.6	4.3	5.1	5.3	5.8	6.2	6.4	-1.2	4.5	0.7
Histidine	2.8	2.7	2.9	2.9	3.5	3.4	2.4	0.6	3.1	0.0
Isoleucine	3.9	3.8	4.5	4.1	4.4	4.7	4.2	0.0	4.2	0.1
Leucine	6.4	6.2	7.3	6.8	7.4	7.5	7.2	-0.3	7.6	-0.6
Lysine	6.2	5.6	6.0	8.3	6.0	5.7	7.5	-1.2	6.2	0.1
Methionine	1.7	1.4	1.7	1.8	2.0	1.7	2.7	-1.0	1.3	0.4
Phenylalanine	4	3.2	3.9	4.0	4.4	4.0	3.9	0.0	5.2	-1.2
Proline	5.3	5.2	4.9	5.1	5.8	6.2	4.2	1.2	6.0	-0.6
Serine	4	4.3	4.0	4.1	4.3	3.9	3.9	0.2	5.2	-1.1
Threonine	3.9	3.9	3.7	3.9	4.2	4.0	4.1	-0.2	3.8	0.1
Tryptophan	-	-	1.5	1.4	1.7	1.3	1.0	0.5	1.4	0.1
Tyrosine	5.7	4.2	5.8	6.0	-	-	3.1	2.3	3.4	2.1
Valine	5.8	5.5	6.1	5.8	6.2	6.9	4.9	1.2	4.5	1.6

2.2.1.6. Vitamin and Mineral Contents

BSFL meal contains notable levels of vitamins and minerals essential for health of many domesticated animals (Table 2.2) (Bonrath and Netscher, 2005; Spranghers et al., 2017). Larval vitamin contents are less dependent on substrate, while mineral contents are linearly proportional to the substrate mineral content and typically high in calcium (Liland et al., 2017; Liu et al., 2017; Shumo et al., 2019; Spranghers et al., 2017). Studies that explore vitamin contents are scarce (Ebeneezar et al., 2021; Liland et al., 2017; Shumo et al., 2019), typically focusing on fat soluble vitamin E contents, which have been reported as 6.7 ± 0.64 mg/100g DM in Liu et al. (2017) and 53.3 ± 3.9 mg/kg DM in Spranghers et al. (2017). Provitamin A carotenoids have been shown to accumulate from the substrate into the larval mass (Borel et al., 2021). B-complex vitamins are present in larvae, though their substrate dependence is unknown (Finke, 2013). Minor levels of D vitamins have been reported (Ebeneezar et al., 2021). The academic community has paid relatively little attention to the vitamin and mineral contents of larval meal, which may be due to their lower concentration compared to protein and lipid contents, and therefore their perceived lower impact on the nutritional quality of the BSFL meal.

Table 2.2 Mineral contents of BSFL reported in literature. Liu et al. (2017) reported values as mg/100g, Spranghers et al. (2017) reported values as g/kg.

Mineral	**Larval content (Liu et al., 2017)** Substrate: Chicken feed	**Larval content (Liland et al., 2017)** Substrate: Processed wheat	**Larval content (Spranghers et al., 2017)** Substrate: Chicken feed
Macro-minerals (g/kg DM)			
Ca	29.0 ± 0.14	8.4 ± 0.4	28.70
Fe	2 ± 0.01	0.21 ± 0.02	0.35
K	-	10.2 ± 0.2	6.16
Mg	-	2.1 ± 0.1	2.65
Mn	-	0.19 ± 0.01	0.22
Na	1.0 ± 0.1	1.0 ± 0.1	0.67
P	3.5 ± 0.1	6.8 ± 0.1	4.99
S	-	-	0.20
Micro-minerals (mg/kg DM)			
Cu	-	7.9 ± 0.2	10
I	-	<Limit of quantification	-
Se	-	0.1 ± 0.0	-
Zn	614.0 ± 17.1	68.0 ± 1.6	160

2.2.1.7. BSFL Lipids

Once mechanically or solvent extracted from the larvae, BSFL lipids have shown promise as an additive in animal feed and human food formulations as well as feedstock for biodiesel (Delicato et al., 2020; Kim et al., 2020; Li et al., 2011b). The lipids are typically high in lauric acid as found in coconut and palm kernel oils; hence researchers have posited a range of use cases wherein BSFL lipids could substitute these materials, including pharmaceuticals, cosmetics, soaps and detergents, and chemical additives (Almeida et al., 2022; Chou et al., 2020; Franco et al., 2022). However, studies on these uses are in their infancy, with studies focusing on characterisation and extraction of the lipids while providing a theoretical discussion of potential uses (Almeida et al., 2022; Matthaus et al., 2019; Rabani et al., 2019). Contrarily, there are ample studies proving the potential of BSFL lipids as feedstock for biodiesel production, an avenue seen as feasible even for larvae developed on low quality substrates such as manures or contaminated post-consumer food wastes (Li et al., 2011a; Li et al., 2011b; Nguyen et al., 2018; Surendra et al., 2016; Zheng et al., 2012a; Zheng et al., 2012b). Despite this, none of the large scale facilities in the Joly and Nikiema (2019) review were post-processing the lipids into biodiesel. Instead, most existing commercial listings market the lipids solely towards animal feed formulations, likely for economic reasons (Circa Biotech, 2023; Enviroflight, 2023; Future Green Solutions, 2023).

The value of BSFL as a source of bioproducts was first identified in terms of their high protein contents, with the lipids as secondary (Bondari and Sheppard, 1981). Hence, less attention has been given to the market potential of the lipids, and wholesale market prices have yet to be publicly established. As an approximate indicator, AgriProtein's 'MagOil' was sold at 1,015 USD per metric tonne as of 2017 as reported in Meat & Livestock Australia and All Energy PL (2017), though it is unknown at what quantity this price was interpreted from. For

comparison, between 2018 and 2023 the coconut and palm kernel oil prices ranged between 635 – 2230 and 542 – 2440 USD per metric tonne, respectively (World Bank, 2023).

2.2.1.8. Fatty Acid Profiles

Studies on BSFL lipids are largely reported in the literature as fatty acid (FA) profiles determined from gas chromatography of transesterified fatty acids (Caligiani et al., 2018; Rabani et al., 2019), from which the lipid quality is judged based on the intended end use. BSFL lipids are dominated by saturated triglycerides (Alagappan et al., 2022a; Liu et al., 2017; Spranghers et al., 2017). There is evidence that the fatty acid profile of BSFL lipids can be altered significantly more than the amino acid profile by varying the substrate fed to the larvae, providing opportunity to 'design' the lipid profile for selected downstream products (Ewald et al., 2020b; Liland et al., 2017; Starcevic et al., 2019). For example, a high content of omega 3 poly-unsaturated fatty acids is often desirable in animal feed formulations, particularly aquafeeds, hence the use of substrates that increase this content is desirable (Barroso et al., 2017; St-Hilaire et al., 2007a). Contrarily, high unsaturated fatty acid contents are undesirable in lipids marketed for biodiesel applications due to their detrimental effects on thermal efficiency and susceptibility to oxidation (Meira et al., 2011; Redel-Macías et al., 2012). Lauric acid appears to dominate regardless of substrate, limiting the potential for 'designed' FA profiles (Ewald et al., 2020a). The dependency of lipid profile on substrate lipid contents is demonstrated most clearly in Spranghers et al. (2017), from which the major fatty acids are compared between BSF prepupae fed different substrates and the substrates themselves in Table 2.3.

Table 2.3 Comparison of fatty acid groups in different substrates and prepupae fed those same substrates summarised from Spranghers et al. (2017) (g/kg fatty acid methyl esters). SFA = Saturated Fatty Acids; MUFA = Mono-Unsaturated Fatty Acids; PUFA = Poly-Unsaturated Fatty Acids; n-6 = omega 6 unsaturated; n-3 = omega 3 unsaturated.

Fatty acid	**Chicken feed**		**Digestate**		**Vegetable waste**		**Restaurant waste**	
(g/kg FAME)	Substrate	Prepupae	Substrate	Prepupae	Substrate	Prepupae	Substrate	Prepupae
C10:0	1.4	14.3	8.5	11.7	2.3	16.3	13.3	20.3
C12:0	14.5	573.5	97.5	436.5	21.3	608.9	154.9	575.6
C14:0	3.3	73.4	43.1	68.7	12.8	94.8	59	71.4
C16:0	160	96.5	236.3	101.2	305.2	87	231.2	102.9
C18:0	25.1	13.6	38.5	17.5	31.8	11.1	67.5	9.8
SFA	215	774	483	648	407	828	541	782
MUFA	255	100	190	191	120	95	290	120
n-6 PUFA	501	116	176	80	320	46	142	80
n-3 PUFA	29	9	71	16	150	23	22	14

Although the fatty acid composition of different substrates influences the fatty acid profiles of the larvae, Table 2.3 indicates that other unidentified properties of the substrates (for example other nutrients) also contribute to the observed variations.(Gold et al., 2020b; Stanley-Samuelson et al., 1988; Starcevic et al., 2019). For facilities intent on producing a consistent quality BSFL lipids within narrow tolerances, the variety of compatible substrates are therefore likely to be restricted while this knowledge gap remains.

2.2.1.9. Techno-Functional Properties

Additional measures of BSFL lipid quality are occasionally investigated, particularly in reference to their influence on biodiesel performance and product formulation (Daylan Amelia Tzompa and Vincenzo, 2017; Li et al., 2011b). These include melting and crystallization behaviour, viscosity, colour, odour and lipid class profiles such as contents of total free fatty acids vs tri- and di-acylglycerides, lipid polarity and sterols (Bogevik et al., 2022; Daylan Amelia Tzompa and Vincenzo, 2017; Matthaus et al., 2019). These characteristics are most significantly determined by the extraction and purification methods employed yet several are also influenced by the substrate used, particularly as a consequence of FA profiles (Bogevik et al., 2022; Daylan Amelia Tzompa and Vincenzo, 2017; Mai et al.,

2019). Several studies have explored the larvae's sterol contents, finding them typically comparable with common vegetable oils yet dependent on the substrate (Boukid et al., 2021; Matthaus et al., 2019; Ushakova et al., 2016). Investigations into further techno-functional properties of the lipids such as emulsification and foaming are in their early stages (Delicato et al., 2020; Mshayisa et al., 2022).

2.2.1.10. Substrate-Residue

Substrate-residue remaining once larvae have been removed consists of a mixture of unconsumed substrate, larval exoskeletons and larval faeces (Yildirim-Aksoy et al., 2020). The mixture is often termed as frass; however, this specifically refers to the larval faeces which is only one component of the residue, hence the term substrate-residue used here. Several use cases have been proposed or this material, most commonly as a plant fertiliser. There are also mentions in literature of its use in more specialised high value products including as a source of antimicrobial compounds and beneficial microbes in animal feed formulations (Arabzadeh et al., 2022; Chavez, 2021; Yildirim-Aksoy et al., 2020). Its quality is typically measured in terms of plant nutrient contents, namely nitrogen, phosphorous and potassium, moisture contents, microbiological stability and composition, and presence of phytotoxins, hormones and other bioactive compounds (Lopes et al., 2022). Nutrient contents of substrate-residue are significantly dependent on the substrate composition (Lopes et al., 2022; Palma et al., 2020).

A notable hurdle for the sale of substrate-residue as fertiliser is its unclear regulatory status regarding microbial stability and determining the need for additional processing such as pasteurisation (Palma et al., 2020) (Insect Protein Association of Australia, personal communication). From consultation with the NSW Environmental Protection Agency (Australia), it was recommended that treatment according to Australian Standard AS 4454 (2012): "Composts, soil conditioners and mulches" be adhered to for the material to be allowed for use without restriction in Australia, particularly in regard to the pasteurisation. This may

reduce the materials marketability, considering much of the purported value of the substrate-residue as fertiliser stems from its contained beneficial microorganisms (Lopes et al., 2022).

2.2.2. Production performance measures

Researchers have used various measures for BSFL production performance trials, typically including survival, larval growth and conversion efficiency. As with differences in production settings (see 2.2.1.2 Proximate Composition), slight variations in the formulas used to calculate these measures, including lack of clarity as to whether the calculations were performed on a wet or dry matter basis, hinder comparison across and reproducibility of studies (Bosch et al., 2020; Hopkins et al., 2021). This section reviews the core production performance measures from literature, outlines how they are measured and provides recommendations as to which are appropriate.

2.2.2.1. Growth Rates and Larval Gain

Larvae follow an 'S' shaped growth curve (Figure 2.2) (Miranda et al., 2020a; Scala et al., 2020), as may be described with a logistic function (Laganaro et al., 2021), typically commencing with a lag phase of slow growth for several days following hatching, prior to a period of rapid mass gain. This gain continues as the larvae develop towards prepupae, at which point there is a stationary phase and mass begins decreasing as larvae pupate. Measuring the growth curve for a population of larvae on a particular substrate is particularly useful for identifying the transition from growth to stationary phases (maximum larval mass) for timing larval harvests in substrate comparison trials. Two methods are used for determining the larval growth curve, either by regularly sampling and weighing larvae from growth containers during development prior to returning them to the container, or by harvesting separate replicate containers at regular intervals (Bosch et al., 2020; Miranda et al., 2020a). The former method is not recommended as handling the larvae may adversely affect performance (Tinder et al., 2017), particularly in cases where larval populations are small. However, the latter method

requires significantly greater numbers of culture containers and therefore increased supplies of neonates, substrate materials and space which can be infeasible.

Larval gain refers to the larval biomass produced during the trial and is often the primary objective for which producers aim to maximise. Larval gain is measured as the difference in mass between the harvested larvae at the end of the trial and the mass of input neonates. Exact harvest points vary widely across studies, with some choosing to harvest at the emergence of a set percentage of prepupae (Cammack and Tomberlin, 2017; Meneguz et al., 2018b; Oonincx et al., 2015; Spranghers et al., 2017), at the emergence of a set percentage of 5^{th} and 6^{th} instar larvae (Tschirner and Simon, 2015), at the point when daily recorded masses begin decreasing (Scala et al., 2020), or simply at a set number of days after commencing the experiment (Danieli et al., 2019; Gold et al., 2020b). Some notable procedural influences on measured larval gain include proximity of the selected harvest time to the true maximum larval mass, whether the larvae were purged, the purging method (e.g. starvation, blanching or through the use of an absorbent), and the extent of drying prior to weighing. The latter two typically only have minor influences on larval gain (personal observation). Larval gain may also be determined on a wet or dry matter basis and in terms of a particular nutrient (Gold et al., 2020b), such as protein, lipid, chitin, vitamins or minerals.

2.2.2.2. Substrate Loss and Reduction

Substrate loss is the difference in mass of input substrate and remaining residue which can be calculated on a wet or dry matter basis. Substrate reduction (aka overall degradation, waste reduction, reduction rate) is calculated as the ratio of substrate loss to substrate input (Bosch et al., 2020; Gold et al., 2020b). To measure substrate loss, a homogenous sample of residue is required for determining residue moisture contents, which can prove challenging in the event that stratification of residue often occurs (personal observation). This may contribute to overestimated residue water contents and in turn overestimating substrate loss and reduction.

Similarly, upon mixing culture container contents, the presence of larvae within the residue can prove challenging to remove, particularly with under-developed larvae (personal observation). While substrate reduction can be useful, it should not replace the reporting of substrate loss as in some studies the total substrate provided is not clear (Meneguz et al., 2018b; Oonincx et al., 2015), particularly those in which the substrate is supplied *ad libitum* to the larvae, hence substrate reduction will be inherently lower than studies using a restricted feeding regime.

2.2.2.3. Substrate Conversion

While absolute measures of larval gain and substrate loss are critical in comparing performance within a study, they are incomparable to other studies using different process parameters without some form of normalisation. Most commonly, this normalisation is to the substrate added as in Bioconversion rate in Gold et al. (2020b) and Bioconversion efficiency in Bosch et al. (2020). However, as with substrate reduction, this becomes incomparable to studies using a varied feeding regime. Hence, alternatives have been adopted from traditional animal agriculture to account for the feed consumed, such as feed conversion ratios (FCR), efficiency of conversion of ingested food (ECI) as is used in other areas of entomology, and efficiency of conversion of digested food (ECD). These correct for either the undigested substrate of the residue, the faecal content of the residue, or both. Unfortunately, distinguishing between undigested substrate, faecal content and exoskeleton remnants from the substrate is not readily performed, requiring either the use of 'nappies' as in Gold et al. (2020a) which are impractical and labour intensive, or the use of indigestible markers which are in the preliminary stage of research in BSFL (Bosch et al., 2020). Hence, reported measures of FCR, ECI and ECD are not truly equivalent to those used in traditional animal agriculture.

A useful compromise is the measure termed the 'bioconversion efficiency corrected for residue' (BER) in Bosch et al. (2020), or the inverse of Gold 2020's 'waste conversion efficiency'. This is calculated as the ratio of substrate loss to the larval gain and is hereon

simply referred to as ‘substrate conversion’. This becomes an especially useful performance measure when using high-cost substrates, as it provides an indicator to the extent of substrate transformed into larval biomass rather than lost in gaseous emissions. Substrate conversion can be modified to calculate the conversion efficiency of select nutrients, most commonly nitrogen or protein, but also lipid, vitamins and minerals conversion efficiencies.

2.2.2.4. Other Performance Measures

Several other performance measures are less commonly reported in the literature, most common of these being larval survival. Survival or mortality may seem critical to report, however, it may be argued that survival is not particularly valuable to a BSFL producer when larval gain is known. Determining larval survival accurately can additionally be challenging when operating at a large scale, with some large-scale investigations omitting the performance measure (Scala et al., 2020). Nevertheless, larval survival can provide an indicator of whether substrate components were actively toxic to larvae as opposed to hindered development, which may be overcome with surplus nutrients. Similarly, low survival yet high larval gain may provide a crude indicator of cannibalism among larvae. Alternate measures of larval growth such as larval length and instar are occasionally used (Harnden and Tomberlin, 2016; Wong et al., 2019). While useful in some circumstances, these are less informative compared with larval mass and whether the larvae have entered the prepupal stage or not, particularly since classifying instars less developed than the 5^{th} instar can prove challenging. Lastly, measures of material circularity, energy efficiency and life-cycle assessments may be used to gauge performance (Guo et al., 2021; Salomone et al., 2017).

2.2.2.5. Potential Cross-Study Metrics

Comparing performance across studies can prove challenging given the variety of performance measures reported (Hopkins et al., 2021). Standardisation will assist this (Bosch et al., 2020), yet quick identification of optimal substrates and process settings for maximum

returns will remain challenging. Simple, comparable metrics for production of larval products using as few inputs as possible would facilitate rapid assessment of performance across studies. Two proposed candidates for this are the average daily mass of larval protein produced per input larvae, and per input substrate mass. Together, these metrics provide a comparison of the primary product output with the primary material expenses (Joly and Nikiema, 2019). These can be further supplemented with equivalent measures of lipid and other larval biomass, in turn facilitating quick visual identification of the most productive substrates and process parameters outlined in the literature. Visualisation of these measures in terms of input larvae from a variety of sources are presented in Figure 2.7.

There are some issues with relying solely on these metrics for comparing larval production. Most studies do not use an optimised feeding regime, instead supplying substrate in excess of larval requirements, often *ad libitum* (Oonincx et al., 2015; Scala et al., 2020). This makes comparisons of production per unit substrate mass impossible across studies without standardisation of feeding regimes, such may be done on a 'per larval mass' basis proportional to growth rates, as in aquaculture (Robaina et al., 2019). Similarly, simplifying non-linear growth curves to a linear function with a singular average growth rate restricts the usefulness of these metrics (Laganaro et al., 2021), particularly due to their sensitivity to variations in harvest times across studies (Banks et al., 2014). Hence, including growth curves and standardising harvests to the point of maximal larval production can be beneficial.

Likewise, when compared across studies, these metrics remain limited by variations in quality of analysis methods employed for determining protein and lipids, particularly differences in the conversion factor (K_P) used to convert total nitrogen to protein content (Hopkins et al., 2021). A simple fix for this is to report average daily total nitrogen rather than protein, however this has not been implemented for Figure 2.7 due to the absence of reported larval nitrogen contents or conversion factors in multiple studies (Danieli et al., 2019; Scala et

al., 2020). Standardisation of the protein conversion factor will allow more comparable reporting of protein content, with the value of 4.76 emerging as a potential candidate (Janssen et al., 2017). However, a recent article has proposed a lower K_P factor of 4.43 (Smets et al., 2021), indicating that research in this area is ongoing. Finally, these measures do not indicate the quantity of residue, however, this seems a small omission given the value of larval biomass is substantially greater than residue on a mass basis (see 2.2.1 Products and Relevant Quality Factors).

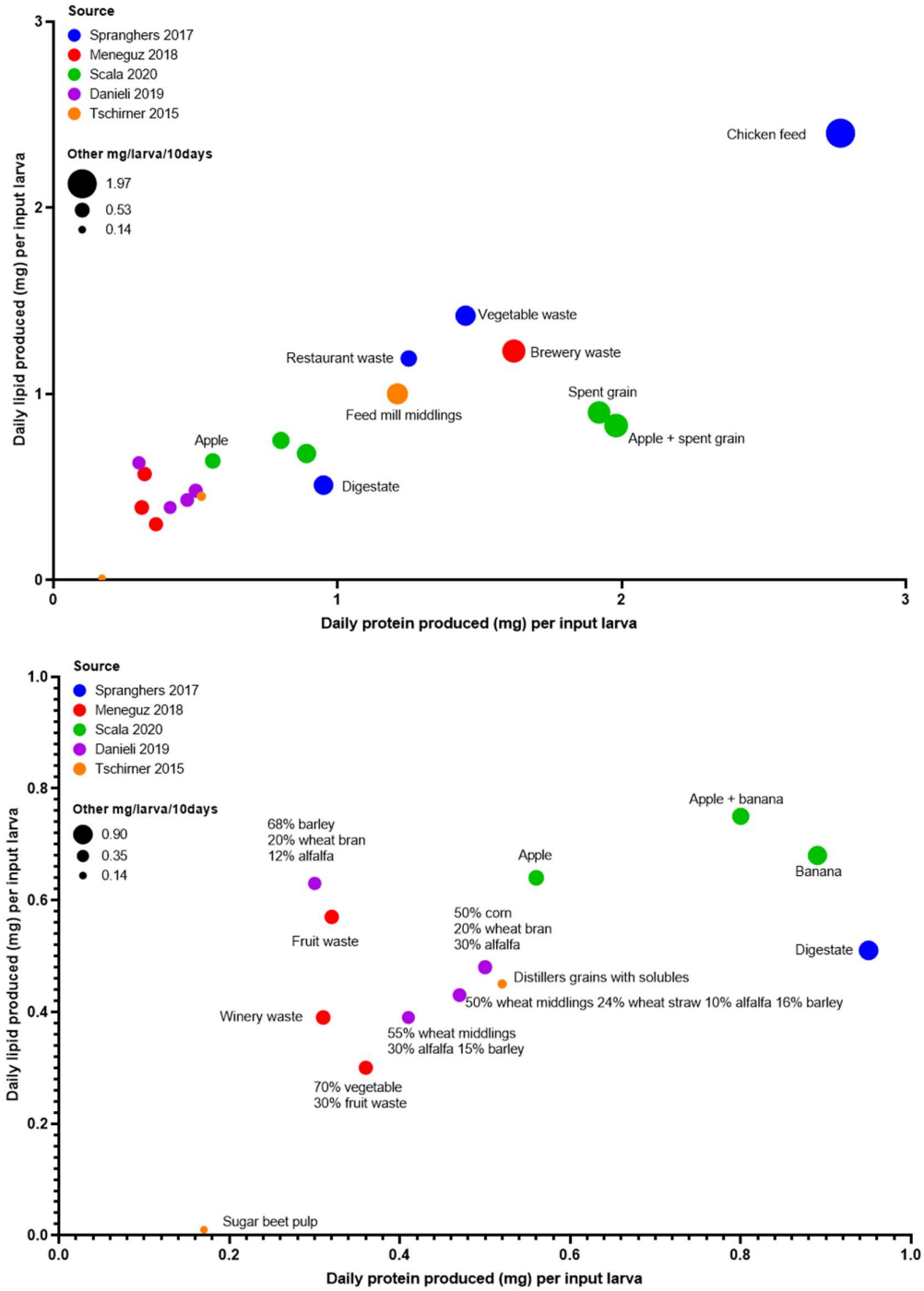

Figure 2.7 Above: Average protein and lipid production per larva added across a range of substrates and studies. Symbol size is proportional to the production of other larval components per larva added. Below: Zoomed in view of less-productive substrates.

2.3. Core Mechanisms of BSFL Production

As an emerging technology, our understanding of the mechanisms involved in BSFL production is incomplete. However, progress has been made in uncovering these mechanisms at the genetic, metabolic, morphological, and engineering scales, helping to establish BSFL production as a promising tool of the circular bioeconomy. This section examines the core mechanisms explored in literature that are relevant to BSFL production at the engineering level, as required to understand and reduce variability in product quality and process performance.

At commercial scales, BSFL production occurs in batch or fed batch, often in trays, facilitating harvest of appropriately developed larvae. In a typical batch arrangement, substrate and young larvae are introduced to the trays then allowed to develop for a set number of days before separation of the matured larvae from the residue and downstream processing (Figure 2.3). Influential process settings such as feeding regimes, environmental conditions and larval numbers are thoroughly discussed in Bosch et al. (2020) and Joly and Nikiema (2019).

Gold et al. (2018) usefully describes the production system as containing two distinct bioreactor regions: the ‘biowaste reactor’ region referring to the area external to the larvae where bacteria and fungi convert substrate, and the ‘fly larva reactor’ regions internal to the larvae where substrate is converted and absorbed along their gut. Process engineers may find value in distinguishing the ‘biowaste reactor’ region as akin to a stirred tank and the ‘fly larva reactors’ as miniature plug flow reactors. Few resource bioconversion studies refer to or acknowledge this distinction of process regions, designing studies with regards to the larvae reactor region (Cammack and Tomberlin, 2017; Danieli et al., 2019). This oversimplification is limiting when attempting to understand the connection between substrate and BSFL product features. Material flows between the two bioreactor regions, with the larvae consuming outputs from the external microbes as well as the microbes themselves and larval excrement, and larval excrement being further converted by external microbes (Gold et al., 2018). The complex

dynamics of these material flows and their influence on BSFL product features remains underexplored. The combination of microbial and larval metabolism result in ammonia release, moisture reduction and temperature and pH increases of the substrate (Cickova et al., 2015).

2.3.1. Larvae Reactors

Starting with the 'larvae reactors' themselves, substrates are partly liquefied via excreted enzymes prior to ingestion (Lalander et al., 2019). Morphological features of the head and mouthparts facilitate rapid larval movement to access and ingest substrate (Bruno et al., 2020). Nutrient sensing happens prior to ingestion, with feeding rates increased by sugars in the substrate and regulated by hormones (Gold et al., 2018; Shen and Cai, 2001). Substrate and microbes enter past the mouthparts along the digestive tract to the midgut, where complex macronutrients are hydrolysed to simpler molecules by digestive enzymes. Microbiota and quantities vary across the midgut in relation to pH and oxygen availability, contributing digestive enzymes (Bruno et al., 2019; Gold et al., 2018). The anterior midgut (pH 6.0) is responsible for most enzymatic hydrolysis of polysaccharides and lipids, while the mid-midgut (pH 2.0) performs most pathogen lysis (Bonelli et al., 2019). The posterior midgut (pH 8.5) handles most hydrolysis of proteins and peptides, further digestion of lipids, and nutrient absorption (Bonelli et al., 2019). Small molecules are absorbed across the peritrophic matrix and intestinal epithelium into the haemolymph (akin to blood in vertebrates) via a combination of diffusion and active transport (Rockstein, 1978; Thompson, 2003). Nutrient absorption is regulated by the concentrations and activities of enzymes in the gut, as well as availability and quality of the food (Gold et al., 2018).

Bonelli et al. (2020) demonstrate that it is in the midgut where we learn how larvae withstand such varied substrates: by adapting the midgut cell morphology and digestive enzyme activities to maximise nutrient recovery from poor substrates, a form of post-ingestion adjustment mechanism. The expression of genes responsible for digestive enzymes additionally

enables adaptation to varied substrates through the adjustment of enzyme activities (Bonelli et al., 2020). The larval fat body also plays a central role in nutrient accumulation influencing larval proximate composition by controlling metabolic processes responsible for larval growth and development in response to nutrient availability (Gold et al., 2018; Pimentel et al., 2017). A model of the metabolic processes involved in larval fat accumulation has been developed based on transcriptome sequencing to describe the variability of fatty acid profiles between larval stages (Zhu et al., 2019b). The author's model identifies key up-regulated and down-regulated genes involved in metabolic pathways such as pyruvate formation, acetyl-CoA biosynthesis, and triacylglycerol biosynthesis, providing a foundation for future research to optimise larval fat accumulation for downstream products.

2.3.2. External Microorganism Reactor

In an appropriately sized cultivation chamber, the 'biowaste reactor' region external to the larvae is typically well mixed by larval movement, enabling aerobic microbial digestion of substrate (Gold et al., 2018). Activity in this region plays a significant role in the substrate conversion and consequent larval growth, as evidenced by sterile substrates causing reductions of all performance measures by 31-46% in Gold et al. (2020d). Microorganisms in this region are diverse and vary in compositions and quantities across substrates, with some contributed from the digestive tract of the larvae themselves (De Smet et al., 2018a). Metabolic outputs from one microbe become inputs for another, modifying the nutrient availability of the substrate. In an ideal system, beneficial microbes would transform recalcitrant substances (e.g. lignin and cellulose) into available nutrients for accumulation into larval biomass (Isibika et al., 2019). Alternatively, beneficial microbes may synthesise amino acids out of nitrogen-containing substrate components (Amorim Franco and Blanchard, 2017), contributing to the larvae's development of protein tissues. Contrarily, detrimental microbes may compete with larvae for available nutrients. Developing methods to encourage proliferation of beneficial

microbes within the 'biowaste reactor' region provides holds potential for substantially improving BSFL production efficiency.

2.4. Future of BSFL Production Research

Recent years have seen numerous quality reviews around BSFL production that propose evidence-based future research directions (Gasco et al., 2019; Huis, 2020; Liu et al., 2019; Raksasat et al., 2020; Ravi et al., 2020; Singh and Kumari, 2019; Sogari et al., 2019). This section discusses these proposed directions and introduces additional themes discovered throughout this review for maturing the technology into a viable tool for the circular bioeconomy.

2.4.1. Addressing Product and Process Variability

A dominant theme in the literature is the need for widespread adoption of standardised procedures for BSFL production and measures of product quality and performance. This will enable more reproducible research and greatly facilitate addressing core challenges to large-scale adoption of BSFL production, most notably its variable product quality and process performance as functions of the substrate (Galassi et al., 2021; Gold et al., 2020b; Ravi et al., 2020). Caruso et al. (2013) suggested for producers to overcome this variability by securing a continuous supply of high-quality growth substrates. However, Joly and Nikiema (2019) noted this to be one of the largest challenges for producers at all scales, as evidenced by their exploration of several commercial BSFL production case studies. This can be explained by the often transient availability of 'high quality' substrates, in part due to seasonality of supply and competition from other waste valorisation processes, such as composting and anaerobic co-digestion (Dahiya et al., 2018; Hagos et al., 2017; Malinauskaite et al., 2017; Ravi et al., 2020). Furthermore, even seemingly equivalent substrates such as brewers' grains from two different generators can vary with regards to influential properties, such as nutritional content or texture. Existing producers are forced to manage this variation however is economically feasible.

BSFL producers tend to overcome the substrate-induced variability challenge using one of two strategies (Gold, 2021):

- By relying on substrate materials otherwise used in animal feeds, prioritising a reliable high-quality product over waste valorisation; or,
- Growing larvae predominantly for waste management, selling them in lowly regulated markets as a bulk, variable quality animal feed.

Inherent to the circular bioeconomy appeal of BSFL production lies in the win-win scenario of producing high quality products using end-of-pipe organic wastes, such as food wastes. While there are benefits associated with both strategies, neither meet this ideal. It may be argued that producing high-quality BSFL protein from traditional animal feeds is more sustainable with regards to resource consumption than alternative animal proteins, yet this is not sufficient for the process to align with the circular economy (EMF, 2013). Per a circular economy, BSFL production would ideally use unavoidable organic wastes which lack existing high value uses, i.e. BSFL production should avoid competing for resources used for animal feed. Strategies bridging the gap between waste management and quality production using inherently variable substrates are therefore required to meet circular economy standards. Several researchers have attempted this by blending multiple wastes and/or through pre-treatments to create a more consistent substrate formulation (Chia et al., 2018b; Danieli et al., 2019; Lim et al., 2019; Rehman et al., 2017), however, there remain unknowns as to which substrate properties to control for optimal production (Gold et al., 2020b).

2.4.1.1. Substrate Formulation

Feed formulation whereby numerous nutrient sources are intermixed and processed together is a practice carried out in agriculture (Cho and Bureau, 2001; Uyeh et al., 2019). Dedicated feed mills prepare formulated feeds for numerous species including fish in aquaculture, poultry, pigs and cattle (Hardy and Brezas, 2022; Suresh, 2016)– adopting a similar approach for BSFL would be advantageous to bioconversion performance and larval

quality (Gold et al., 2018; Raksasat et al., 2020). Blending wastes in this way may yield the additional benefit of expanding the range of wastes as nutrient sources for BSFL, such as the blending of recalcitrant wastes with more palatable ones (Raksasat et al., 2020). However, it is unclear how recalcitrant wastes would be digestible without the presence of appropriate decomposition enzymes.

Nevertheless, one notable challenge with organic wastes as nutrient sources in feed formulation is that wastes (e.g. food wastes, manures and agricultural residues) are inconsistent and impure, often containing large amounts of fibre and other poorly digestible components relative to the desired nutrient (e.g. protein, carbohydrate), as compared to traditional animal feed ingredients (e.g. grains, soybean meal, fishmeal). Additionally, some wastes have very high-water contents (e.g. whey from dairy processing) and anti-nutritional factors (see section 2.3.2.6 Anti-Nutritional Factors). Blending wastes to target particular protein and carbohydrate levels, as is typically done in BSFL blending studies (see section 2.3.2.1 Protein and Carbohydrates), can therefore result in compromised levels of these other components (e.g. recalcitrant fibres and lipids) (Gold et al., 2020b). This aids in explaining variability seen in studies using blends targeting particular protein and carbohydrate levels (Cammack and Tomberlin, 2017; Danieli et al., 2019). While increasing the number of various nutrient sources could alleviate the need for such compromises, it becomes logistically infeasible to source, transport, store and appropriately pre-treat a wide variety of nutrient sources. Further research is therefore needed to determine the fewest substrate properties to control for through substrate formulation and pre-treatments that enable acceptably reliable performance and product quality.

Identifying optimal substrate properties will require further understanding of process mechanisms (Liu et al., 2019). Greater characterisation of substrate properties in bioconversion studies are required to achieve this, particularly in regard to nutrients with

known biological functions in the larvae (Gold et al., 2018). Bosch et al. (2020) builds on this need for substrate characterisation, providing guidelines for standardising quantitative bioconversion studies to enable "apples to apples" comparisons across studies, as discussed in the section 2.2.1.2 Proximate Composition. This standardisation is especially important considering the variety of BSF genotypes that exist (Kaya et al., 2021) and that interaction effects on life history traits have been found between genotypes and substrates (Sandrock et al., 2022). Additionally, understanding the roles of microbes, both external and internal, and their interactions with the larvae, have been highlighted as potentially rewarding in managing the variability challenge (Gold et al., 2018; Law and Wein, 2018; Liu et al., 2019) (see section 2.3.3 Substrate Microorganisms).

Variation in safety is another significant feature that varies across substrates (see section 2.2.1.4 Safety), with key concerns regarding unknown levels for transfer of pathogens and bio accumulative substances from substrates through to final products (Cickova et al., 2015). Lack of favourable safety confirmations are responsible for current precautionary legislation, restricting the use of BSFL products for inclusion in many animal feeds (such as pig feeds) and therefore reducing market size (Surendra et al., 2020; Wang and Shelomi, 2017). DiGiacomo et al. (2019) highlights that safety protocols must be developed in tandem with mass rearing facilities and the optimisation of substrate features. This is particularly crucial given optimisation of substrate features is likely to include the addition of select microbes, with microbial safety being a concern for operators and downstream animal feed consumers (Gold et al., 2018).

2.4.1.2. Modelling Substrate Nutrient Correlations

Raksasat et al. (2020) notes the potential value of statistical modelling in identifying the correlations between nutritional components of substrates and BSFL production performance and quality, correlations that Joly and Nikiema (2019) agrees are missing from

current literature. Sripontan et al. (2020) begins to address this through their development of a BSFL growth model based on benchtop experiments with 10 substrates, with the aim of using the model for evaluating diet quality. The authors found the Richards model (a variation of logistic model accommodating asymmetry around the point of inflection in the growth curve) to be best fitting, with correlations in the growth parameters (max body width, growth limit, growth rate and time to max growth rate) to the diets' estimated protein and carbohydrate contents. It was concluded that the balance of these nutrients was responsible for improving performance.

Unfortunately, direct quantitative correlation between growth parameters protein and carbohydrate contents is lacking. Additionally, there are discrepancies in results with several diets performing better in some growth parameters than the proposed 'high-quality' diets, conflicting with their conclusions. Interpreting what constitutes a high-quality diet is made challenging with the absence of traditional growth parameters, such as survival and larval weights. As discovered in Gold et al. (2020b) and discussed prior, protein and carbohydrate contents of waste-based substrates alone are insufficient to comprehensively describe larval performance for assessing substrate quality. Nevertheless, Sripontan et al. (2020) work provides a foundation for future studies on the analysis of diet quality through modelling. Meta-analyses of existing literature data would further comprehensively identify correlations between substrate quality and BSFL production factors, as well as identify conflicting findings between studies, highlighting gaps in our understanding.

2.4.2. Scaling Up

For BSFL products to play a significant role in the circular bioeconomy, they must be mass producible and cost competitive with existing alternatives (e.g. soybean and fishmeal). Cickova et al. (2015) emphasised this need for further research on industrial scale production

of larvae. This is still an area with limited publicly available research; however, some publications are emerging.

2.4.2.1. Non-linear Scaling from Laboratory to Industry

Miranda et al. (2020b), Scala et al. (2020) and Yang and Tomberlin (2020) present the challenge of benchtop-scale data scaling non-linearly to industrial levels, adding another dimension to the challenge of product and process variability. This likely requires commercial producers to undergo expensive in-house research and development, establishing facility-specific scaling factors from small testing scales to large scale.

Scala et al. (2020) discovered industrial scale resulted in significantly shorter larval development times (-6 to 24 days) and 2 to 50 times larger larval masses relative to several benchtop studies, though this should be interpreted with caution since factors other than scale varied between the studies (e.g. diet per larva). Yang and Tomberlin (2020) found industrial scale production resulted in a significant increase in mean larval survival (+28.2%) and biomass conversion (+2.7%) relative to lab scale, noting the importance of container size and total larval number. A possible mechanism for this may be the dramatic increase in temperature of the substrate during processing with high density and large numbers of larvae (e.g. 10,000), while benchtop studies typically with less than 1,000 larvae maintain temperatures closer to the surrounds (Yang and Tomberlin, 2020). Actively matching temperatures of lab scale experiments with those found in industrial conditions may more closely allow lab scale findings to translate to industry.

The recent work by Yakti et al. (2022) contributes to this research by examining the impact of rearing scale and larval density on larval growth, bioconversion efficiency, and nutritional profile. Survival and biomass conversion was greater in the large scale treatments compared with the smaller scales, in line with the findings of Yang and Tomberlin (2020). Higher temperatures were also observed in larger scales, impacting larval composition such as

crude protein and fat concentrations. Both scale and density influenced concentrations of various elements including S, Mg, K, P, Fe, Cu, Al, B and Co, in addition to several free amino acids, underscoring the complexities involved in scaling from lab to industry. Novel reactor designs and/or adaptive heat exchange systems may assist in managing temperatures in large scale production.

2.4.2.2. Mass Colony Management and Breeding

The need for industrialised mating and egg production are another theme in the literature (Cickova et al., 2015; Law and Wein, 2018), which Liu et al. (2019) describes as in the 'exploratory phase'. While individual females have been reported to lay upwards of 1000 eggs (Joly and Nikiema, 2019), this varies dramatically across studies. Additionally, ratios of females to males and mating success can be problematic in egg production (Liu et al., 2019). Such problems in egg production are symptoms of insufficient knowledge of the adult fly biology (Cickova et al., 2015). Colony management, such as described in Dortmans B.M.A. et al. (2017), are typically conducted in batch with steps of breeding, larval development and pupation occurring in separate cages and environmental conditions. Integration and upscaling of such activities in this format would be challenging. Law and Wein (2018) further point out the challenge of automating these separate life cycle stages, while Ravi et al. (2020) looks to automation as the solution to improve colony outputs.

Surendra et al. (2020) touches on the potential of genetic modification of colonies to facilitate mass production of neonates. However, there is a risk that negative perceptions of genetic modification may negatively impact the marketability of BSFL products, especially those intended for human consumption (Cui and Shoemaker, 2018). A less rapid yet more palatable alternative may be selective breeding of BSFL strains for desired traits, such as greater egg clutches. Indeed, management of genetic diversity in mass rearing colonies is already an integral task for the sustainability of the industry (Hoffmann et al., 2021). Greater

understanding of neonate production will be critical for BSFL products to be a successful component of the circular bioeconomy. A step toward this is the recent review article by Meneguz et al. (2023) which reviews knowledge on adult biology for use in optimising breeding-systems.

2.4.2.3. Process Automation

Process automation is one technological innovation researchers are looking towards to enable large scale production (Cickova et al., 2015; Ravi et al., 2020; Surendra et al., 2020). Large commercial BSFL production companies such as Enterra and Agriprotein are already embracing automation (Joly and Nikiema, 2019), since labour costs can account for between ~15-40% of total operating costs (Joly and Nikiema, 2019). Automation is typically most cost effective at high volumes and in areas where labour is expensive (Law and Wein, 2018).

Mathematical modelling can improve operation and automation at scale, with digital twins providing unprecedented decision support and optimisation tools in manufacturing industries (Bécue et al., 2020; Preut et al., 2021). Padmanabha et al. (2020) describes development of mathematical models as lacking in the case of BSFL production, mentioning the potential aid of such models in automation of HVAC, harvesting, and feeding activities. Building on literature data and previous models by Chia et al. (2018a) and Diener et al. (2009), they have produced a comprehensive growth model using existing Von Bertalanffy growth and dynamic energy budget models specifically designed for BSFL. The authors' model accounts for temperature, feeding rate, moisture content and airflow, and while it is stated that feed quality is also accounted for, it is unclear where or how it is included. Models factoring in substrate properties such as nutritional features are absent from the literature, presenting a significant research opportunity.

2.4.3. Novel High Value Products

Exploring novel high value products from black soldier fly larvae (BSFL) fractions is a recurring theme in research (Almeida et al., 2020; Liu et al., 2019; Ravi et al., 2020; Surendra et al., 2020). Beyond the previously discussed product applications (see 2.2.1 Products and Relevant Quality Factors), studies have uncovered the potential of high value products including:

- Antimicrobial peptides for killing antibiotic-resistant bacteria (Müller et al., 2017; Park et al., 2014; Park et al., 2015; Vogel et al., 2018)
- Novel enzymes for unlocking nutrients from recalcitrant wastes (De Smet et al., 2018b; Lee et al., 2014; Müller et al., 2017)
- Vitamin and mineral supplements (Bessa et al., 2020; Borel et al., 2021)
- Melanin for biocompatible electronics (Migliaccio et al., 2019; Ushakova et al., 2017)

Of particular value are the range of potential antimicrobial peptides found in BSFL, thoroughly introduced in Müller et al. (2017), outlining four classes (defensins, cecropins, attacins, and diptericins) capable of causing cell lysis through multiple methods of action. Chitin in the BSFL cuticle additionally presents numerous opportunities in bioproducts, particularly once de-acylated to chitosan, including in biomedical, food, pharmaceutical, cosmetic, and various industrial applications (Müller et al., 2017; Rinaudo, 2006). Many of these applications have been proven with chitin sourced from crustaceans (Rinaudo, 2006). However, large scale producers have yet to incorporate processing steps for valorising larval chitin (Joly and Nikiema, 2019). The exoskeletons of pupated BSF adults provide a comparatively pure source of chitin, facilitating extraction and downstream use (Henry et al., 2015).

Such high value products widen the market of BSFL-based products and could aid in the establishment of the BSFL production industry (Ravi et al., 2020). However, there is a risk that such products may shift the economics such that producers use non-waste substrates in

favour of the added reliability and quality control, losing the valorisation aspect that is so appealing from a circular bioeconomy perspective. To address this issue, incentives may need to be developed for producers to prioritize using waste substrates whenever possible to maintain the circularity of the system. Despite this risk, the discovery of high value products from BSFL has sparked significant interest in the field and provides further justification for continued research and development.

2.5. Influence of Substrate on BSFL Qualities and Performance

As discussed in section 2.2.4.1 Addressing Product and Process Variability, understanding the substrate-dependent drivers of product and process variability is critical to the establishment of BSFL production as a scalable tool of the circular bioeconomy. This section reviews the existing literature pursuing this goal, specifically studies which identify substrate properties through which to control this variability. Studies can be grouped by how they approach the BSFL production process, most dominantly as either a form of composting, or as traditional animal agriculture. Whilst BSFL production does not fall neatly into either camp (see section 2.2.3 Core Mechanisms of BSFL Production), these approaches are useful simplifications since the process shares many mechanisms with both. However, neither describe the process sufficiently enough to explain the variation. Additionally, these approaches mostly ignore the complexity imparted by substrate microorganisms. As such this section is structured around substrate properties relevant to composting and animal nutrition, followed by sections on substrate microorganisms.

2.5.1. Substrate as Composting Material

Composting refers to the aerobic process of converting heterogenous solid organic materials into carbon dioxide, water, a microbially stable soil-like material largely through the action of mesophilic and thermophilic microorganisms. The similarity of converting solid organic materials into nutrient dense biomass including residues with similar properties and

uses to compost likely explains use of the term ‘BSFL composting’ in the literature (Ermolaev et al., 2019; Jamilah et al., 2022; Lalander et al., 2020; Lindberg et al., 2022a; Paton et al., 2023; Singh et al., 2021). Substrate properties relevant in composting are typically broader and simpler to measure and control for than those used in animal nutrition, such as protein and carbohydrate contents. Studies approaching BSFL production akin to a composting process explore substrate properties such as particle size, moisture content, volatile solids (VS), carbon (C) to nitrogen (N) ratios and pH.

2.5.1.1. Particle Size and Moisture Content

Particle size is an important consideration in composting, interacting with the moisture content of the substrate. Smaller particles provide greater specific surface area for enzymes and microorganisms to act on, hence, pre-treatments reducing substrate particle size can be beneficial. However, such actions risk reducing porosity and pore size distributions which may influence the system’s ability to maintain aerobic conditions and support colonies of beneficial microorganisms (Tremier et al., 2009).

Palma et al. (2018) observed the same mechanism in BSFL production, finding that excess moisture reduces oxygen penetration. Smaller particle sizes result in smaller pores, which fill with water more quickly due to increased capillary action, and retain water due to stronger capillary forces, thereby reducing oxygen diffusion rates. Formation of anaerobic regions via this mechanism may aid in explaining the finding that use of a 6.35 mm screen resulted in greater larvae growth than use of a 4.00 mm screen when using almond hulls as substrate in Palma et al. (2019). However, this was a preliminary study lacking replicates hence conclusions cannot be drawn. This interaction of particle size and moisture content provides further justification for setting the substrate’s moisture content according to its water holding capacity as in Danieli et al. (2019).

Additionally, this mechanism may aid in explaining reductions in BSFL survival and growth observed in some studies at larger scales and densities (Jones et al., 2019; Miranda et al., 2020b), wherein higher temperatures reduce the oxygen holding capacity of water, potentially creating detrimental anaerobic regions in substrates with adversely low particle sizes. This may be compounded by excessive substrate depths which can impact survival (Guidini Lopes et al., 2023). Use of supplementary continuous mixing or forced aeration systems may be beneficial in such conditions (Palma et al., 2019). Lastly, it is likely that interaction effects exist between particle size and other substrate properties, such as lignin content which is known to block access to more readily digestible hemicellulose and cellulose (Tuomela et al., 2000).

2.5.1.2. Volatile Solids

Volatile solids (VS) are occasionally used as a measure of substrate quality (Czekala et al., 2020; Isibika et al., 2021; Isibika et al., 2019; Lalander et al., 2020; Lindberg et al., 2022a; Lindberg et al., 2022b), stemming from the study by Lalander et al. (2019). In this study, a generalised linear regression model was implemented to demonstrate that substrate VS content and protein feeding ratio were the main influential properties on larval production, as opposed to total nitrogen, C/N ratio, pH and carbohydrate to protein ratios. It is important to note that VS provides a measure of the ash content of a substrate, as it refers to the difference in mass between a sample and the inorganic residue remaining after volatilisation. The correlation between VS and larval production can therefore be explained by the fact that minerals and other inorganic compounds in ash are only required in small quantities for larval and microbial nutrition. Hence, substrates with lower ash content and higher VS tend to have higher concentrations of usable nutrients. A high VS content is therefore a requirement of a suitable substrate for BSFL production, and all substrates would ideally consist of predominantly VS. Consequently, while VS may be helpful in identifying poorly suitable substrates, its usefulness

is limited when comparing between more suitable substrates. Information on the composition of the VS is required to optimize nutrient availability and larval growth on suitable substrates.

Contributing to the observed correlation between VS and larval growth is the fact that the substrates with lowest VS also appear to be those typically considered as low quality (e.g. manures, faeces, and sludges). These materials contain higher levels of recalcitrant materials such as fibres, since the more easily digestible nutrients have been removed by the source organism (i.e. animals for manures, humans for faeces, microorganisms for sludges). Hence, larval production is understandably worse on these substrates, regardless of if the VS content was equivalent in all substrates. The authors acknowledge the likelihood of other substrates properties such as fats, a component of VS, also influencing performance.

2.5.1.3. Carbon and Nitrogen

It is valuable to understand why measures of carbon and nitrogen are used in composting to understand their adoption in BSFL production. Carbon and nitrogen are the most commonly limiting elements for microbial decomposition in composting. Carbon is metabolised as an energy source and leaves the system as CO_2, while nitrogen is used for microbial protein production (Hubbe et al., 2010). Obtaining a balanced C/N ratio is essential for successful composting, as excess nitrogen results in rapid microbial growth often causing significant quantities of heat and oxygen depletion, resulting in detrimental anaerobic conditions (Hubbe et al., 2010). Hence, C/N ratio is a useful measure of substrate quality for composting.

Critically however, the optimal C/N ratio depends on the bioavailability of both elements, which is influenced by the substances containing the elements, as well as physical particle size and resulting available surface area for microbial exposure (Hubbe et al., 2010; Zhu et al., 2019a). Organic nitrogen (e.g. nitrogen contained in protein) is more slowly available than inorganic (e.g. ammonium nitrate) and becomes available at a rate similar to the

growth rate of the microbes so they are used efficiently, whereas inorganic is available faster and must be dosed in series and in lower amounts (Hubbe et al., 2010; Rynk et al., 2022). Carbon available in simple short monomers or oligomers are available more rapidly than carbon trapped in fibrous components of hemicellulose, cellulose and lignin (Hubbe et al., 2010; Rynk et al., 2022). Lignin (crosslinked phenylpropane) is the most recalcitrant of the fibres and is interwoven with the other fibrous components, reducing surface area available for enzymatic attack and therefore its content significantly limits biodegradability of substrates (Hubbe et al., 2010; Rynk et al., 2022). As such, optimal C/N ratios vary depending on the substrate components, particularly lignin contents.

This ratio typically does not vary substantially beyond the range of 25:1 and 30:1 when using traditional sources of carbon and nitrogen, such as woody materials and manures. This is in part due to the variety of microorganisms available in a compost system capable of consuming various C and N sources (Rynk et al., 2022). Additionally, composting is often framed as a waste management process rather than a production process as the value from gate feeds often overshadows that of the product (Coker et al., 2022). Product quality specifications for compost are therefore typically broad, with wider ranges than in more specialised products, allowing the use of a variety of different C and N sources. This is not the case for BSFL production intended as a tool for the circular bioeconomy (see ‘Bioeconomy: designing products from organic resources’).

Nevertheless, numerous studies explore the influence of substrate C/N ratio on BSFL production in disregard of bioavailability. Lalander et al. (2019) cultivated larvae on several waste-based substrates and used principal component analysis to identify which substrate properties were the most important factors influencing larval production. Given the variations in bioavailability, C/N did not appear to directly correlate to any of the measured response variables. This is understandable given that changes in substrate protein content have a

substantially greater effect size than changes in carbohydrate content (Barragan-Fonseca et al., 2021). Moreover, while total N is likely a reasonable proxy for protein content (assuming absence of non-protein nitrogenous compounds), total C is a poor estimate of readily digestible carbohydrates since it is present in recalcitrant fibrous compounds as well. Therefore, protein to non-fibre carbohydrate ratio may be a more worthwhile factor to assess.

Similarly, Singh et al. (2021) used waste-based substrates (mixed waste, restaurants waste, fruit waste, vegetable waste) finding fresh prepupal and larval weights varied across substrates. The mixed waste substrate yielded the highest bioconversion, which the authors concluded to be in part due to an optimal C/N ratio at 21.96. However, there appears to be no correlation between C/N ratio and the observed bioconversion performance, since the second-best performance was found from restaurant waste at the lowest C/N of 14.7, followed by fruits at the highest ratio of 23.0 and finally vegetables – the worst performing - at 18.8. Measured differences in nutritional contents across the substrates used, such as fat, ash and mineral contents, as well as those unaccounted for such as fibre contents, may be more appropriate contributors to the observed results.

Palma et al. (2019) avoids the challenge of variations in bioavailability of C and N sources, testing several C/N ratios from 16 to 45 using almond hulls (high in acid detergent fibre) and urea as the only C and N sources. Increasing C/N ratios increased larval quality in terms of methionine and cysteine contents, however this benefit was countered by decreased larval dry weights and yields up to the maximum ratio tested of 45. Interestingly, substrate reduction was negatively correlated with larval growth, with higher C/N ratios up to 45 resulting in greater substrate reduction. Given the recalcitrant nature of the carbon source, these increased C/N ratios may have been approaching the optimal ratio for microbial decomposition of the substrate, at the expense of larval nutrition. However, this result should be interpreted with caution considering the small sample sizes and large variations within treatments.

While carbon bioavailability is typically the main consideration in modifying the C/N ratio of composting substrates, Lu et al. (2021) demonstrate that nitrogen bioavailability may also be a property of concern in BSFL production, particularly when using inorganic sources as may be found in biowaste leachates. They examined nine sources of nitrogen including organic (urea, uric acid, soybean flour, fish meal, L glutamic acid, L aspartic acid) and inorganic species (NH_4Cl, $NaNO_3$). Small differences were found across organic treatments, while inorganic sources were poorly tolerated by the larvae with respect to all measured performance and quality variables. This aligns with the knowledge of inorganic N sources requiring dosage in series in composting due to the rapid availability for microbial action. They further explore C/N ratios using urea as the nitrogen source added to food waste, testing ranges from 10 and 21 finding max larval protein and lipid bioconversion at between 18 and 16. It is unclear the mechanism by which urea addition up to C/N of 18 improves larval production, though it was likely through enabling beneficial actions of microorganisms, such as amino acid synthesis for larval metabolism.

Overdosing of organic nitrogen sources can also be problematic in BSFL production. A study using pig manure and corncob as sources of N and C respectively found ratios of between 20 and 30 yielded statistically similar results on a fresh larval weight basis, while larval weights reduced outside this range. Nitrogenous emissions were higher from low C/N given the greater quantities of nitrogen-rich manure used (Pang et al., 2020b). Another study explored measures to counter the toxic effects of excessive nitrogen through the use of biochar, finding improvement up to 86% in dry yield over the control (Beesigamukama et al., 2020). This was postulated to be due to adsorption of ammonium ions preventing them from entering the liquid phase, thereby reducing loss as ammonia gas, and reducing availability for nitrifying bacteria.

C/N may be a useful measure of substrate quality comparing between substrates containing equivalent sources of C and N, as in the case of Pang et al. (2020b), Lu et al. (2021) and Palma et al. (2019). However, this scenario seems uncommon in an industrial BSFL production facility where C and N sources likely vary, requiring more detailed analyses as to the bioavailability of these elements. Similarly, it is unclear under which conditions an optimal C/N ratio would encourage beneficial rather than detrimental microorganisms. Hence, C/N ratio alone is unlikely to be a reliable and transferable predictor of substrate quality.

2.5.1.4. pH

pH is monitored in composting primarily as means of monitoring the decomposition process, since composting microorganisms prefer pH between 5.5 and 8. Organic acids are produced in the early stages which lower the pH, enabling fungal growth which aid in breakdown of fibrous components, prior to the acids neutralising producing final compost at between 6 and 8 pH generally. BSFL appear to similarly reduce the pH initially from starting pH levels of between 11 and 4 to levels between 4 and 5, before increasing to between 7 and 9 (Ma et al., 2018). Meneguz et al. (2018a) explored the role of pH in BSFL production, finding that with Gainesville diets modified to varied starting pHs between 4.0 and 9.5, weights differed between treatments in the early few days of the experiment, but no significant differences were observed in final larval weights once growth had plateaued. This is theorised to be due to the larvae expending resources to manipulate the pH of substrate, resources which would otherwise contribute towards larval development (Meneguz et al., 2018a). Ma et al. (2018) used an expanded range of initial pHs to more extreme levels than would be expected to be found in typical organic substrates, only finding significant final weight reductions at pH levels 2.0 and 4.0 and development time increases at pH 2.0 (from 24 to 28 days), as well as differences in weights earlier in the study.

Contrarily, Pang et al. (2020a) concluded there were significant changes in final larval weights (total culture time 10 days) as a function of changing pH of the substrates, with higher pHs resulting in larger larvae weight. From the growth curves presented in Pang et al. (2020a), it appears the larvae were actively growing and had not reached the plateau stage unlike those cultured in the other two studies, and as such, it may be that the larvae across treatments would have reached the same maximal weights if left to continue developing, as in the other two studies. As such, controlling the pH around 11 may be of value if the intention is to harvest larvae before they have reached their maximum possible weights, however, for most BSFL operators with the intention maximise larvae weight, current studies indicate that pH is reasonably insignificant as long as it sits approximately above 4.

2.5.2. Substrate as Animal Feed

Some studies approach BSFL production akin to traditional animal agriculture, with some studies referring to the larvae as 'mini-livestock' (Dorper et al., 2021; Rehman et al., 2017). In such studies, substrate quality is measured in terms of properties commonly controlled for in animal nutrition, namely macromolecule contents of carbohydrates, fats, proteins, minerals and vitamins (Cohen, 2015). This is a sensible approach given BSFL share similarities in nutritional requirements to farmed animals, however, it is limited in its disregard for the contribution of microorganisms external to the larvae in the substrate's digestion.

Of the properties relevant to animal nutrition, protein (P) and non-fibre carbohydrate (C) contents are commonly considered the most influential factors after moisture content in BSFL production – as in several animal feed formulations - and therefore most valuable to optimise for in substrate formulations (Cohen, 2015; Gold et al., 2020b; Gold et al., 2018; Hardy and Brezas, 2022; Maiolo et al., 2020; Uyeh et al., 2019). Additional properties including lipids, dietary fibre, anti-nutritional factors, and texture are of importance in animal nutrition and therefore merit exploration in BSFL production, hence their review in this section.

Despite their requirement for various physiological functions (Cohen, 2015), vitamins and minerals are not included in this section due to the lack of investigations into their influence on BSFL production and invertebrate nutrition generally (see 2.2.1.6 for further information).

2.5.2.1. Protein and Carbohydrates

Proteins (P), namely their composing amino acids, are essential for production of tissues and biomolecules in animals, including BSFL (Cohen, 2015). While carbohydrates (C) are not essential, they supply a significant portion of an animal's energy needs alongside fats (Cohen, 2015). Carbohydrates additionally consist of the bulk of BSFL's natural diet and non-fibre content of many wastes deemed suitable in substrates for BSFL production (Barragán-Fonseca, 2018). Investigating protein and carbohydrate consumption is additionally a fundamental stage of the well-established geometric framework for identifying nutritional requirements (Simpson and Raubenheimer, 2012). As such protein and carbohydrates are important macronutrients in BSFL production and have received significant attention in the literature.

However, attempting to quantitatively isolate the effects of P and C on BSFL production is challenging due to conflicting findings in the literature, likely in large part a consequence of the lack of standardised experimental methods as discussed in section 2.2.1.2 Proximate Composition. Additionally, studies that use chemically complex substrates in setting the P and C contents - such as real wastes - often unintentionally vary secondary substrate properties between treatments (e.g. fibre, lipids, anti-nutritional factors or texture), creating confounding variables. While this approach provides an appropriate proxy for BSFL production at commercial scale, it creates difficulty in isolating the contribution of P and C contents on the observed responses. This challenge is not solved by using purified P and C sources as they are potentially more bioavailable than real waste-based sources, thereby generating correlations that are not transferrable to a commercial scale. Alternative methods

such as using the 'Design of Experiments' approach and extensive monitoring of substrate properties may therefore be required to more completely account for confounding variables (Eriksson et al., 2000). Despite these limitations preventing conclusive quantitative relationships between P and C contents with BSFL production, P and C contents have proven to be substantially influential on BSFL production in the literature.

First to explicitly explore P and C contents in substrates were Cammack and Tomberlin (2017), exploring a range of P and C contents from 7 to 35%, finding no significant differences in prepupal weight in response to varying the ratio of the two nutrients. However, a balanced ratio of 21% protein to 21% carbohydrate yielded the fastest larval development, feed conversion efficiency and survival out of the artificial diets. This remained inferior to the performance of the Gainesville control diet (Table 2.4) for which several factors may be responsible. Most notably, the substrate was fed to the control larvae twice as often as the other treatments. Additionally, the lack of vitamins and minerals in the ingredients of the treatment substrates. Further contributors may have been the increased bioavailability of the pure P and C sources possibly increasing microbial competition; the insufficiency of total carbohydrate contents, or limiting of specific amino acids, microbes, lipids, fibre, or texture. The varied contents of cellulose would have additionally altered the texture across treatments. Hence, it is difficult to conclude the influence of P and C from this study.

Protein contents appear to have an optimum for maximising larval weights, yet not necessarily larval growth rates (Table 2.4). Barragán-Fonseca et al. (2018) found larval lipid content increased due to increasing dietary protein from 10 to 24%, implying that energy from excess protein may be stored as fat. Similarly, Beniers and Graham (2019) found that high protein contents up to 28% increased larval weights and lipid contents yet decreased survival. Contrary to Danieli et al. (2019), Barragán-Fonseca et al. (2018) found larval protein content

is less affected by dietary nutrient concentration as larval crude fat content, the former ranging from between 42 and 44% over the tested substrates, while the latter ranged from 20 to 32%.

As for carbohydrates, Beniers and Graham (2019) found dietary carbohydrates had little significant influence on larval weights or lipid content. In contrast, Danieli et al. (2019) and Barragan-Fonseca et al. (2019) found higher carbohydrate content up to 68% and 55% respectively resulted in higher larval lipid contents. In fact, higher larval lipid content was the only significant difference between treatments when accounting for survival-adjusted total yields of larval dry matter, protein and lipid in Danieli et al. (2019).

There is some evidence that the total protein and carbohydrate content may be more important for larval development than the ratio of the two or the interaction effect (Barragan-Fonseca et al., 2019). Expanding the range of P and C tested, Barragan-Fonseca et al. (2021) recommended substrates contain a total P and C content between 25 and 50% at ratios of between 1:2 and 1:4. This combined P and C content is substantially lower than the optimum found in Beniers and Graham (2019) of 66% and Barragan-Fonseca et al. (2019) of 79%. This implies the sources of P and C are important with regards to their quality, likely in terms of bioavailability, amino acid composition and P and C compositions (Table 2.4) (Barragán-Fonseca et al., 2018).

Table 2.4 Summary of literature values on BSFL production and composition as a function of substrate P and C contents. Total dry larval mass produced has been calculated using the assumption of 70% moisture in the case of Cammack and Tomberlin (2017). Values for Barragan-Fonseca et al. (2019) were estimated from figures.

Source	Diet description (relative to diets in the same study)	Diet P %	Diet C %	P+C %	Total dry larval mass produced (g)	Larval P %	Larval Lipid %	Development time (days)	Initial larvae added	Ingredients
Cammack and Tomberlin (2017)	High P Low C	35	7	42	1.99	Not reported	Not reported	33	125	P (3:1:1 ratio casein : bacteriological peptone : egg albumin), C (1:1 ratio sucrose : dextrose), cellulose
	Mid P Mid C	21	21	42	2.02	Not reported	Not reported	32	125	P (3:1:1 ratio casein : bacteriological peptone : egg albumin), C (1:1 ratio sucrose : dextrose), cellulose
	Low P High C	7	35	42	1.96	Not reported	Not reported	35	125	P (3:1:1 ratio casein : bacteriological peptone : egg albumin), C (1:1 ratio sucrose : dextrose), cellulose
	Control (Gainesville)	15	62	42	3.59	Not reported	Not reported	22	125	50% wheat bran, 30% alfalfa meal, 20% corn meal
Danieli et al. (2019)	High P Mid C	14	56	70	2.28	34	33	22	90	15% barley, 55% wheat middlings, 30% alfalfa
	Low P High C	11	68	79	2.55	22	47	22	90	68% barley, 20% wheat bran, 12% alfalfa
	Low P Low C	11	51	62	2.54	34	33	22	90	16% barley, 50% wheat middlings, 10% alfalfa, 24% wheat straw
	Control	11	60	71	2.77	35	32	22	90	50% corn, 20% wheat bran, 30% alfalfa
Barragan-Fonseca et al. (2019)	High P High C	24	55	79	8.20	43	33	20	100	63% chicken feed, 14% casein, 23% starch
	High P Low C	24	35	59	6.60	42	32	19	100	63% chicken feed, 14% casein, 3% starch, 21% cellulose
	Low P High C	10	55	65	7.70	43	27	18	100	63% chicken feed, 23% starch, 14% cellulose
	Low P Low C	10	35	45	5.30	45	19	15	100	63% chicken feed, 3% starch, 34% cellulose

While relations between substrate P and C influence BSFL compositions and performance measures as most evidently displayed in the works by Barragán-Fonseca, when normalised against the development time and number of input larvae (the value of which is discussed in 2.2.2.5 Potential Cross-Study Metrics), the influence becomes less substantial. Mean daily dry larval biomass produced per input larva was calculated for the substrates in Table 2.4 and partial least squares regression was performed to produce contours reflecting the influence of substrate P, C contents and the study source on the outputs (Figure 2.8). The study source was most significant on this metric, with Barragan-Fonseca et al. (2019) yielding the most larval biomass per input larva per day, demonstrating the importance of standardising culture conditions. Additionally, substrate carbohydrate contents were shown to be the most influential on BSFL production, supporting the theory of excess carbohydrate energy stored in larval biomass as fat. Contrarily, substrate protein contents appear to have only a slight influence on BSFL production within the range explored.

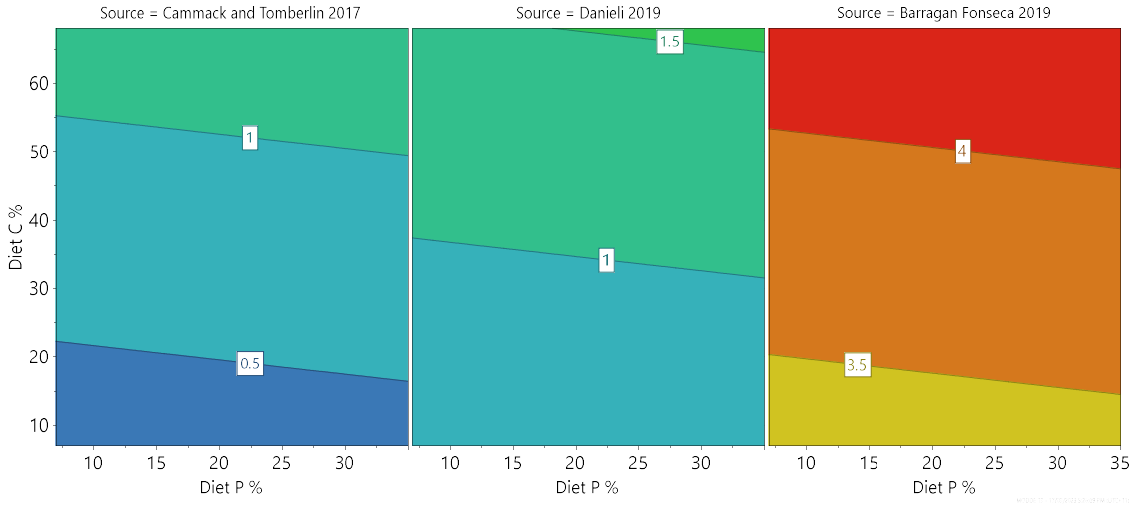

Figure 2.8 Mean daily dry larval biomass produced per input larva (mg) as a function of substrate protein and carbohydrate contents. Contours developed using partial least squares regression in MODDE software.

While P and C contents are clearly useful indicators of a substrate's performance, they do not tell the full story. Woods et al. (2019) demonstrates this with two meridic diets containing near identical protein and digestible energy, differing only with one of the diets

containing animal protein in the form of blood meal at 10%. All else equal, the blood meal diet increased larval survival from 69 to 91%, theorised to result from a seemingly miniscule increase in methionine content from 0.32 to 0.54%. This shows the challenge of formulating substrates according to only crude protein contents without consideration of amino acid profiles, of which studies are few (Koethe et al., 2022).

2.5.2.2. Lipids

Lipids serve roles as building blocks for animal tissues, cell membranes, hormones, nutrient transporters and dense stores of energy (Cohen, 2015). Sterols are required for development of insect cell membranes yet are unable to be synthesised by insects, making them essential lipids which must be sourced from the substrate (Cohen, 2015). Studies exploring the sterol requirements of BSFL are lacking, possibly due to the challenge of incorporating the non-polar immiscible compounds into the substrate (Cohen, 2015; Woods et al., 2019).

Studies have demonstrated fatty acids composing lipids within a substrate are either bioaccumulated within larval tissues, transformed into alternative saturated fatty acids or metabolised for energy expenditure (Ewald et al., 2020b; Li et al., 2022). The bioaccumulation potential has generated some interest in recovering nutritionally valuable poly-unsaturated fatty acids from wastes such as fish offal as a means of fortifying larval fatty acid profiles, however the conversion efficiencies are low (Barroso et al., 2017; El-Dakar et al., 2021b; Erbland et al., 2020; Oonincx et al., 2019; Putra et al., 2021; St-Hilaire et al., 2007a). Additionally, unsaturated fatty acids are prone to oxidation into toxic products, limiting their value in BSFL substrates without use of oxidation preventing measures such as antioxidants (Cohen, 2015; Nguyen et al., 2013).

Given their value as dense energy stores, substrate lipids can improve substrate conversion, larval gain and larval lipid contents, to an extent (Gold et al., 2020b; Oonincx et al., 2015). It is tempting to ascribe the dominant influence of lipids as their contribution as

energy, allowing the measure of substrate energy content rather than explicitly measuring lipid and carbohydrates. However, several studies have demonstrated this to be an oversimplification. Bellezza Oddon et al. (2022) used iso-energetic diets and found increasing substrate lipid contents using cocoa butter from 1 to 4.5% increased larval size, demonstrating lipid-derived energy corresponds to larger larvae than carbohydrates. Similarly, Li et al. (2022) tested six substrate lipid sources which not only altered larval fat contents and fatty acid profiles, but significantly varied larval growth, concluding palmitic, stearic and alpha-linoleic acids is beneficial for larval growth. Given their hydrophobic nature, lipids additionally may have indirect effects on BSFL production by altering substrate texture.

2.5.2.3. Dietary Fibres

Dietary fibre has complicated consequences for animal nutrition, with various competing and interacting mechanisms. There is little literature on this subject in insects. Of the few studies that explore fibre in BSFL production, Rehman et al. (2017) demonstrated the potential for all insoluble fibre components to be degraded by BSFL at varied levels, with observed fibre reductions in mixtures of chicken and cow manures ranging between 49 and 67% for cellulose, 49 and 59% for hemicellulose and 30 and 42% for lignin. In mixtures of restaurant waste and rice straw, Zheng et al. (2012a) reported BSFL treatment reduced 27.9% of cellulose, 32.6% of hemicellulose and 0.8% of lignin. It is unclear to what extent these digestions equate to improvements in BSFL growth from the existing literature, as other factors are changed simultaneously in these studies. These reductions are largely considered to be a function of lignocellulolytic enzymes produced by microorganisms either external to the larvae or within the larvae gut, hydrolysing fibrous components into mono and oligosaccharides available for metabolism by the larvae or further by microorganisms (Gold et al., 2018). Studies in humans and meat animals, such as broiler chickens and pigs, provide further insights into

mechanisms of relevance to BSFL digestion, as may be broadly distinguished as physical or chemical.

2.5.2.4. Physical Mechanisms Influencing Fibre Digestion

Addition of fibre to BSFL substrates or animal feeds dilutes readily digestible proteins, amino acids, starches, sugars, lipids and micronutrients. In homogenous substrates, larvae are unable to selectively avoid consuming fibre in favour of these readily digestible nutrients, requiring them to intake more total substrate to meet nutrient requirements than in a substrate with a lower fibre concentration (Li et al., 2021). When the rate of substrate intake is at its maximum, the growth rate is reduced as more time is required for the animal to ingest similar quantities of key nutrients and energy (Li et al., 2021). Similarly, dietary fibre (DF) can restrict access to readily digestible nutrients for both enzymatic and fermentative digestion. Intact plant cell walls create a mechanical barrier to intracellular nutrients for some insects (Clissold et al., 2004). Readily digestible nutrients may also be contained in the fibrous plant cell wall matrix themselves such as soluble proteins (O'Connor et al., 2010) and made inaccessible by indigestible cell wall components, particularly lignin (Siqueira et al., 2017). Lignin degradation is minimal during BSFL production (Liu et al., 2018; Ramzy et al., 2022), likely due to the short culture times and aerobic mesophilic conditions of BSFL.

Substrate texture is another important physical property influenced by dietary fibre contents due to altering water holding capacity and viscosity. Insoluble dietary fibre (IDF) is typically non-viscous and increases water holding capacity (Boulos et al., 2000; McRorie and McKeown, 2017). Soluble dietary fibre (SDF) often increases water holding capacity and substrate viscosity in a way dependent on the fibre's physical structure as opposed to its chemical composition (Boulos et al., 2000; McRorie and McKeown, 2017). Long linear chain fibre polymers that pack together - as in the case of apple pectin - increase viscosity more than highly branched polymers such as inulin and fructo-oligosaccharides (Canteri et al., 2012;

McRorie and McKeown, 2017; Nevara et al., 2021). However, DF content alone is insufficient to predict water holding capacity and viscosity of a substrate, as other substrate components such as starches and lipids, and the substrate matrix itself, are also involved (Brachet et al., 2015).

Substrate texture influences larval mobility and consequent ability to intake substrate (Fischer et al., 2021; Woods et al., 2019). Larvae move by forming an undulating wave with their bodies, pushing off their rear segment then lifting and stretching their middle segments to position their head forward, anchoring into the substrate from which the rest of the body is pulled forward (Giannetti et al., 2022). Substrate that is non-viscous and watery may reduce mobility by preventing larvae from anchoring their bodies from which they can push and pull their body forward. Contrarily, substrate that is highly viscous may require larvae to expend more energy during movement to overcome the increased drag (Goldstein and Jeffreys, 1929).

Furthermore, digesta texture has consequences for transit time, feed intake, satiety and nutrient digestibility (Lalander et al., 2020), with mixed reports as to its effects. Hooda et al. (2011) investigating fibre in swine nutrition found highly viscous feed increased nutrient digestibility, particularly amino acids, by way of increasing gut residence times, providing more opportunity for nutrients for enzymatic digestion and absorption. This contrasts with Li et al. (2021), noting highly viscous SDF decreased protein digestibility and aggravated epithelium cells. Reduced passage rate in swine and poultry also reduces total feed intake, resulting in less total nutrients consumed and made available for digestion (Smith, 2021). In humans and non-ruminant animals, the increased viscosity of digesta from soluble fibres is known to improve glycaemic control (McRorie and McKeown, 2017) by delaying glucose absorption (Montagne et al., 2003). Key nutrient signalling pathways involved in growth regulation in vertebrates are conserved in insects, such as the insulin and insulin-like growth factor signalling pathway, and the target of rapamycin pathway (Koyama and Mirth, 2018).

However, there are significant differences across species in the effects of these pathways on growth, and it is therefore unknown if these mechanisms notably influence BSFL performance and composition (Koyama and Mirth, 2018).

2.5.2.5. Chemical Mechanisms Influencing Fibre Digestion

Incompletely hydrolysed fibres are fermented by microorganisms under anaerobic conditions, such as those found in the posterior gut in various mammals (Montagne et al., 2003; Wang et al., 2019; Williams et al., 2017). These conditions produce organic acids and short chain fatty acids (SCFAs), known to be nutritionally significant to intestinal cells and influential on gut hormones (Canfora et al., 2015; Kim et al., 2018; Koh et al., 2016; Li et al., 2021). The extent of gut microbial fermentation into SCFAs can be significant, with a study showing approximately 70% of the total polysaccharide energy content (predominantly fibres) was converted into SCFAs via microflora in ruminants (Xu et al., 2021). Such fermentation has also been found to occur in insect species (Odelson and Breznak, 1983; Santo Domingo et al., 1998; Zheng et al., 2017). The BSFL gut contains anaerobic sections, and analyses of gut microbiota have found genera capable of producing the same fermentation products, namely *Firmicutes* and *Bacteroides* (Gold et al., 2018). Furthermore, SCFAs have been found in BSFL faeces in higher concentrations than in the substrate, indicating this fermentative digestion is present in BSFL (Gold et al., 2020a). However, there is a lack of studies investigating commonly fermentable dietary fibres in BSFL substrates, despite these fibres occurring in various wastes used in BSFL substrates (Montagne et al., 2003; Wang et al., 2003).

Soluble fibres are generally considered more rapidly and completely fermentable than insoluble fibres, and cellulose and hemicellulose less resistant to fermentation than lignin (Montagne et al., 2003; Williams et al., 2017). However, this is an oversimplification. Fermentability and the profile of fermentation products of any fibre fraction is dependent on the fibre's chemical structure, substrate matrix and microorganisms present (Bai et al., 2022;

Montagne et al., 2003; Nevara et al., 2021). Indigestible lignin is known to restrict access to otherwise fermentable plant cell wall components (Liu et al., 2021a; Siqueira et al., 2017). Substrate texture, as discussed above, can also shift the balance between enzymatic and fermentative digestion (Hooda et al., 2011).

Animal studies show that SCFAs, notably butyrate (Zhang et al., 2022), can reduce adiposity and body weight gain in the form of fat (Canfora et al., 2015), thereby reducing total fat content. SCFAs have also been found to increase leptin secretion (a satiety hormone) in mice and bovine adipocytes, alter lipolysis in rodents and humans, and alter insulin sensitivity and glucose metabolism in human skeletal muscle cells (Canfora et al., 2015). In Drosophila, dietary acetic acid was found to slow larval development, increase substrate consumption and increase lipid content (Kim et al., 2018). In pigs, butyric and propionic acids stimulate secretion of digestive enzymes (Suiryanrayna and Ramana, 2015). If similar processes occur in BSFL, substrate fibre fermentation may notably impact performance and composition.

2.5.2.6. Anti-Nutritional Factors

Anti-nutritional factors (ANFs) are substances which negatively influence the health and growth of animals when consumed, hence feed formulations attempt to minimise their contents (Ancuța and Sonia, 2020). Beside those which additionally pose safety concerns (see 2.2.1.4 Safety), ANFs are underrepresented in studies on BSFL production. A selection of ANFs which commonly restrict the use of food wastes in traditional animal feed are outlined in Table 2.5. They may be accumulated from the substrate or be produced by the larvae (such as chitin) in response to substrate properties, thereby restricting the range of feasible substrates (Somroo et al., 2019). Contrarily, BSFL may prove resistant to common ANFs, allowing use of contaminated materials in BSFL substrates as an effective valorisation pathway.

Studies on the influence and bioaccumulation potential of several key ANFs in BSFL have shown varied results. Somroo et al. (2019) found trace levels of tannin, oxalate and

phytate in BSFL when fed soybean curd residue and an unknown artificial feed, yet initial contents of these ANFs in the substrates were unclear. Callegari et al. (2020) discovered phytase activity produced by gut microbiota of BSFL, indicating the potential for degradation of this ANF. Tannins are present in various plant-based wastes, acting as defence mechanisms against insect attacks. Despite their prevalence, their influence on BSFL is under-examined, with Barragán-Fonseca (2018) stating only that tannins did not affect BSFL performance without discussion. Spent coffee grounds have slowed BSFL growth rates, potentially as a function of residual caffeine content (Permana and Ramadhani Eka Putra, 2018), and high copper contents have reduced BSFL survival (Cai et al., 2017). Other compounds, such as enzyme inhibitors, limonene, lectins, polyphenols, and volatile fatty acids, have shown antagonistic effects on various insect species and earthworms, warranting investigation in BSFL (Baran et al., 2018; Barragán-Fonseca, 2018; Hemming and Lindroth, 2000; Karr, 1989; Macedo et al., 2015).

Mycotoxins, such as aflatoxins, which are responsible for the contamination and subsequent removal of crops from the food chain neither accumulated in BSFL tissue nor hindered growth compared with controls (Bosch et al., 2017; Purschke et al., 2017). By-products from ethanol production such as spent brewer's grains and anaerobically stored organic wastes can contain notable levels of ethanol which may otherwise restrict use of such substrates in traditional animal feeds. However, this contamination likely does not affect larval quality or performance, given the regularity of using such substrates in BSFL studies with no noted consequences (Jucker et al., 2019; Scala et al., 2020). Additionally, *Drosophila* flies are known to be naturally resistant to ethanol enabling them to feed on decaying fruits (Fry, 2014). Organic wastes contaminated with mycotoxins or ethanol may therefore be suitable in BSFL substrates despite prohibition in traditional animal feeds.

Table 2.5 Potential constraints associated with the use of the above waste streams as animal feed.

Material	Possible constraints as animal feed
Apple pomace	Ethanol, pesticides (Alibes et al., 1984).
Bananas	Tannins, mycotoxins, pesticides (Heuzé V. et al., 2016).
Bread, dough and other baked products	Mould and mycotoxins including zearalenone and botulism (Heuzé V. et al., 2018a).
Brewer's grains	Mycotoxins (Asurmendi et al., 2013).
Cheese whey (liquid)	Need for pasteurisation, high water content (Schingoethe, 1976).
Fish offal (trimmings)	Nematodes, thiaminase content, histamine content (Buchmann and Mehrdana, 2016) (Food and Agriculture Organization of the United Nations, 1986).
Grain mill and starchy products (e.g. wheat bran, pasta)	Wheat storage proteins including albumins, acidosis potential from sugars, mycotoxins, phytic acid (phytates) (Ravindran et al., 1994).
Grape marc (seeds, skins, pulp)	Tannins, copper toxicity, pesticide residues (Heuzé V. and Tran G., 2017).
Lees (dead yeast)	Ribonucleic acids (causes high uric acid in blood of ruminants which effects metabolism) (Ferreira et al., 2010).
Oranges	Mineral imbalance, mycotoxins, limonin, lectins, tannins, pesticides, volatile fatty acid (VFA) content (Heuzé V. et al., 2018b).
Potatoes	Glycoalkaloids (α-solanine and α-chaconine) cause death in ruminants (particularly high in potato peel), protease inhibitor, pesticides, bypass starch (Ncobela et al., 2017).
Spent coffee grounds	Caffeine, tannins, polyphenols, oxalates and high amounts of potassium may result in low palatability, feed intake, protein digestibility and nitrogen retention. Mycotoxins (Choi and Koh, 2017) (Ballesteros et al., 2014).
Tomatoes	Tomatine (glycoalkaloid), pesticides, tannins (Heuzé V. et al., 2015).

2.5.2.7. Texture

Texture, as heavily influenced by fibre contents, is an important aspect of animal nutrition, influencing intake rates particularly in animals lacking mouthparts for mechanical breakdown of substrate such as BSFL (Bruno et al., 2020; Teferedegne, 2000; Valverde et al., 2008). However, no studies have been found that study the influence of physical attributes such as texture (e.g. viscosity, firmness, gumminess), with the standardisation protocols presented in Bosch et al. (2020) only mentioning the need "to create a texture that leads to an efficient digestion by the larvae". This appears inadequate, considering the findings of Woods et al. (2019) where the addition of small quantities of pig's brains (predominantly lipids) resulted in drastic reductions of larval survival, theorised to be a function of the physical properties of the substrate. It is possible that chitin contents may vary in response to hard or gritty substrate textures, since other insects employ thicker exoskeletons to move through such substrates

(Merzendorfer and Zimoch, 2003). Such physical properties may help account for the unexplained differences found between waste blends of equivalent macronutrient contents, such as in Gold et al. (2020b), as well as differences between studies using purified diets and those using waste blends.

2.5.3. Substrate Microorganisms

Knowledge from traditional animal nutrition provides a solid groundwork for understanding the influence of substrate on BSFL production, however it does not account for the involvement of microorganisms (see 2.2.3.2 External Microorganism Reactor). As such, while a substrate may theoretically be formulated per the nutritional requirements of larvae, it is likely to be altered by these microorganisms thereby rendering optimisation efforts futile on an animal nutrition basis. Indeed, recent studies have shown the influence of microorganisms on BSFL production to be substantial (De Smet et al., 2018b; Gorrens et al., 2021).

Symbiotic microorganisms – as sourced from the larvae themselves, the substrate or other contaminating sources – may benefit the insects by synthesising otherwise unavailable nutrients such as amino acids and vitamins, improving substrate properties such as texture, or through the provision of digestive enzymes (Cohen, 2015). Ao et al. (2021) noted that some of the taxa found in the BSFL gut such as *Rhizobiales*, *Burkholderia* and *Bacteroidales* are capable of synthesizing amino acids, along with creating bioavailable polypeptides from free amino acids. Furthermore, microorganisms found in the larval gut are known to be involved in the digestion and fermentation of plant-based polymers. Ng et al. (2019) isolated genera of *Enterococcus*, *Staphylococcus*, *Bacillus*, *Providencia* and *Morganella*, finding the consortia produced amylase, cellulase, lipase and protease, enzyme classes that have been confirmed in other studies (De Smet et al., 2018b). Gorrens et al. (2021) found altering the substrate by introducing fibre additives induced bacteria, proposed for use as potential symbionts. This hints at the potential for selecting particular microbes as inoculants (or probiotics) to improve

substrate properties. Contrarily, adverse microorganisms may cause disease, compete for nutrients or produce compounds such as anti-nutritional factors which are detrimental to the larvae's development (Cohen, 2015). Gold et al. (2020d) calls for studies that isolate members of identified taxa in larval guts to determine their true potential to influence BSFL production, which would enable improvement of in vitro digestion models (Gold et al., 2020c) and development of pro-biotics.

There are transfers of microorganisms between larvae and substrate (Gold et al., 2020d), prompting investigation into the flexibility of the larval gut microbiome in response to varied substrates, producing mixed results. Complicating the investigation is the fact that larval gut microbiota vary across studies and geographies (Shelomi et al., 2020). Commonly reported larval gut bacterial phyla include *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Gammaproteobacterial* (Ao et al., 2021; Caruso et al., 2013). Klammsteiner et al. (2020) theorise a core consortium of microbes from genera *Actinomyces*, *Dysgonomonas* and *Enterococcus* are indigenous to the larvae and facilitate the metabolism of varied substrates, similar to findings from Wynants et al. (2019). Similar communities were also found between larvae fed different substrates in Jeon et al. (2011), though the relative quantities of each taxon varied dramatically. The review by Sontowski and van Dam (2020) found no confirmation that the gut compositions of dipteran species were driven by the diet.

Contrarily, Greenwood et al. (2021) indicated that the diversity of the larval gut microbiome is influenced by the feed substrate as well as larval genetic background, and a potential interaction between these factors. Similarly, Boccazzi et al. (2017) conclude substrate determined gut fungi genera, supported by Tanga et al. (2021) who found varied fungal and bacterial gut communities in larvae fed different substrates. The fungi genera *Pichia*, *Geotrichum* and *Trichosporon* have additionally been found to dominate larval fungal communities in a substrate dependent manner, theorised to be due to their excretion of

antimicrobial compounds eliminating the competition (De Smet et al., 2018b). These contradictory findings across studies may be partly explained by differences in sampling locations from the larval gut as well as different larval strains in use. Additionally, De Smet et al. (2018b) notes low confidence in the findings from metagenetics studies identifying and quantifying microbial numbers.

Attempting to separate the contributions of egg associated microbes from substrate associated microbes, researchers have sterilised substrates prior to feeding, finding reduced production performance relative to unsterilised (De Smet et al., 2018b; Gold et al., 2020d; Schreven et al., 2021). However, the use of autoclaving may have altered other substrate properties influencing BSFL performance, such as disrupting fibrous components. This is a fundamental challenge in efforts to dissuade microbial growth on BSFL substrate, given techniques such as altering substrate pH, water activity, temperature and use of antibiotics may negatively alter substrate properties important for BSFL development (Cohen, 2015).

De Smet et al. (2018b) note an absence of studies investigating microorganisms from industrial scale rearing setups, theorised to vary from lab studies due to the typically less controlled and less hygienic conditions. Variations may also arise from differences in abiotic conditions such as substrate temperatures during BSFL development, since high volumes and densities of larvae increase temperatures relative to the settings of lab scale experiments (Scala et al., 2020). Large inactivation of pathogenic microbes in substrates by the larvae have been discovered and are dependent on temperatures (higher typically resulting in more inactivation) and substrate compositions, potentially indicating less microbial diversity in substrates in industrial settings.

2.5.3.1. Altering Substrate Properties through Microbial Pre- and Co-treatments

Given the positive contributions of microbial symbionts in BSFL production, a research trend has developed around manipulating the substrate microbial populations with selected

inoculants to improve the substrate properties and/or the larvae's abilities to convert the substrate into larval biomass. Intentional pre- and co- treatments of substrates (Figure 2.9, occasionally referred to as ex-situ or in-situ fermentations in literature) share similarities to solid state fermentations (Howdeshell and Tanaka, 2018; Kuttiyatveetil et al., 2019), and have been found to be capable of substantially improving BSFL production, both in terms of product quality and performance measures. Selecting species and conditions (such as temperatures, processing times, oxygen levels) that favour symbiotic over adverse microbial activity remains a challenge.

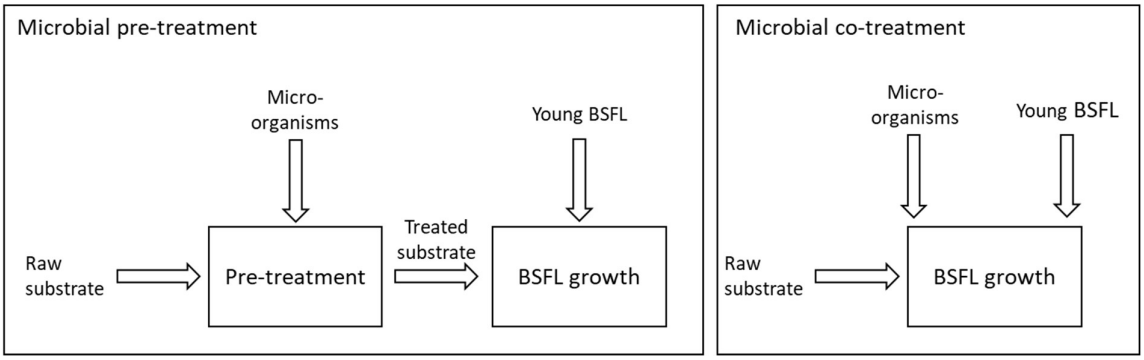

Figure 2.9 Block diagram comparison of microbial treatments.

Pre-treatments entail the inoculation and microbial conversion of the substrate prior to larval feeding. This can take significant time (Mohd-Noor et al., 2017; Wong et al., 2019), depending on the microbes and substrate used, however has the benefit of avoiding potential negative interactions between the microorganisms and the larvae. The simpler approach is co-treatment, wherein the microbial inoculant is added simultaneously to the young larvae, akin to the well-established practice of using probiotics in animal feeds (De Smet et al., 2018b). This appears a more economically viable option considering the time savings, however, has the possible disadvantage of direct competition, such as the larvae digesting the introduced inoculant prior to it improving substrate properties (Wong et al., 2021). There are no known studies on the direct comparison between pre and co-treatment with the same microbial inoculant and substrate, with the exception of Wong et al. (2021) who found ex-situ rather than

in-situ fermentation coconut endosperm waste with *Rhizopus oligosporus* improved larval growth rates and substrate reduction. Further investigations are needed comparing pre- and co-treatments in substrates of varied qualities. An overview of such studies is provided in Table 2.5.

Table 2.6 Overview of larval production studies that employ microbial treatment.

Source	Inoculant	Substrate	Pre or co treatment	Inoculation and treatment method	Findings relative to untreated control
Callegari et al. (2020)	Bacillus licheniformis, Stenotrophomonas maltophilia	Apple, pear, orange	Co	17 million CFU/g substrate	Increased larval growth rate and yield
Franks et al. (2021)	Rhodococcus rhodochrous	Gainesville diet: 30% alfalfa meal, 20% corn meal, 50% wheat bran	Co	415 thousand CFU/g substrate	Increased larval growth rate, yield and substrate conversion. Altered larval fatty acid profiles
Hasnol et al. (2020)	RidX bacteria consortia + enzymes	Coconut endosperm	Co	2.5 mass %	Increased larval growth rate, yield and protein and lipid contents
Kooienga et al. (2020)	Arthrobacter AK19, Bifidobacterium breve, Rhodococcus rhodochrous	Gainesville diet: 30% alfalfa meal, 20% corn meal, 50% wheat bran	Co	600 thousand CFU/g substrate	Arthrobacter AK19 and Rhodococcus rhodochrous 21198 increased larval growth rate, yield and substrate conversion at benchtop and industrial scales, Bifiobacterium breve decreased larval yield and substrate conversion
Mazza et al. (2020)	Kocuria marina, Lysinibacillus boronitolerans, Proteus mirabilis and Bacillus subtilis	Chicken manure	Co	1 vol/mass %	Increased larval yield, protein and lipid content and substrate reduction
Rehman et al. (2019)	Bacillus MRO_2 isolated from pig manure	40% dairy manure, 60% chicken manure	Co	1 million CFU/g substrate	Increased larval yield, substrate conversion and lipid and protein conversions. Significant reductions in substrate hemicellulose, cellulose and lignin
Somroo et al. (2019)	Lactobacillus buchneri	Soybean curd residue	Co	1 million CFU/g substrate	Increased larval yield, substrate conversion, protein and lipid contents
Wong et al. (2020)	Saccharomyces cerevisiae	Coconut endosperm	Co	2.5 mass %	Increased larval growth rate, yield, substrate conversion and protein content
Xiao et al. (2018)	Bacillus subtilis species	Chicken manure	Co	1 thousand CFU/g substrate	Increased larval growth rate, yield and substrate conversion
Yu et al. (2011)	Bacillus subtilis species isolated from BSFL gut and substrate	Chicken manure	Co	1 million CFU/g substrate	Increased larval growth rate and yield
Zheng et al. (2012a)	RidX bacteria consortia + enzymes	30% rice straw, 70% restaurant waste	Co	0.5 mass %	Increased larval yield, lipid content and protein conversion, significant reductions in substrate cellulose and hemicellulose
Wong et al. (2021)	Rhizopus oligosporus	Coconut endosperm	Co and Pre	1 mass %, 72h, open to atmosphere	Pre-treatment increased larval yield, lipid yield, protein and lipid conversion and substrate reduction. Co-treatment reduced larval growth rates, yield and substrate reduction
Gao et al. (2019b)	Aspergillus oryzae	Maize straw	Pre	Inoculation ratio 1:4000, 24h, open to atmosphere	Altered larval fatty acid profile, reduced substrate lignocellulose

Isibika et al. (2019)	Trichoderma reesei, Rhizopus oliosporus, bacteria	Banana peels	Pre	1 mass %, 7 to 21 days, unknown oxygen conditions	Increased larval yield and substrate conversion
Kuttiyatveetil et al. (2019)	Lactobacillus plantarum, Aspergillus oryzae	Borage, flaxseed meal	Pre	Open to atmosphere	Increased larval yield, protein and lipid conversion
Li et al. (2015)	Saccharomyces cerevisiae	Hydrolysed rice straw	Pre	48h anaerobic ethanol fermentation	Xylose increased larval lipid yield
Mohd-Noor et al. (2017)	Native microbes (self-fermented)	Coconut endosperm	Pre	4 weeks, anoxic conditions	Increased larval growth rate, yield, lipid and protein yields and conversion efficiencies
Qi et al. (2019)	Trtichoderma viride, Saccharomyces cerevisiae	Corn, wheat straw	Pre	3 mass %, 4 days, unknown oxygen conditions	Increased larval yield and substrate conversion (house fly larvae, not BSFL)
Wong et al. (2019)	Mixed-bacteria powder	Coconut endosperm	Pre	0.5 mass %, 14 days, anoxic conditions	Increased larval growth rate, yield and protein conversion, organic acids released from substrate

A key focus in literature is investigating the potential for microorganisms to improve production by increasing nutrient bioavailability in substrates (Ravi et al., 2020), notably carbon and nitrogen sources. Fibre and non-protein nitrogen are two substrate components targeted for this given they largely rely on microbial enzymes and actions to make them available for larvae metabolism (Cohen, 2015). Fibres are broken down into monomeric constituents and/or fermented into organic acids available for larval metabolism, as described in section 2.3.2.3 Dietary Fibre. Inoculants from termite guts such as species of *Spirochaetes* or *Fibrobacteres* may be beneficial for this (Auer et al., 2017). Similarly, non-protein nitrogen can be used as a nitrogen source for microbial synthesis of amino acids and peptides available for larval metabolism, as may be enhanced by select inoculants (Ao et al., 2021). De Smet et al. (2018b) notes that microbial inoculants could additionally alter antimicrobial activity and vitamin content. These mechanisms help explain how microbes can influence BSFL production at a high level, however more work is needed to understand variations in the extent of effects, particularly as a function of substrate properties, treatment methods and the selected microbes chosen. Additionally, whether the possible benefits from microbial treatments are cost effective remains unknown.

In light of the challenge in securing access to quality BSFL substrate materials (Joly and Nikiema, 2019), microbial treatments may have significant impact on the BSFL industry by enabling greater inclusion of more accessible and inexpensive materials, such as those with high fibre contents (Liu et al., 2019; Ravi et al., 2020; Surendra et al., 2020). Additionally, even 'high quality' raw materials currently explored for BSFL treatment such as food wastes contain variable amounts of fibre components, whose contents contribute to varied production across substrates (Gold et al., 2020b). Assuming microorganisms can be selected to avoid consuming the readily available nutrients (Callegari et al., 2020), microbial treatments may help bring consistency to such 'high quality' substrates, improving nutrient profiles as is

performed in sileage (Ewald et al., 2020b; Muck, 1988). By improving larval yields and substrate conversion, microbial treatments may facilitate economically viable BSFL production on smaller scales, enabling greater uptake of the BSFL bioconversion process and furthering the creation of a circular bioeconomy. Research on microbial substrate treatments will have to be performed alongside confirmations of microbe-related safety (refer to section 2.2.1.4 Safety) to reassure the industry.

2.5.4. Knowledge Gaps Relevant for Formulating Substrates using Organic Wastes

Numerous knowledge gaps restrict the formulation of predictable and consistently high-performing substrates from varied organic wastes. Most salient of these is understanding the variation of nutrient availabilities across waste materials, particularly carbon and nitrogen, as influenced by the nutrient source (i.e. chemical structure) and interaction with adjacent substrate components (e.g. interconnectedness with lignin). Experimental setups which better control for confounding variables are required to explore this area and identify larval nutritional requirements, such as by using better chemically defined substrates, microbial controls, and texture modifiers. Similarly, impacts of anti-nutritional factors borne from waste materials such as tannins are under accounted for. These substrate properties have consequences on optimal substrate nutrient contents and ratios for maximising larval production performance and composition. Addressing these knowledge gaps will facilitate systematic exploration of microbial and alternative treatments to further increase larval production efficiency and enable inclusion of a wider range of substrate materials. This is especially important considering wastes that are suitable for use in substrates are not standardised and in some cases are highly affected by seasonality, thus forcing the insect industry to resort to the use of high-quality animal feeds such as chicken feed. Alongside investigations into nutritional properties, further studies into chemical contaminants are required to ensure product quality from a safety standpoint when using waste materials in substrates.

2.6. Conclusion

Black soldier fly larvae production has significant potential as a tool of the circular bioeconomy. However, its deployment is restricted by variable larval-product quality and production performance, under-establishment of optimal operation procedures at industrial scale, and under-exploitation of novel high value products. Moreover, scaling up BSFL production will require attention to automation, mass colony management, and the potential for deriving novel high-value products.

The variability of larval quality and production performance is a critical barrier, requiring greater understanding of the core mechanisms involved, namely the interplay between larvae, microorganisms, and substrate properties. This necessitates careful experimentation investigating the roles of substrate nutrient contents, physical features, and safety considerations. Filling these gaps will enable creation of comprehensive predictive models to assist in formulating substrates using organic wastes, without compromising larval quality or production performance, thereby maximally realising the technology's potential for the circular economy. This chapter serves as a starting point for such future investigations and aims to contribute to the field by providing a comprehensive overview of both the current state and future research directions in BSFL production.

Chapter 3. Understanding Dietary Carbohydrates in BSFL Substrates

3.1. Introduction

As explored in Chapter 2, recent studies have investigated the importance of several macronutrients, notably of crude protein and non-fibre carbohydrate contents and ratios within the feedstock (Barragán-Fonseca, 2018), as commonly controlled in animal feed formulations (McDonald, 2002). Others have approached BSFL treatment more akin to a composting process, investigating broader factors of volatile solids and carbon to nitrogen ratios (Ayilara et al., 2020; Kumar et al., 2010). Formulations of organic wastes for optimal amino acid profiles have also been explored (Gold et al., 2018; Lalander et al., 2019). However, these factors alone do not sufficiently explain the variations in process performance, lipid contents and fatty acid profiles that are seen between blends of different wastes made to contain similar nutrient profiles (Gold et al., 2020). A more nuanced characterisation of the nutrient content of wastes is required. Of the numerous variables that differ between waste streams, the types of carbohydrates (polyhydroxy aldehydes, ketones and their derivatives) within the waste have been identified as likely significant contributors to BSFL treatment performance, larvae lipid contents and fatty acid profiles (Chippendale and Reddy, 1974; Rockstein, 1978). This chapter therefore investigates the effects of select carbohydrate types, commonly found in organic wastes, on BSFL treatment performance, larvae lipid contents and fatty acid profiles. The primary hypothesis is that, within an otherwise nutritious substrate, the types of carbohydrates can be significant in influencing these aspects of BSFL treatment. To the authors' knowledge, this has not been explicitly studied before and will valuably build on the existing knowledge base around BSFL treatment.

3.1.1. Variability in Waste Carbohydrate Profiles

Current commercial practice of using BSFL in production and waste treatment use pre-consumer food wastes as the bulk of the substrate, given their broad nutritional suitability and regulatory acceptance (Fowles and Nansen, 2020; Gold et al., 2018). Many of these food wastes contain significant amounts of carbohydrates, including various fibres (typically non-starch polysaccharides), starches and sugars (Table 3.1). Pre-consumer food wastes differ chemically via their comprised monomers, linkage types and sizes (Asp, 1996; Englyst and Englyst, 2005), varying their usability as nutrient sources for organisms. The most frequently encountered waste streams are outlined in Table 3.1 and form the carbohydrates of interest in this study.

Table 3.1 Carbohydrate contents of wastes commonly considered for BSFL treatment.

Waste generator type	Significant organic wastes produced	Notable carbohydrates (ordered by prevalence)	References
Bakeries	Baked products, flour, bread	Wheat starches approx. 60 dry mass%, simple sugars, fructans, hemicelluloses (heteroxylans), cellulose	(Biesiekierski et al., 2011; Hor et al., 2021)
Beverage manufacture	Co-products (e.g. fruit pomaces), raw ingredient wastes	Simple sugars (glucose, sucrose, fructose, xylose), starches, fructans, hemicelluloses	(Perussello et al., 2017)
Breweries	Brewers grains, trub, yeast	Starches, hemicelluloses (arabinoxylans), xylose, cellulose	(Mandalari et al., 2005)
Cafes	Coffee grounds, post-consumer food scraps	Cellulose approx. 9 wet mass%, hemicelluloses approx. 37 wet mass% containing galactose, arabinose, glucose and mannose, various	(Ballesteros et al., 2015)
Confectionery	Off-spec products, ingredients, off cuts	Simple sugars, corn starches, pectins, polyols	(Zumbé et al., 2001)
Dairy	Whey, dairy off-cuts, milk solids	Lactose, galasctose, oligosaccharides	(Urashima et al., 2013)
Feed mills	Grain waste (non-endosperm), oilseed waste, off-spec batches	Starches, hemicelluloses	(Saha et al., 2009)
Feedlots	Manures	Hemicelluloses, cellulose	(Wen, 2004)
Food processing (other)	Off-spec products, ingredients, off cuts	Simple sugars, corn, potato and wheat starches	(Dey et al., 2021)
Grain milling	Bran, germ, pollard, mill mix/middlings, grain dust, screenings	Cellulose, hemicelluloses (glucofructans), wheat starches, free sugars	(D'Appolonia and Rayas-Duarte, 1994)
Malt processing	Culms, dust, screenings	Hemicelluloses, starches, simple sugars	(Pereira et al., 1998)
Markets	Fruits and vegetables	Simple sugars approx. 10 wet mass% (glucose, sucrose, fructose, xylose), starches, pectins, fructans, hemicelluloses	(Zia et al., 2020)
Post-consumer sources	Food scraps, organic fraction of mixed waste	Various	
Seafood processing	Fish off-cuts/offal, organic liquids, scales	Glycogen	(Kilborn and Macleod, 1920)
Sugar milling and refining	Molasses, filter cake/mud, clarifying agents	Simple sugars (sucrose, fructose, glucose), non-starch polysaccharides	(Hashizume et al., 1966)
Wineries	Grape marc, lees	Hemicelluloses approx. 50 dry mass% (heteroxylans), simple sugars approx. 18 dry mass% (glucose, fructose), pectins, cellulose	(Corbin et al., 2015)

3.1.2. Known Carbohydrate Effects on Insect Digestion

Dietary carbohydrates are used as an energy source and material for tissue development in insects, with the usability of any particular carbohydrate varying across insect species (Cohen, 2015). In nature, carbohydrates typically form most of the diet for black soldier fly larvae; carbohydrates also make up the bulk of many organic wastes (Barragán-Fonseca, 2018;

Gold et al., 2018). Nutritional availability is dependent on the carbohydrate's physico-chemical properties, the composition of the meal with which they are consumed and biological variations across the consuming individuals (Englyst and Englyst, 2005). From this literature, there is a strong indication that carbohydrate types within insect diets, including within non-fibre carbohydrates, are a significant influence on insect growth, and by extension, on the performance of BSFL.

To begin investigating the macro-level effects of dietary carbohydrates on treatment performance, lipid contents and fatty acid profiles, it is important to understand the BSFL's digestion process. A summary diagram on the process of digestion in BSFL is provided in Figure 3.1. Solid foods are first liquefied prior to ingestion by the larvae (Lalander et al., 2019). Nutrient sensing happens before ingestion, with feeding rates regulated by hormones and increased by sugars in the diet (Gold et al., 2018; Shen and Cai, 2001). Enzymes are produced by either the larvae or associated microbes located externally or within the larvae digestive tract. These enzymes hydrolyse complex carbohydrates into simple sugars of mono and disaccharides which are transported across the peritrophic membrane and intestinal epithelium into the haemolymph (similar to vertebrate blood) via a combination of diffusion and active transport (Rockstein, 1978; Thompson, 2003). Transport of small carbohydrates is regulated by the concentrations and activities of enzymes in the gut, as well as availability and quality of the food (Gold et al., 2018). Upon transport into the recipient cell, each simple sugar then undergoes a series of enzymatic reactions following metabolic pathways that are regulated by hormones, ultimately yielding energy-carrying intermediates such as ATP and various metabolites (Gold et al., 2018; Waterhouse, 1957), as well as by-products such as CO_2. These metabolites provide building blocks largely used for structural carbohydrates such as chitin (in the insect exoskeleton) and in lipid and glycogen synthesis.

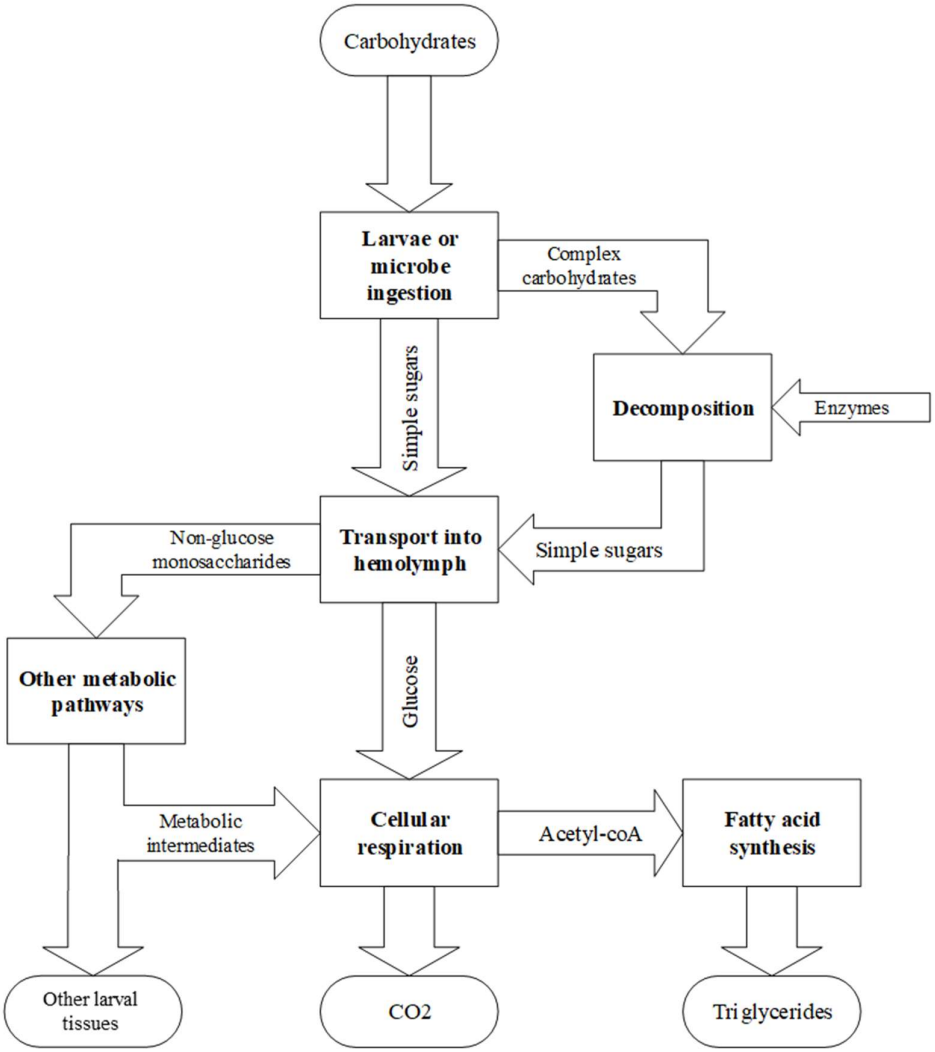

Figure 3.1 The dominant carbohydrate pathways through black soldier fly treatment.

Lipid content and fatty acid profiles in insects are influenced by metabolic shifts in glycogenesis and lipogenesis in the fat body, which in turn are influenced by dietary carbohydrate content and profiles (Inagaki and Yamashita, 1986; Rockstein, 1978). In a study on diapausing mosquitos, an artificial diet supplemented with sucrose was found to result in increased glycogen branching and accumulation with a reduction in lipids, while pure glucose resulted in both increased glycogen and lipid accumulation with longer fatty acid chains (Chang et al., 2016). When fed glucose at 1% mass in a diet with sucrose at 10% mass, the glucose was used solely for lipid synthesis as triglycerides, indicating an interactive effect between the sugars on their metabolic fates (Chang et al., 2016). Given sucrose is not absorbed across the

peritrophic membrane in the mosquito species studied and requires hydrolysis into fructose and glucose first, the presence of fructose could be a contributory factor in the observed effects. The authors infer that the composition of the diet activates insulin like peptides and adipokinetic hormones which regulate carbohydrate metabolism (Chang et al., 2016).

Similarly, mobilisation of glycogen and lipid energy stores in the fat body of *D melanogaster*, as regulated by adipokinetic hormone released from adipocytes (fat cells), has been shown to be affected by concentrations of glucose and trehalose in the haemolymph, in turn influenced by the diet (Arrese and Soulages, 2009). Should similar mechanisms occur in black soldier fly larvae, the ratios of sucrose, glucose and other simple sugars in the diet may therefore be influential in determining the lipid content and fatty acid profile within the produced larvae. This is important since the fatty acid profile determines the insect lipid's suitability for particular downstream markets, with for example, a high content of unsaturated fatty acids reducing the lipid's suitability for biodiesel production due to the oxidation potential (Ewald et al., 2020b; Kim et al., 2020a; Lin and Chiu, 2010; Surendra et al., 2016; Wong et al., 2019). Similarly, BSFL lipids high in saturated fatty acids could be less suitable for inclusion in animal feeds as they may be detrimental to the quality of animal-derived products such as meat or eggs (Gasco et al., 2019; Kim et al., 2020b; Melenchón et al., 2021; Park et al., 2021).

3.2. Methods

3.2.1. Experiment Design

A simple screening design was used wherein chicken feed-based diets were formulated to contain 20% dry mass of a target carbohydrate, providing surrogate 'waste substrate' diets with substantial volumes of the target carbohydrates. Two additional diets were formulated, one where the 20% additive was simply more chicken feed (referred to as 'Pure Chicken Feed') and the other where it was sunflower oil. The diet with sunflower oil was included to compare whether any observed variations from the carbohydrate treatments were as significant as those

from changing the macronutrient content (in this case the macronutrient content was changed by increasing crude fat, providing an alternative and denser energy source). A benchmark of chicken feed with no additive (i.e., containing only 80% of the mass of other diets) was used as a control. Carbohydrate additives were selected according to their prevalence in common wastes used as substrates in BSFL treatment (Table 3.1) and as representatives of varied carbohydrate properties.

A linear experimental design was chosen composed of 13 unique runs with three identical samples of each (n = 3). The independent variables were the treatment additives as listed in Table 3.2. Dependent variables were percent survival, waste reduction, bioconversion, waste conversion efficiency, crude lipid content and fatty acid profile. A graphical overview of the experiment is provided in Figure 3.2.

Table 3.2 Treatments (unique runs) used in this study.

Unique runs/additives	**Grouping**
Chicken feed (Benchmark – no treatment additive)	
Pure Chicken Feed (where the treatment additive was simply more chicken feed)	Complete diet – for comparing larval gain
Sunflower Oil	Triglycerides
D Glucose	Non-fibre carbohydrate (NFC) – monosaccharide
Sucrose	NFC – disaccharide
D (-) Fructose	NFC – monosaccharide
Corn starch	NFC – polysaccharide
Wheat starch	NFC – polysaccharide
D (+) Galactose	Hemicellulose constituent – hexose
D (+) Mannose	Hemicellulose constituent – hexose
D (+) Xylose	Hemicellulose constituent – pentose
D (-) Arabinose	Hemicellulose constituent – pentose
Xylan from beechwood (90% xylose residues)	Fibre – hemicellulose

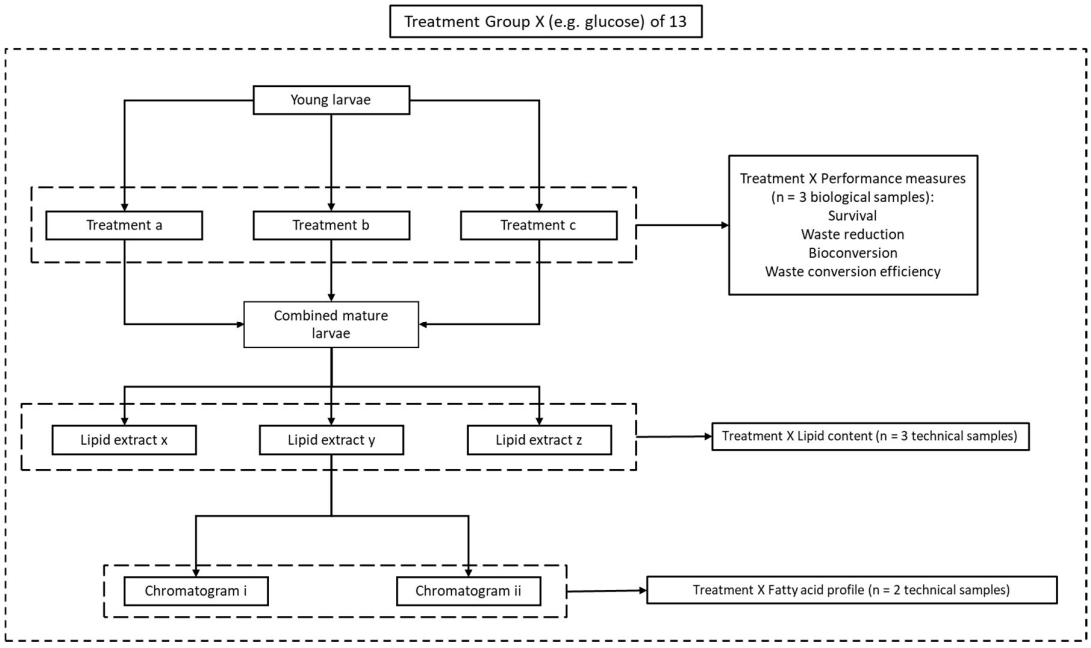

Figure 3.2 Overview of the experiment design. This was repeated for each of the 13 unique treatment groups as listed in Table 3.2.

While the D conformation is the most commonly found enantiomer in nature of most carbohydrate monomers, this is not the case for arabinose, instead occurring more commonly in the L conformation (Rahim et al., 2017). It has nevertheless been included for this study due to its occurrence in arabinogalactans of mycobacterial cell walls and some plant tissues (Holtzapple, 2003), as well as its availability at the time of the experiment. Treatment carbohydrates will hereon be referred to without indication of their enantiomerism or chirality (e.g., 'D (+) xylose' will be simply referred to as 'xylose').

Protocols for larvae handling, culturing, harvesting and performance determination were developed from Eawag's Organic waste treatment guide, Gold et al. (2020) and Barragán-Fonseca (2018), with modifications per local constraints which are elaborated below. Once harvested, larvae were analysed for crude lipid content using a modified folch extraction method (Folch et al., 1957) before transesterification of the lipid extracts (Sukhija and Palmquist, 1988) and fatty acid analysis via GC-MS.

3.2.2. Culturing and Harvest

3.2.2.1. Young Larvae Preparation

5-day old *Hermetia illucens* larvae were purchased from Future Green Solutions, arriving at 7 days old. Young larvae arrived in a wet mixture of moist feed (spent brewers' grains) and residue, requiring separation. The mixture was placed in a narrow container with a small lamp positioned above the surface and left to sit for several minutes as the larvae to moved down to the bottom of the container. When larvae were no longer visible on the surface, the top layer of residue and feed was scraped off with a spoon and disposed of. This was repeated until mostly larvae were left with minor remnants of feed. These larvae and remnants of feed were moistened with tap water and allowed to develop for 2 days in the environmental chamber (Value Scientific environmental control chamber model SHP-360 modified with several humidifiers) at 28 ºC and 60 – 80% relative humidity, before rinsing clean any residue, drying with paper towels, and weighing. Two random samples of approximately 2 g each were weighed and counted using a hand tally to estimate the total number of young larvae. Both samples were frozen for later analysis. Images of the young larvae preparation phase are presented in Figure 3.3.

Figure 3.3 Left: Young larvae during preparation after most residue was removed. Right: Young larvae after all residue removed.

3.2.2.2. Culturing

Unmodified corn starch, wheat starch and D-(-)-Arabinose were sourced from Sigma Aldrich, Sucrose, D-(+)-Mannose and D-Glucose from Chem Supply, D-(+)-Galactose and D(+)-Xylose from Merck, D(-)-Fructose from AXAJ chemicals, and Sunflower oil from a local grocery store (Coles). Culture containers (16 oz PET cups with lids, Lombard The Paper People with lids enclosed by Cyclone Miniweave fibreglass fly mesh secured over the opening with aquarium-safe silicone) were pre-weighed and filled with 80% chicken feed (Organic Backyard Layer Mash, Enfield Produce Pet and Garden Supplies, 16% protein, 7% fibre) and 20% treatment additive such that there were 205 mg initial dry feed per larva (16.4 g per treatment container). The exception to this were the benchmark containers, for which there were no additives (13.1 g chicken feed only per benchmark container). Each container was swirled by hand to combine ingredients before adding tap water to reach a moisture content of 60% and swirling again until visually homogenous. 80 young 9-day-old larvae were manually counted, weighed, and dropped into a container before closing the lid and positioning into the environmental chamber (28 ºC 60-80% RH) for culturing. 2.8 larvae were added per cm^2 of container base area, within the typical range of 0.1-3.3 noted by (Barragán-Fonseca, 2018). The average mass of an individual larva at the start of culturing was 6.05 ± 0.89 standard deviation mg wet mass, determined from 39 random samples (1.24 ± 0.18 standard deviation mg dry mass). Every three days the containers were randomly re-arranged to control for possible unknown effects of position in the environmental chamber. Images of the culturing phase are presented in Figure 3.4.

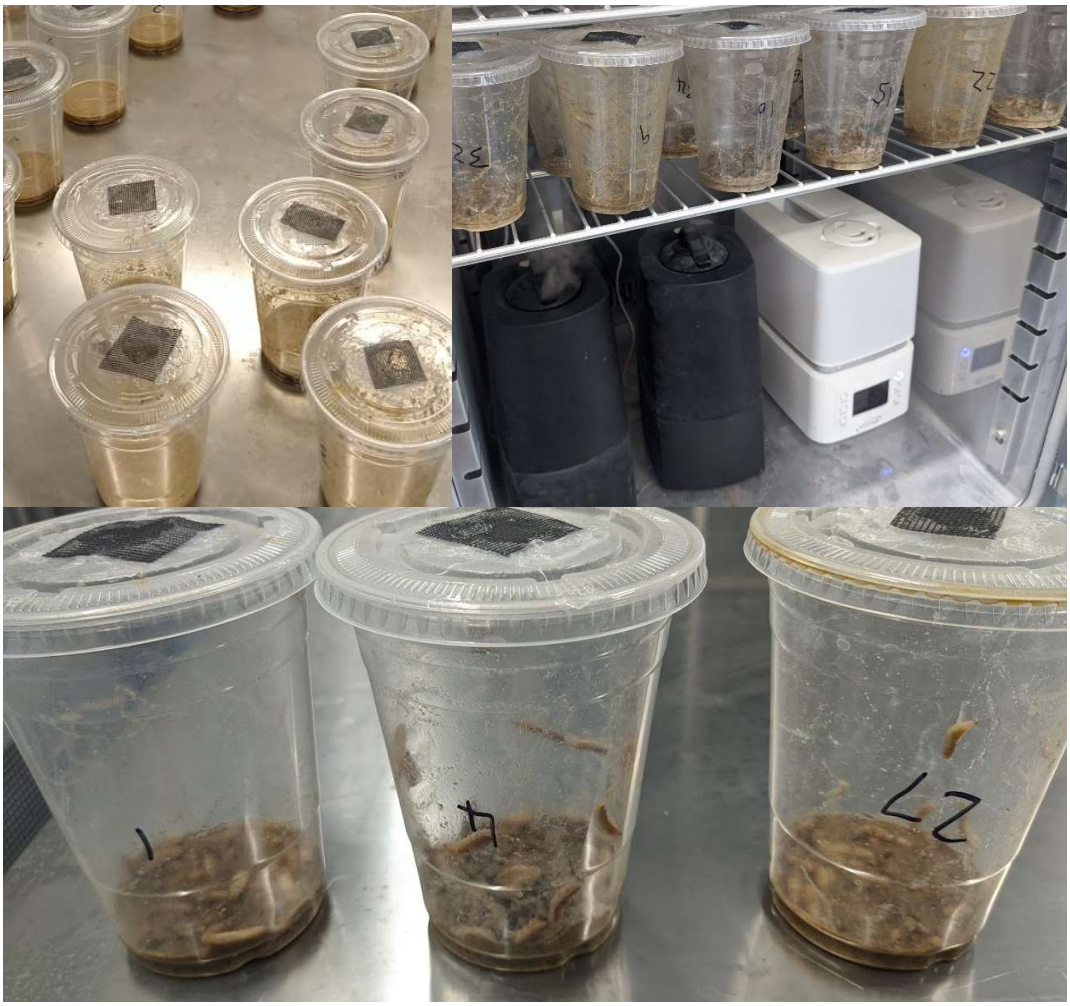

Figure 3.4 Top Left: Culture containers after filling with feeds and young larvae. Top Right: Culture containers in environmental chamber with extra humidification. Bottom: Culture containers after three days showing larvae activity.

3.2.2.3. Harvest

After 9 days of culturing (18-day-old larvae), each container was weighed and small random scoops of residue ~1 g were taken and frozen at -20 ºC for later moisture determination, ensuring no larvae were in the samples. Residue was removed by soaking the larvae in tap water for several minutes before rinsing in a 1mm sieve. Cleaned larvae were hand counted, noting if any had become black prepupae, then allowed to purge their digestive tract contents by leaving them with no food on dry paper towels for 24 hours. Once purged, the larvae underwent a final rinsing with tap water, were pat dry with paper towels, weighed and placed

in a freezer at -20 ºC to kill and store them for later analysis. Images of the harvest phase are presented in Figure 3.5.

Figure 3.5 Top: Culture containers at harvest. Middle: Fresh larvae after harvest, purging and cleaning. Bottom Left: Larvae mixed with residue prior to harvest. Bottom Middle: Fresh larvae after harvest. Bottom Right: Dried larvae.

3.2.3. Performance Calculations

Performance of each treatment was calculated in terms of larval survival, dry waste reduction and bioconversion and dry waste conversion efficiencies, with formula sourced from (Gold et al., 2020). Dry matter contents used in these calculations were determined by mass difference from drying in a laboratory oven at 70 ºC for 48 hours.

3.2.3.1. Survival

Refers to the percent of input larvae that survive until the end of the experiment, calculated using equation 1.

$$Survival\ rate\ (\%) = \frac{n_f}{\mathrm{n}_0} * 100 \qquad 1$$

Where n_f refers to the number of larvae that survive to the end of the experiment and n_0 refers to the number of larvae initially input into the experiment.

3.2.3.2. Waste Reduction

Refers to the percent reduction in the initial dry substrate mass, calculated using equation 2. Low waste reduction values result in greater mass of frass and unconsumed substrate that may require further treatment. While none of the substrates used in these experiments are wastes, the term waste reduction is used for simplicity and ease of comparison with other works.

$$Waste\ reduction\ (\%) = \frac{S_0 - R_f}{S_0} * 100 \qquad 2$$

Where S_0 refers to the total input dry substrate (g) and R_f refers to the total dry residue at the end of the experiment (g).

3.2.3.3. Bioconversion

Refers to the gain in larval dry mass over the mass of initial substrate fed (also referred to as yield), calculated using equation 3. High bioconversion result in more larval biomass and marketable product per unit of input waste, either in terms of lipids, protein, ash and/or chitin.

$$Bioconversion\ (\%) = \frac{\left(L_f * n_f - L_0 * n_0\right)}{S_0} * 100 \qquad 3$$

Where L_f refers to the mean dry mass of an individual larva at the end of the experiment (g) and L_0 refers to the mean dry mass of an individual larva at the beginning of the experiment

(g). The term on the numerator is referred to in the literature as the total larvae gain (Gold et al., 2020).

3.2.3.4. Waste Conversion Efficiency

Refers to the efficiency of waste conversion into larval biomass, also termed efficiency of conversion of ingested food. It provides an indication of how much consumed substrate was accumulated as larval biomass rather than lost in the generation of energy and CO_2, calculated using equation 4.

$$Waste\ conversion\ efficiency\ (\%) = \frac{\left(L_f * n_f - L_0 * n_0\right)}{S_0 - R_f} * 100 \qquad 4$$

3.2.4. Lipid Contents and Fatty Acid Profiles

Larvae from replicate runs were of similar masses and it was therefore assumed that variations between their lipid contents and fatty acid profiles would be negligible. Larvae from replicate runs were therefore combined and mixed before further analysis.

3.2.4.1. Crude Lipid Determination

Crude lipid was determined in triplicate following the Folch method (Folch et al., 1957) with minor modifications. Briefly, samples of dry larvae were homogenised in an IKA A 11 basic analytical mill before mixing with mortar and pestle. 6 mL extraction solution of DCM (AJAX chemicals) and methanol (Sigma Aldrich) (2:1) was added to ~300 mg homogenised larvae sample in a 20 mL glass vial, vortex mixed for 30 seconds at high speed and centrifuged at 2000 RPM for 4 minutes. 4 mL liquid was aspirated into a 15 mL polypropylene centrifuge tube, ensuring no transfer of solids. This liquid was washed with 0.2 volume of 0.9 mass% NaCl solution (Chem Supply), vortex mixed for 10 seconds at high speed and centrifuged at 2000 rpm for 4 minutes. 2 mL of the lower DCM phase containing the lipids was aspirated using a pre-wetted micropipette tip, ensuring not to disturb the aqueous layer or interface. This was left to evaporate in a fume hood for 24 hours leaving a light-yellow oil. Crude lipid % was

then calculated from the mass of this recovered oil scaled up by ~2 (calculated exactly for each sample) to account for incomplete transfer of lipid-containing intermediates and divided by the mass of dry larvae used for extraction. One of the three extracts was used as the source of oil for fatty acid profiling. The process was repeated for each unique run.

3.2.4.2. Fatty Acid Analysis

Fatty acid profiles were determined via GC-MS following esterification of the extracted lipids. Samples of oils from Folch extraction were esterified to fatty acid methyl esters following a modified method from Sukhija and Palmquist (1988) that uses acid catalysed methanolic hydrogen chloride. Briefly, 3 mg of extract was weighed into a 20 mL glass vial and 150 ul of methyl heptadecanoate solution (Sigma Aldrich) in dry toluene (AJAX chemicals) (2 mg/mL) was added as the internal standard. 2,850 ul of dry toluene was added and vortex mixed until all lipid was dissolved. Methanolic HCl was prepared by slowly dripping 1-part acetyl chloride (Sigma Aldrich) into 10-parts cool, dry methanol, cooled in an ice bath behind a safety shield. 180 ul of freshly prepared methanolic HCl was added to the lipid solution slowly and allowed to sit for 1 minute before tightly capping and vortex mixing at slow speed for 10 seconds. The vial was then vented, tightly capped, and placed in a hot water bath at 70 ºC for 1 hour. After cooling to room temperature, 1 mL of 6 mass% K_2CO_3 solution (Sigma Aldrich) was added and vortex mixed at high speed for 5 seconds before centrifuging at 2000 RPM for 1 minute. 1 mL of the top toluene layer containing the fatty acid methyl esters (FAMEs) was then aspirated into a 2 mL GC-MS vial, ready for analysis. The process was repeated for each extracted crude lipid sample.

GC-MS was used to analyse several dilutions of the Supelco FAME 37 mix from Sigma Aldrich to generate retention factors of each fatty acid methyl ester and confirm peak identities. The prepared FAME solutions from each unique run were then analysed with a Perkin Elmer Clarus 680 GC 30 m column (Perkin Elmer Elite 5 ms - 0.25 mm ID x 0.25 um film thickness)

with SQ 8 C positive ion EI (70 eV) quadrupole mass spectrometer. Helium at a flowrate of 1.3 mL/min was used as the carrier gas. 0.5 uL sample was injected at a 50:1 split into the port preheated to 250 ºC. After 30 seconds for oven equilibration at 90 ºC, the GC oven program was held at 90 ºC for 2 mins, ramped up 10 ºC/min to 190 ºC, held for 1 minute, ramped up 3 ºC/min to 250 ºC then held for 5 minutes (total GC time 38 minutes). The GC-MS interface and MS source temperatures were 250 and 150 ºC respectively. MS scan settings included a solvent delay from 0-3 minutes before scanning from 45-550 m/z over 200 ms with 100 ms interscan delay.

TurboMass software Version 6.0.0.1811 was used for peak detection and integration for each chromatogram. Peaks that were not associated with a fatty acid methyl ester were removed manually by visual inspection of the chromatogram before quantification. Relative retention factors for each fatty acid methyl ester to the internal standard were determined from the FAME 37 mix calibration curves and concentrations of each FAME/100 g calculated using equations 5 and 6:

$$X_{RRF} = \frac{X_{RF}}{IS_{RRF}} = \frac{\frac{Xarea}{Xconcentration}}{\frac{ISarea}{ISconcentration}} \quad (5)$$

$$Xconcentration = Xarea * \left(\frac{ISconcentration}{ISarea}\right) * \left(\frac{1}{X_{RRF}}\right) \quad (6)$$

Where RF is the retention factor, RRF is the relative retention factor, X is the FAME of interest and IS is the internal standard.

3.2.5. Data Analysis

Data was analysed using the software package GraphPad Prism version 9.0.2 (161) and Microsoft Excel version 2008. Equal variances and normality of residuals were assumed prior to analysis. The assumption of equal variances was checked using the Brown-Forsythe test while the normality of residuals was checked using the Shapiro-Wilk test with significance

determined at $P < 0.05$. Significant differences between the means of treatment groups and that of the benchmark was determined using ordinary one-way ANOVA with post-hoc Dunnett test to correct for multiple comparisons. The post-hoc Dunnett test was selected since the aim was to compare the treatments with that of the benchmark (many-to-one comparison), rather than compare between all treatments (many-to-many comparison). Independent variables were the treatment additives and dependent variables were each of the performance measures and crude lipid contents. All error bars represent standard deviations where the sample size was three in all cases except fatty acid analysis where sample size was two (Figure 3.2).

3.3. Results and Discussion

3.3.1. Larval Performance

The absolute performance measures of each treatment along with the benchmark are presented in Figure 3.6 and in the appendices Chapter 3. The benchmark larvae performed similarly to previous studies in terms of survival and dry waste reduction, with typically higher bioconversion and waste conversion efficiencies (Table 3.3). The D (-) arabinose treatment drastically lowered performance across each category excluding survival and results from this treatment have hereon been excluded from presentation to facilitate comparison among the other treatments.

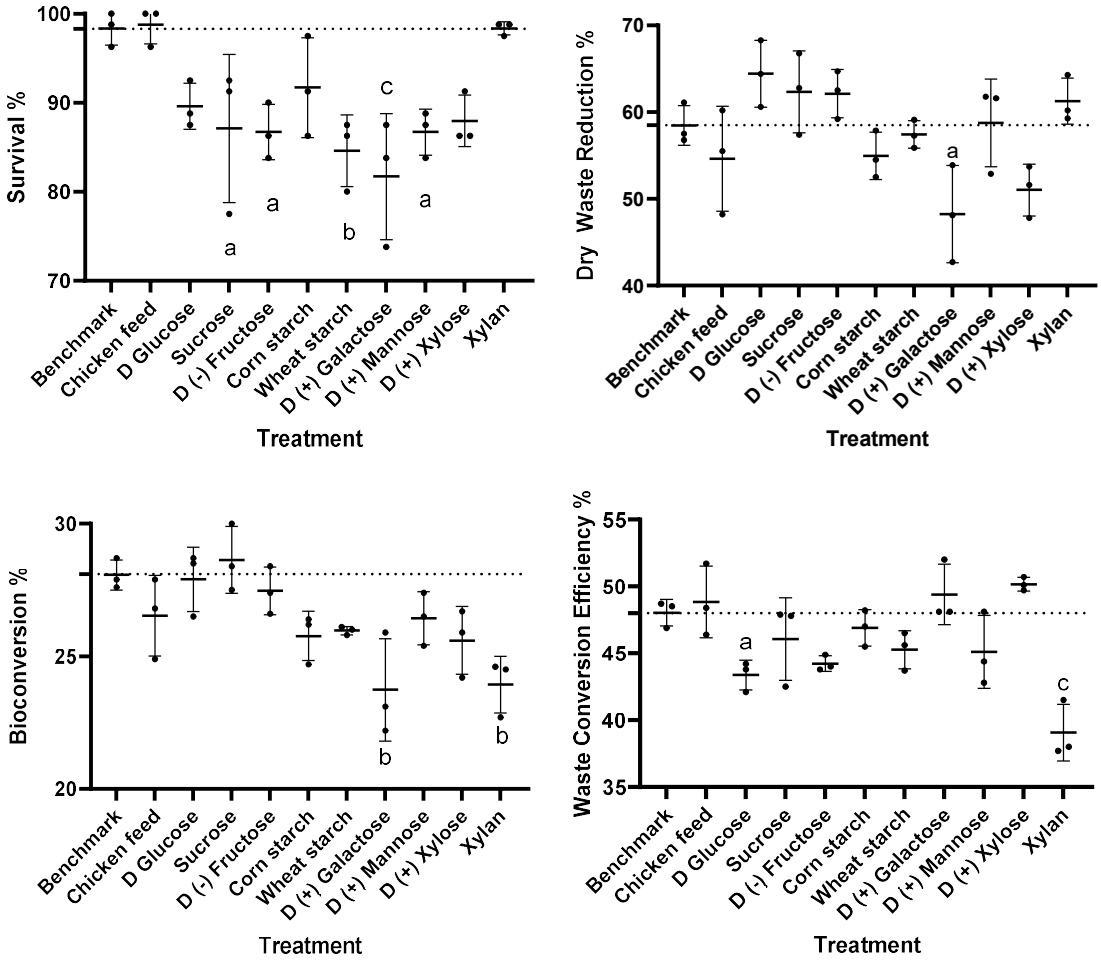

Figure 3.6 Comparison of larvae survival (top left), dry waste reduction (top right), bioconversion (bottom left) and waste conversion efficiency (bottom right) from treatments compared to the chicken feed benchmark. Error bars indicate standard deviations. Letters indicate significant differences to the benchmark (a: adjusted $P < 0.05$; b: adjusted $P < 0.01$; c: adjusted $P < 0.001$).

Table 3.3 Comparison of performance results from the benchmark of this study with those from literature.

Treatment	Survival %	Dry Waste Reduction %	Dry Bioconversion %	Dry Waste Conversion Efficiency %	Source
Chicken feed for 9 days - 170 mg DM/larva one time feeding - 60% feed moisture - 2.83 larvae/cm2	98.3 ± 1.9	58.5 ± 2.3	28.1 ± 0.5	48.0 ± 1.0	This study
Gainesville, chicken feed and CSMA diets, 22-24 days - approx. 650 mg DM/larva staggered feeding - 70% moisture - 300 larvae/L	96.0-97.8	65.3-64.71	6.5*	18*	(Tomberlin, 2003)
Poultry feed for 9 days - 240 mg DM/larva staggered feeding - 60% feed moisture - 2 larvae/cm2	97.9 ± 2.1	67.7 ± 6.9	21.0 ± 2.4	32*	(Gold et al., 2020)
Poultry feed 16 days to 50% prepupa - 40 mg DM/larva every second or third day - 60% feed moisture - 0.6 larvae/cm2	93.0 ± 2.9	84.8 ± 3.6	12.8 ± 0.7*	-	(Lalander et al., 2019)
Chicken feed for 12 days to first prepupa - 930 mg DM/larva staggered feeding - 74% feed moisture - 1000 larvae/5.5 L	-	-	9.1*	-	(Spranghers et al., 2017)
Vegetable waste diet for 22 days until 50% prepupae - 1000 mg DM/larva one time feeding - 60% moisture - 100 larvae/750 mL	93.7 ± 6.2	-	3.65 ± 0.46*	-	(Barragán-Fonseca, 2018)
Varied conditions	-	26.2-68.0	-	-	(Diener et al., 2011)
Varied conditions	-	54.6**	22.9**	-	(Banks et al., 2014)

* - approximated by authors of this study. ** - on wet mass basis

Survival was high across all runs ranging from 74 – 100%. This is similar to reported values in literature (Table 3.3). The benchmark and chicken feed yielded near identical mean survival at 98.3 and 98.8% as expected, while all containers with additives had lower means except for those treated with xylan showing a mean of 98.3%. Sucrose, fructose, and mannose treatments had significantly lower survival than the benchmark, averaging -11.7 ± 4.8% lower (adjusted $P < 0.05$). Wheat starch, galactose and arabinose decreased further on average by -14.0 ± 4.4%, -17.0 ± 7.2% and -17.3 ± 2.5% from the benchmark (Figure 3.6). Since these treatments contained the same nutrients as the benchmark with the additional carbohydrate added, it appears that these carbohydrates actively reduced survival. This is unexpected particularly for the simple hexose sugars and starches such as fructose and wheat starch respectively which are typically considered beneficial sources of energy supporting insect survival (Rockstein, 1978). These results also differ from the study by (Gold et al., 2020) that used substantially varied waste substrates yet showed little variation in survival. It is possible that other components present in the native wastes alleviate the toxic effects observed here (Hodges and Minich, 2015). Alternatively, the added carbohydrates may have been more available for digestion by competitive or otherwise inhibiting micro-organisms, resulting in the reduced survival (Flint et al., 2007). This could also help explain why the hemicellulose xylan allowed for such high survival over the simpler sugars and starches, since it is typically less easily digestible by micro-organisms. Further investigation into the sugar concentrations of the substrate during BSFL treatment would be of value in supporting this hypothesis.

The largest variations between carbohydrate treatments were observable in the dry waste reduction measure. However, large variations between replicates resulted in only the galactose and arabinose additives showing significant differences to the benchmark (Figure 3.6), with -17.7 ± 9.5% (adjusted $P < 0.05$) and -50.7 ± 1.2% (adjusted $P < 0.001$) of the benchmark respectively.

D (-) arabinose greatly impeded the larvae and micro-organisms capacity to reduce the mass of initial substrate material, likely due to them not containing the enzymes capable of metabolising the rare D enantiomer. This is speculative, requiring confirmation with studies using substrates containing the L enantiomer. For comparison, the treatment with added sunflower oil also reduced dry waste reduction relative to the benchmark at a similar level of galactose (-16.3 ± 3.5% adjusted $P < 0.01$). This differs from Gold et al., 2020 who noted that lipid content of feed correlated positively with waste reduction (Gold et al., 2020). They also found that fibre content correlated negatively with waste reduction which agrees with the hemicellulose components xylose, arabinose and galactose reducing waste reduction in this study, yet contrasts with the results from the xylan treatment.

Comparing between carbohydrate treatments themselves (Figure 3.6), the common sugars of glucose, fructose and sucrose yielded the greatest dry waste reduction measures, as expected given their ease of metabolism by both insects and microbes. Starch additives showed minimal variation from the benchmark, similarly for mannose and xylan. Interestingly, the xylan additive had higher waste reduction than its dominant constituent xylose, possibly due to the presence of more easily digestible monomers within the polysaccharide structure such as glucuronic acid side chains or L-arabinose (Bastawde, 1992). Alternatively, its presence may have provided a source of xylo-oligosaccharides, acting as a prebiotic supporting symbiotic microbial digestion of the chicken feed (Baker et al., 2021; Carvalho et al., 2013). The impacts of hemicelluloses including its prebiotic and anti-nutritional potentials in BSFL treatment require further exploration.

Few treatments showed statistically significant variation in bioconversion performance (Equation 3) with most performing similar to the benchmark of 28.1 ± 0.5% (Figure 3.6). Galactose, xylan and arabinose treatments yielded significant variations relative to the benchmark, with large reductions of -14.7 ± 6.8%, -14.7 ± 3.8% (adjusted $P < 0.01$) and -63.0

± 2.6% (adjusted $P < 0.001$) respectively. These results align with findings of Gold et al., (2020) who reported fibre content negatively correlated with bioconversion (Gold et al., 2020). These reductions are likely in part due to the larvae's inability to produce the necessary enzymes for metabolism, and therefore may be alleviated with pre-treatment such as inoculation with appropriate companion bacteria (De Smet et al., 2018).

Bioconversion is the ratio of larval gain against the amount of substrate added and it is therefore important to note that the treatments had larger amounts of input substrate relative to the benchmark. Variation in larval gain is best assessed by comparing treatments with the chicken feed treatment (Figure 3.6), to which only the arabinose treatment was statistically different.

Average individual mass of larva at harvest from the pure chicken feed treatment (57.67 ± 3.52 mg) was on average higher than that of the benchmark (49.12 ± 0.76 mg), as was the larval gain, indicating that the extra substrate did result in more larval biomass. However, the bioconversion performance of the benchmark was on average higher and therefore a more efficient feeding rate for producing larval biomass in terms of substrate required.

Except for galactose and xylose, all carbohydrate additives reduced waste conversion efficiency relative to the benchmark (Figure 3.6). Glucose, xylan and arabinose treatments were significantly lower than the benchmark by -9.7 ± 2.1% (adjusted $P < 0.05$), -19.0 ± 4.4% and -25.3 ± 5.5% (adjusted $P < 0.001$) respectively, with these carbohydrates therefore upregulating the conversion of substrate into gaseous emissions (Figure 3.1). The experimental methods used in this study do not enable identifying the mechanisms behind these effects, requiring further investigations into the dominant metabolic pathways involved.

For comparison, the sunflower oil additive greatly improved waste conversion efficiency by 19.3 ± 4.0% relative to the benchmark, indicating it was bioaccumulated into the larval biomass rather than metabolised for energy generation, in line with previous studies on

lipid bioaccumulation from the diet in BSFL (Barroso et al., 2017; Gold et al., 2020; Liland et al., 2017; St-Hilaire et al., 2007).

Waste conversion efficiency is a measure of the fraction of reduced substrate that ends up as larvae, providing an indication of the mass loss via gaseous emissions from microbial and larval metabolism (Figure 8 and Figure 3.1). A recent study by (Parodi et al., 2020) quantified the carbon and nitrogen emissions from a BSFL treatment unit. They identified that most emissions were from loss of substrate carbon via production of CO_2, over a third of which was from microbial activity ((Bekker et al., 2021) found that microbial CO_2 emissions may even exceed those from the larvae in some conditions). High waste reduction with low waste conversion efficiencies, such as seen from the xylan treatment, therefore reflect large CO_2 emissions. This may be important to consider for BSFL treatment operators concerned with carbon losses through gaseous emissions.

3.3.2. Larval Composition

3.3.2.1. Lipid Contents

Larval lipid contents from the benchmark were 41.10 ± 0.02%, within the range of previous studies (Danieli et al., 2019; Ewald et al., 2020a; Wong et al., 2020). Crude lipid contents varied significantly in few treatments (Figure 3.7). Addition of wheat starch significantly increased larval lipid contents relative to the benchmark by 12.57 ± 2.98% (adjusted $P < 0.001$), whereas galactose and xylan reduced larval lipid content by -15.01 ± 1.37% and -27.49 ± 3.40% (adjusted $P < 0.001$) respectively (the arabinose treatment was excluded from crude lipid analysis given its substantially low performance relative to all other treatments).

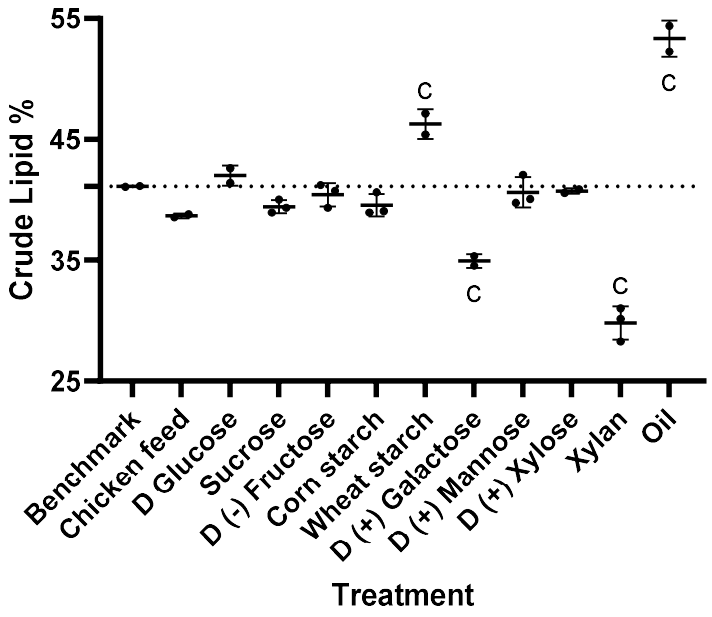

Figure 3.7 Comparison of larvae crude lipid contents from treatments compared to the chicken feed benchmark. Error bars indicate standard deviations. Letters indicate significant differences to the benchmark (a: adjusted $P < 0.05$; b: adjusted $P < 0.01$; c: adjusted $P < 0.001$).

Despite knowledge of the conversion of excess carbohydrates into lipids (Li et al., 2015), the free sugars glucose, sucrose and fructose had no significant influence on larval lipid contents. This may be due to the rapid consumption of these sugars by microorganisms in the substrate, which may have greatly reduced these sugar concentrations before the young larvae had developed enough to effectively compete. This hypothesis can be investigated by adding the sugars to the substrates after the larvae have developed for several days and by monitoring the sugar concentrations in the feed and larval growth over time.

The effect of wheat starch increasing the larval lipid contents was unexpected, particularly considering there were no similar responses from the corn starch treatments. This result should be taken with a grain of salt considering the low statistical power resulting from analysis of the combined wheat starch larvae replicates, rather than from each replicate individually. There are few clear differences between typical intrinsic properties of wheat and corn starches, with close similarities in amylose content, granule sizes, crystalline content and side chain lengths of amylopectin (Horstmann et al., 2017; Martens et al., 2018). Such

properties have been identified to influence digestion rates of starches in mammals (Martens et al., 2018), however they appear unable to explain the difference in larval lipid contents observed between the two starch treatments. A possible explanation may be that the mortalities in the wheat starch treatments were consumed by the remaining larvae (Logan et al., 2021), bioaccumulating their lipid contents, yet similar increases in lipid contents were not observed in the other treatments for which survival was low. Further investigation including a more detailed analysis of the starch properties and larger sample sizes will be needed to conclusively understand the influence of wheat starch on larval lipid contents.

The anti-nutritional effects observed from the galactose treatment likely indicates limited functionality of the Leloir pathway responsible for metabolising galactose into glucose-1-phosphate able to enter glycolysis (Daenzer and Fridovich-Keil, 2017). Therefore, a significant effect of the addition of galactose may have simply been the dilution of more digestible nutrients in the substrate, including amino acids and carbohydrates, requiring the larvae to consume more substrate to meet their nutritional needs. The resultant slowing of larvae development may explain these larvae's reduced lipid contents, with a previous study showing less developed larvae typically have lower lipid contents (Liu et al., 2017).

A similar explanation may account for the reduction in crude lipid content from the xylan treatment, however unlike galactose, xylan simultaneously reduced waste conversion efficiency without influencing dry waste reduction, indicating another mechanism rather than simply delayed development. A possible complementary explanation may be that xylan supported the development of xylanase producing microbes which competed with the larvae for the remaining carbohydrates in the substrate, reducing their availability for fatty acid synthesis (Gold et al., 2018).

For comparison, the sunflower oil treatment greatly increased lipid content by 29.77 ± 3.67% (adjusted $P < 0.001$) more than the benchmark, likely a result of bioaccumulating the

oil rather than an increase in fatty acid synthesis as can be inferred from the fatty acid profile (Table 3.4) (Danieli et al., 2019). All other treatments were not statistically significant from the benchmark, contrary to the finding by Li et al. (2015) that the presence of xylose in the feed increased larval lipid content relative to a control. A comparison of the crude lipid contents across treatments is provided in Figure 3.7.

3.3.2.2. Fatty Acid Profiles

Across carbohydrates treatments, there were minimal variations in fatty acid (FA) profiles of the larvae at the end of culturing (Table 3.4). The esterification of one of the wheat starch lipid samples failed due to a faulty vial cap and due to time constraints was therefore excluded from the fatty acid analysis. Young larvae (without undergoing the experiment) showed a fatty acid profile very dissimilar to those of the matured larvae from the experiment, notably containing a much higher content of unsaturated fatty acids including omega 6 linoleic acid. In the mature larvae, lauric acid dominated in all treatments as anticipated from literature findings, present at concentrations similar to or higher than previous studies (Barroso et al., 2017; Hoc et al., 2020; Meneguz et al., 2018; Spranghers et al., 2017). Lauric acid content of larvae from each carbohydrate treatment was nearly identical to the benchmark of 69.00 ± 1.29% of the total FAMEs (Table 3.4). It therefore appears that of the targeted carbohydrates, the inclusion of 20 dry mass% does not significantly alter the fatty acid profile of mature BSFL in the culture conditions tested. Conversely, the addition of sunflower oil greatly changed the FA profile with C18 omega 6 linoleic fatty acids becoming the dominant species and a reduction in C10 (capric), 12 (lauric) and 14 (myristic) FAs. Since sunflower oil is predominantly C18 omega 6 linoleic acids, this further supports the hypothesis that the larvae bioaccumulated the oil rather than metabolise it for energy. Similar findings have been made with unsaturated fatty acids bioaccumulating from the larvae diet (Barroso et al., 2017; Liland et al., 2017; St-Hilaire et al., 2007). This may be important in facilitating quality assurance

given the dependence of suitable downstream applications of the lipids on their fatty acid profile. Larvae fed the wheat starch diet were not included in the fatty acid analysis due to failure of the vial lid used in its esterification.

Table 3.4 Fatty acid profiles presenting detected fatty acid methyl esters as mass% of the total fatty acid methyl esters ± standard deviations.

	Fatty acid abbreviation*							
Treatment/additive	10:0	12:0	14:0	16:0	16:1n-7	18:0	18:1n-9 cis	18:2n-6
Chicken feed 80% (Benchmark)	2.18 ± 0.01	69.67 ± 1.30	4.19 ± 0.01	8.58 ± 0.04	2.44 ± 0.00	1.99 ± 0.00	4.69 ± 0.06	6.27 ± 0.03
Pure Chicken Feed	1.39 ± 0.01	69.00 ± 1.29	4.69 ± 0.01	9.21 ± 0.04	1.18 ± 0.00	1.23 ± 0.00	5.34 ± 0.07	7.97 ± 0.04
Sunflower Oil	-	35.82 ± 0.67	2.28 ± 0.00	8.45 ± 0.04	1.50 ± 0.00	2.32 ± 0.00	9.33 ± 0.12	39.59 ± 0.18
Young larvae	-	32.52 ± 0.61	4.07 ± 0.01	18.21 ± 0.08	2.35 ± 0.00	5.78 ± 0.01	11.47 ± 0.15	25.60 ± 0.12
D-Glucose	2.63 ± 0.01	70.17 ± 1.31	4.87 ± 0.01	8.62 ± 0.04	2.35 ± 0.00	1.77 ± 0.00	4.08 ± 0.05	5.52 ± 0.03
Sucrose	2.56 ± 0.01	71.66 ± 1.34	4.62 ± 0.01	7.98 ± 0.04	1.89 ± 0.00	1.94 ± 0.00	4.06 ± 0.05	5.29 ± 0.02
D (-) Fructose	2.26 ± 0.01	69.80 ± 1.31	5.29 ± 0.01	7.56 ± 0.03	1.83 ± 0.00	1.45 ± 0.00	4.80 ± 0.06	7.00 ± 0.03
Corn starch	1.39 ± 0.01	69.36 ± 1.30	5.31 ± 0.01	9.62 ± 0.04	2.94 ± 0.00	1.59 ± 0.00	4.76 ± 0.06	5.04 ± 0.02
D (-) Arabinose	3.60 ± 0.02	60.60 ± 1.13	4.66 ± 0.01	9.06 ± 0.04	2.39 ± 0.00	1.84 ± 0.00	8.34 ± 0.11	9.51 ± 0.04
D (+) Galactose	2.18 ± 0.01	68.49 ± 1.28	4.29 ± 0.01	9.33 ± 0.04	1.16 ± 0.00	2.11 ± 0.00	5.46 ± 0.07	6.97 ± 0.03
D (+) Mannose	3.00 ± 0.01	71.98 ± 1.35	4.83 ± 0.01	7.56 ± 0.03	1.59 ± 0.00	1.12 ± 0.00	4.37 ± 0.06	5.55 ± 0.03
D (+) Xylose	2.67 ± 0.01	70.47 ± 1.32	5.25 ± 0.01	7.72 ± 0.03	1.33 ± 0.00	1.64 ± 0.00	4.80 ± 0.06	6.13 ± 0.03

*Abbreviations and common names: C10 capric, C12 lauric, C14 myristic, C16 palmitic, C16:1n-7 palmitoleic, C18 stearic, C18:1n-9 cis oleic, C18:2n-6 linoleic.

3.3.3. Implications for Commercial BSFL Production

The treatments in this study – each containing 20% dry mass of the carbohydrate of interest in an otherwise nutritious substrate – approximate 'worst case' scenarios where wastes high in the carbohydrate of interest are used as a key input to BSFL treatment. For example, substrates overloaded with grape marc or coffee grounds may contain equivalently high xylan contents as those found in the xylan treatment of this study, similarly with bakery products resulting in high starch contents and fruit and vegetable wastes resulting in high sugar contents (Table 3.1). This has been chosen to elicit the maximum likely effect of each carbohydrate of interest and therefore provide valuable insights for improved BSFL substrate formulation from wastes. Such insights are required to facilitate more reliable process performance and ultimately facilitate BSFL treatment as a substantive tool of the circular economy (Lalander et al., 2020). It should be noted however that, the use of pure, chemically defined carbohydrates in this study represents a simplified model that is unlikely to capture the full complexity of real-world waste materials used in commercial BSFL production. Additionally, the potential synergistic effects between different types of carbohydrates and other constituents within real-world waste materials may significantly impact BSFL production and warrant further exploration. Hence, further investigation using real-world waste materials is required to validate and extend the findings of this study to practical, commercial settings.

Due to the performance reducing effects of their carbohydrate contents, wastes that potentially should not be included at high concentrations in BSFL substrates without appropriate pre-treatments include milk and dairy products high in galactose (Daenzer and Fridovich-Keil, 2017), along with those containing high amounts of xylans and their constituents, such as coffee grounds, grape marc, grain mill and malt processing residues (such as cereal brans and culms), some food processing residues, and manures (Table 3.1). Contrarily, wastes containing significant amounts of the simple sugars glucose, sucrose, and

fructose, as well as starches, can likely be used at higher proportions, however pilot testing with real-world waste materials at different inclusion rates is crucial to assess the practical implications on BSFL production and to ensure that larval survival rates are not adversely affected. Exemplar wastes include side streams from sugar milling and refining, fruit and vegetable wastes (such as from markets and pomaces from beverage manufacture), confectionery and food processing residues (such as from high sugar breakfast cereals, pastas and corn chips), and bakery products (such as waste breads and pastries) (Table 3.1).

With the exceptions of products high in wheat starch (such as pastas and breads), the results of this study do not provide evidence that particular wastes could be selected to increase larval lipid contents or alter their fatty acid profiles due to their contained carbohydrate types. Instead, if the production of BSFL lipid for downstream uses (such as biodiesel) is the objective, it would be beneficial to include lipid containing wastes directly (such as used cooking oils) (Zheng et al., 2012). Alternatively, wastes containing galactose and xylans – as mentioned above – may aid in producing BSFL lower in lipid contents and therefore more suitable in many direct-to-animal feed applications (Schiavone et al., 2017), however this likely would come with the cost of reduced larval survival, waste reduction and bioconversion performance if included in too high amounts. Pilot studies should be performed to identify optimal inclusion rates of such wastes, compromising performance loss with larval lipid reduction.

3.4. Conclusions

This work presents the first investigation on the importance of specific dietary carbohydrates on black soldier fly larvae in terms of treatment performance and larvae quality to aid in formulating appropriate feed blends. The hypothesis was proven partially correct, that carbohydrate types can be significant influences of BSFL treatment performance and lipid contents, yet no evidence was found to indicate they have a substantial influence on fatty acid

profiles. Such an investigation had not been performed before and builds on the body of knowledge underpinning BSFL treatment, improving its potential as a tool in the circular economy.

Performance relative to the chicken feed benchmark was largely resilient to the added carbohydrates at a significance of $P < 0.05$, with the main exception of hemicellulose constituents galactose and arabinose which significantly reduced survival, bioconversion, waste reduction, and in the case of arabinose, waste conversion efficiency. The hemicellulose xylan was significantly detrimental to bioconversion and waste conversion efficiencies The simple sugars sucrose, fructose and mannose, as well as wheat starch, each reduced survival. Crude lipid content was significantly increased in the wheat starch and sunflower oil treatments while galactose and xylan treatments reduced crude lipid. Fatty acid profiles appeared completely independent of the carbohydrate treatment, while the addition of sunflower oil greatly increased lipid content and altered the fatty acid profile to contain more C18 omega 6 and oleic fatty acids at the expense of lauric acid. The consequences of these results for BSFL treatment of real wastes are discussed. Future research should investigate whether similar observations are made with wastes of equivalent carbohydrate profiles and explore methods of mitigating the anti-nutritional effects of hemicellulose-sourced carbohydrates.

Chapter 4. Understanding Dietary Fibres in BSFL Substrates

4.1. Introduction

From the treatments selected in Chapter 3, BSFL process performance and composition were most sensitive to the presence of hemicellulose constituents, as well as the hemicellulose xylan, a form of dietary fibre. These findings, coupled with those in Chapter 2 of the various mechanisms by which fibre influences animal nutrition, prompt further investigation into the influence of dietary fibres in BSFL substrates. Despite the significant presence of fibre in organic wastes used in substrates, such as brewers' grains, fruits and vegetable wastes, and agri-residues (Table 4.1), fibre is a significantly understudied component in BSFL substrates.

Table 4.1 Fibre contents of various organic wastes used in BSFL substrates.

Waste	SDF (%)	NDF (%)	ADF (%)	Crude fibre (%)	Lignin (%)	Data sources
Apple pomace	14.6 [1]	65.1 ± 7.4 [2]	57.7 ± 8.0 [2]	36.0 ± 6.8 [2]	26.8 ± 3.3 [2]	[1]Sudha et al. (2007) [2]Heuzé V. et al. (2020)
Banana peel	13.0 – 21.7 [1]	22.9 [2]	14.5 [2]	9.9 [3]	7.0 [2]	[1]Phatcharaporn et al. (2010) [2]Happi Emaga et al. (2008) [3]Heuzé V. et al. (2016a)
Citrus pulp	20.0 – 30.6 [1]	21.6 ± 2.1 [2]	15.9 ± 1.9 [2]	14 ± 0.9 [2]	2.5 ± 1.6 [2]	[1]Pacheco et al. (2021) [2]Heuzé V. et al. (2018)
Soybean curd residue	2.0 [1]	33.6 [2]	22.3 [2]	16 [2]	0.8 [3]	[1]Li et al. (2019) [2]Yoshihara and Yokoyama (2021) [3]Rehman et al. (2017a)
Grape pomace	9.5 [1]	60.8 ± 8.2 [2]	52.6 ± 7.9 [2]	24.7 ± 3.6 [2]	33.7 ± 6.4 [2]	[1]Valiente et al. (1995) [2]Heuzé V. and Tran G. (2017)
Rice straw	-	69.1 ± 4.2	42.4 ± 3.5	35.1 ± 3.1	4.8 ± 0.8	Heuzé V. and Tran G. (2015)
Poultry manure	-	43.8	25.9 ± 13.5	18.5 ± 10.2	7.9	Heuzé (2012)
Cattle manure	-	64.1	43.9	22.4	14.5	Wilkinson (1978)
Maize cobs	2.8 [1]	87.8 ± 3.9 [2]	46.3 ± 3.8 [2]	34.9 ± 3.6 [2]	6.3 ± 1.3 [2]	[1]Njideka et al. (2020) [2]Heuzé V. et al. (2016b)
Human faeces	-	27.9	19.5	-	-	Gold et al. (2020)
Cottonseed hulls	~0 [1]	85.3 ± 5.8 [2]	63.0 ± 4.9 [2]	53.0 ± 7.5 [2]	19.1 ± 3.2 [2]	[1]Hsu et al. (1987) [2]Heuzé V. et al. (2015)
Spent brewer's grains	3.9 – 9.6 [1]	56.3 ± 5.7 [2]	21.9 ± 3.1 [2]	15.8 ± 2.1 [2]	5.4 ± 1.9 [2]	[1]Naibaho et al. (2021) [2]Heuzé V. et al. (2017)
Canteen waste	-	36.2	22.8	-	-	Gold et al. (2020)
Wheat milling by-products	~1 – 3.5 [1]	51.7	22.1	-	-	[1]Ranhotra et al. (1990) [2]Gold et al. (2020)
Soybean hulls	13.8	67.0	49.3	-	2.3	Dust et al. (2004)

Footnote: SDF refers to soluble dietary fibre, NDF to neutral detergent fibre, ADF to acid detergent fibre. All values in terms of dry mass. Errors are standard deviations where available data sources are greater than two. Values with a ~ in front refer to approximations. Cells with a dash (-) indicates unavailable data. Superscript numbers within rows correspond to data sources in the last column.

Quantitative bioconversion studies often report crude fibre, measuring only a fraction of the most recalcitrant components of cellulose and lignin (Figure 4.1), and typically considered an anti-nutritional factor (Myrie et al., 2008). However, other fibre components, such as hemicellulose and soluble dietary fibres, have beneficial effects in mammalian nutrition (Dhingra et al., 2012; Smith, 2021). Variations in fibre contents and sources may therefore contribute to the irregular process performance and larval composition found in BSFL production studies (Gold et al., 2020; Lalander et al., 2019). Additionally, fibre contents may be a useful indicator of substrate digestibility, as is the case in other farmed animals (Fahey et al., 2018).

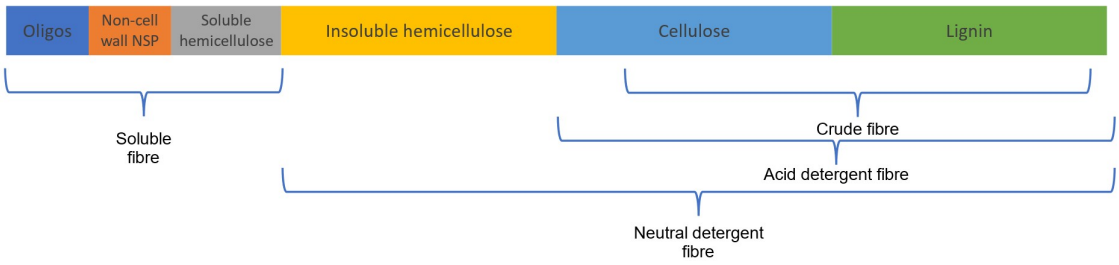

Figure 4.1. Dominant components of total dietary fibre (TDF) in typical ratios and commonly reported measures, developed from Montagne et al. (2003) and Smith (2021). Oligos refer to oligosaccharides including fructo-, galacto- and manno-oligosaccharides. NSP refers to non-starch polysaccharides including pectins, fructans and gums. Hemicelluloses include xylans, glucans, mannans and arabinans.

4.1.1. Influence of Dietary Fibre on BSFL Performance and Composition

Of the few studies that explore fibre in BSFL production, Rehman et al. (2017a) demonstrated the potential for all insoluble fibre components to be digested at varied levels, with observed fibre reductions in mixtures of chicken and cow manures ranging between 49 and 67% for cellulose, 49 and 59% for hemicellulose and 30 and 42% for lignin. In mixtures of restaurant waste and rice straw, Zheng et al. (2012) reported BSFL treatment reduced 27.9% of cellulose, 32.6% of hemicellulose and 0.8% of lignin. It is unclear to what extent these digestions equate to improvements in BSFL growth from the existing literature, as other factors are changed simultaneously in these studies. The literature considers these reductions to be largely a function of lignocellulolytic enzymes produced by microorganisms either external to the larvae or within the larvae gut, hydrolysing fibrous components into mono and oligosaccharides available for metabolism by the larvae or further by microorganisms (Gold et al., 2018b).

The present study fills an important gap in our understanding of fibre influence on BSFL performance and composition, as well as the digestibility of fibre types prevalent in organic wastes used as substrates for BSFL production. Given the various mechanisms involving dietary fibre in BSFL production, it is hypothesised that BSFL performance and composition cannot be reasonably anticipated from a simple singular measure of fibre contents, such as crude fibre. An experiment is performed using five chicken feed-based substrates

containing varied fibre additives. Responses are measured in terms of larvae growth, substrate reduction, feed conversion ratio (FCR), fibre reduction and larvae composition.

4.2. Materials and Methods

4.2.1. Experiment Design

A growth trial was designed to simulate co-digestion of a balanced larval substrate (chicken feed, Organic Backyard Layer Mash, Enfield Produce Pet and Garden Supplies, 16% protein, 5% lipid, 7% fibre) with an added fibrous material (Table 4.2). Three fibre types were chosen as additives, namely apple pectin (Morlife AUS), washed apple fibre (NOW supplements) and pure cellulose (Sigma Aldrich), reflecting sources of soluble fibre, mixed fibre and insoluble fibre respectively. Washed apple fibre – as may be sourced from juice pulping waste (Koutsos et al., 2015) – contains a complex of insoluble hemicellulose, cellulose and lignin, and soluble pectin. Pure cellulose was selected due to its use as a filler material in BSFL production studies (Barragan-Fonseca et al., 2021), despite not typically existing in food wastes without lignin and hemicellulose. Each treatment was performed in triplicate (i.e. three containers per treatment).

Table 4.2 Compositions of treatment substrates. Each treatment was performed in triplicate.

Treatment	Abbreviation	Dry mass (g)	Added components (g)				Moisture (%)	Dry mass composition (%)				Measured fibre content (%)			
		Total	Chicken feed	Washed apple fibre	Pectin	Cellulose		Chicken feed	Washed apple fibre	Pectin	Cellulose	SDF	IDF	SDF/IDF Ratio	TDF
Control	CTL	15.81	15.81	0.00	0.00	0.00	62	100	0	0	0	12%	16%	0.78	28%
High soluble fibre	SOL	18.96	15.81	0.00	3.15	0.00	65	83	0	17	0	26%	13%	1.99	39%
Very high soluble fibre	HSO	22.12	15.81	0.00	6.30	0.00	67	72	0	28	0	36%	11%	3.21	47%
High insoluble fibre	INS	22.05	15.81	6.23	0.00	0.00	65	72	28	0	0	16%	30%	0.54	47%
High cellulose	CEL	22.19	15.81	0.00	0.00	6.37	64	71	0	0	29	9%	38%	0.23	46%

Each substrate contained identical levels of chicken feed (200 mg dry matter/larva) to supply macro and micronutrients. Substrates were supplied in excess of the larvae's needs over the trial duration, as determined from preliminary trials (unpublished data). Differences in total dry mass contents were solely due to the fibre additives. Given that successful BSFL substrates formulated from real wastes are unlikely to have soluble fibre levels above 20% and insoluble fibre levels above 40%, the treatments were formulated to be near these 'maximum realistic' levels, except for the HSO substrate. The HSO substrate was included to provide a treatment with the same total dry mass available per larva as in the insoluble fibre treatments for easier comparison, despite such a level being unlikely to occur in real waste-based substrates.

There were slight differences in moisture contents due to the fibrous substrates requiring more water to become fully wet compared with the control. Past studies on moisture contents show this is not expected to have a significant impact on the results (Lalander et al., 2020; Palma et al., 2018).

4.2.2. Larvae Culturing and Harvest

Prior to commencing the experiment, eggs (purchased from Mobius Farms) were placed on fly screen mesh and positioned above a small quantity of ground chicken feed at 60% moisture and placed into the incubator at 28 ºC and 60-80% relative humidity. Over 12 days, young larvae hatched and consumed the chicken feed.

PET cups (16 oz) with fly-mesh covered lids were used as culture containers (Lombard The Paper People, with domed lids enclosed by Cyclone Miniweave fibreglass fly mesh secured over the opening with aquarium-safe silicone) (Figure 4.2). Substrates were prepared and added to each container as outlined in Table 4.2, prior to seeding with 80 randomly selected 12-day old larvae with mean individual weights of 1.5 mg dry mass, yielding a larval density of 2.8 larvae/cm^2. Containers were incubated in an environmental chamber (Value Scientific environmental control chamber model SHP-360 modified with several humidifiers) at 28 °C

and 60–80% relative humidity for 12 days. Every two days the container positions were randomly shuffled to account for local variations in environmental conditions. To harvest, larvae were sieved from the residue, washed with tap water and returned to the environmental chamber to purge their gut contents for 24 hours. Once purged, they were washed with tap water, dried with paper towels and placed in a domestic freezer for 24 hours. Larvae and residue samples were oven dried at 105 ºC and frozen until further analysis. An image of the larvae culture containers during harvest is provided in Figure 4.2.

Figure 4.2 Larvae culture containers with fly-mesh lids during harvest of BSFL.

4.2.3. Process Performance

Process performance was measured in terms of larval survival, mean larval gain, substrate loss and FCR, calculated per equations 1-4. Sample calculations are provided in the appendices Chapter 4. Composition and mean larval gain of crude protein, lipid and ash were used as measures of product quality. All calculations are in terms of dry mass. Ratio measures of process performance as used in Chapter 3 were avoided in this study due to the recommendations made in Bosch et al. (2020).

$$Survival\ (\%) = \frac{n_f}{n_0} * 100 \quad (1)$$

Where n_f refers to the number of larvae that survive to the end of the experiment and n_0 refers to the number of larvae initially input into the experiment.

$$Mean\ Larval\ Gain\ (mg)\ = (L_f * n_f - L_0 * n_0) \quad (2)$$

Where L_f refers to the mean dry mass of an individual larva at the end of the experiment (mg) and L_0 refers to the mean dry mass of an individual larva at the beginning of the experiment (mg).

$$Substrate\ Loss\ (\mathrm{g}) = S_0 - R_f \quad (3)$$

Where S_0 refers to the total input dry substrate (g) and R_f refers to the total dry residue at the end of the experiment (g).

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{Substrate\ consumed}{Larval\ gain} = \frac{S_0 - R_f}{L_f * n_f - L_0 * n_0} \quad (4)$$

4.2.4. Larval Composition

Crude lipid of each sample (3 biological replicates per treatment) was measured in duplicate following the Matyash method, outlined in Eggers and Schwudke (2016) with minor modifications. This method was selected in place of the Folch method used in Chapter 3 due to the organic phase remaining at the top, facilitating aspiration with less effort required to avoid contamination from the interface and aqueous phase. Briefly, samples of dry larvae were homogenised in an IKA A 11 basic analytical mill before further crushing with mortar and pestle. Matyash extraction solution (9,000 µL) of methyl-tert-butyl-ether (Merck) and methanol (Sigma Aldrich) (10:3, v/v) was added to ~300 mg homogenised larvae sample in a 15 ml centrifuge tube and continuously shaken at room temperature for 1 hour. Distilled water (1,800 µL) was then added, and vortex mixed for 30 seconds at high speed before centrifuging at 2000 RPM for 10 minutes. The upper organic phase (2,000 µL) was aspirated into a 4 mL

polypropylene centrifuge tube. This was left to evaporate in a fume hood for 24 hours leaving a light-yellow oil. The total crude lipid content was calculated from the mass of this recovered oil scaled up by 3.46 to account for the partial recovery of the organic phase and the average mass of the blanks was subtracted. This value was divided by the mass of dry larvae used for extraction and the mean value of duplicates taken as the percentage crude lipid content.

Nitrogen analysis used a vario MACRO cube CHNOS Elemental Analyzer (Elementar Analysensysteme GmbH, 2017) at the XRF laboratory of the Solid State & Elemental Analysis Unit at the University of New South Wales. Crude protein was determined as the larval nitrogen content scaled up by a conversion factor of 4.76 (Janssen et al., 2017).

Ash was determined as the carbon-free residue remaining after sample ignition in a tubular furnace at 525 ºC for 5 hours. Chitin and other components were calculated as the difference between total larval mass and the combined protein, lipid, and ash contents.

4.2.5. Sequential Fibre Extraction

Fibre fractions were determined sequentially as depicted in Figure 4.3 (Van Soest et al., 1991). Sequential analysis allowed for reduced volumes of neutral and acid detergent solution, which were confirmed to yield equivalent results as in the full digestions for the samples used (data not shown). The process is presented in Figure 4.3.

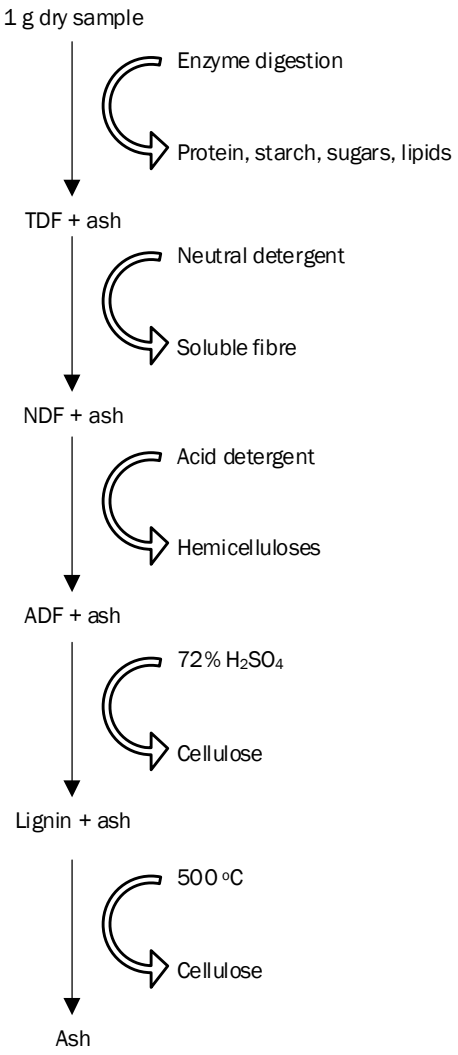

Figure 4.3 Flow diagram presenting the sequential fibre extraction process.

4.2.5.1. Total Dietary Fibre

Total dietary fibre was screened to determine soluble and insoluble fibre contents of samples using the assay kit TDF100A-1KT from Sigma Aldrich as per a modified version of the standard manufacturer protocol. Samples of residues and feeds were ground by hand using a mortar and pestle. 50 ml of pH 6.0 phosphate buffer was added to each of two 1 g samples in tall beakers. 0.1 ml of amylase was added to each beaker and mixed well, prior to covering with aluminium foil and incubation for 15 min in a boiling water bath after internal beaker temperatures reach 95 ºC, agitating every 5 minutes. Once cool, pH was adjusted to 7.5 ± 0.2 by adding 10 ml of 0.275 N NaOH solution to each beaker. Protease solution (50 mg/ml) in

phosphate buffer was prepared immediately prior to use and 0.1 ml added to each beaker, prior to covering with aluminium foil and incubation for 30 min in a water bath at 60 ºC with continuous agitation. Once cool, pH was adjusted to between 4.0 and 4.6 by adding 10 ml of 0.325 M HCl to each beaker. Amyloglucosidase (0.1 ml) was then added to each beaker and incubated as with the protease. Four volumes of 95% ethanol were added to each beaker and allowed to set overnight to precipitate out the soluble dietary fibre. The suspension and precipitate were filtered and washed with three 20 ml portions of 78% ethanol, two 10 ml portions of 95% ethanol, and two 10 ml portions of acetone. Samples were dried at 105 ºC and weighed to determine total dietary fibre with ash. Samples were corrected for ash after acid-detergent lignin digestion.

Typically, total dietary fibre is corrected for residual protein content, as determined in duplicates via the kjeldahl procedure. This step was omitted due to the presence of unknown amounts of chitin from the shed exoskeletons of the larvae which would incorrectly result in greater nitrogen contents and therefore overestimate residual protein by an unknown amount. As such, the total dietary fibre results here may be slightly overestimated since they include the mass of any residual protein. One study has shown the acid-detergent insoluble crude protein content of distiller grains is low, approximately 5% dry matter, which is likely more than the residual content in the residue samples of this study (De Boever et al., 2014).

4.2.5.2. Neutral Detergent Fibre

Neutral detergent fibre was extracted from the total dietary fibre extracts in the same crucibles, following a modified method of Van Soest et al. (1991). Amylase digestion was omitted, as this was already performed in the total dietary fibre extraction. Sodium sulfite was also omitted as it is primarily used to remove keratinaceous residues of animal origin and is known to attack lignin which was determined in following steps (Van Soest et al., 1991).

Neutral detergent (ND) solution was prepared in two steps, firstly by the addition of 4.0 g NaOH, 14.6 g EDTA, 4.56 g sodium hydrogen phosphate (sodium phosphate dibasic) and 6.81 g sodium tetraborate decahydrate to 450 ml of reverse osmosis (RO) water and mixed until dissolved. 30 g sodium lauryl sulfate was then added and once dissolved, 10 ml triethylene glycol added. The volume was brought to 950 ml with RO water prior to mixing. pH to between 6.95 and 7.05 with conc. HCl or NaOH and dilute to 1,000 ml with RO water.

ND solution (20 ml) was added to each crucible sealed at the base with a rubber stopper and the contents stirred, prior to covering with foil and incubation in oven at 105 ºC for 20 minutes from start of boiling. Crucibles were filtered by vacuum and the process repeated two more times using fresh ND solution. After a total of 60 minutes of incubation, crucible contents were washed by soaking with 20 ml hot distilled water for 2 minutes before draining. This washing was repeated until no more foaming was observed, prior to three equivalent washes using acetone. Samples were vacuum dried then returned to the oven to dry overnight. Dried samples were removed and allowed to cool in a desiccator before weighing. Soluble fibre was determined as the difference between TDF and NDF weights (Van Soest et al., 1991). Due to the use of sequential extraction on TDF, NDF is considered equivalent to IDF in this analysis (Mertens, 2003).

4.2.5.3. Acid Detergent Fibre

Acid detergent fibre was extracted from neutral detergent fibre extracts in the same crucibles, following a modified method of Van Soest (1973). Acid detergent (AD) solution was prepared by adding 20g cetyl(trimethyl)ammonium bromide to 1 L of 0.5 mol/L of sulfuric acid and agitated until dissolved.

AD solution (20 ml) was added to each crucible sealed at the base with a rubber stopper and the contents stirred, prior to covering with foil and incubation in oven at 105 ºC for 20 minutes from start of boiling. Crucibles were filtered by vacuum and the process repeated two

more times using fresh AD solution. After a total of 60 minutes of incubation, crucible contents were washed by soaking with 20 ml hot distilled water for 2 minutes before draining. This washing was repeated until no more foaming was observed, prior to three equivalent washes using acetone. Soaking and washing was repeated using acetone until no more colour was removed. Samples were vacuum dried then returned to the oven to dry overnight. Dried samples were removed and allowed to cool in a desiccator before weighing. Insoluble hemicelluloses were determined as the difference between NDF and ADF weights (Van Soest et al., 1991). Samples were corrected for ash after acid-detergent lignin digestion.

4.2.5.4. Acid Detergent Lignin and Ash Content

Acid detergent lignin (hereon referred to as 'lignin') was determined on the acid detergent fibre samples in the same crucibles, following Van Soest (1973) with the exclusion of asbestos. Asbestos was excluded due to its safety risks and since celatom (diatomaceous earth) was already in use as a filtering aid. Crucibles were ashed at 500 ^{o}C for 90 minutes in a tube furnace until carbon free.

4.2.6. Data Analysis

Data were analysed using the software package GraphPad Prism version 9.4.1 (681) and Microsoft Excel version 2210. All data sets had normally distributed residuals and equal variances, as tested by the Brown-Forsythe and Shapiro-Wilk tests respectively with significance determined at $P < 0.05$. Survival data failed the test for normal distribution of residuals and was therefore analysed using the Kruskal Wallis and Dunn's multiple comparisons tests. FCR data failed the test for equal variances and was therefore analysed with Brown-Forsythe and Welch ANOVA, corrected with Dunnett's T3 multiple comparisons test. Significant differences in groups mean larval gain, substrate loss and larval composition between the treatments were determined using ordinary one-way ANOVA with Tukey's test

to correct for multiple comparisons. All error bars for these data represent standard deviations where the sample size was three.

Fibre digestion was analysed in two steps. Firstly, multiple unpaired t-tests were performed for each fibre fraction comparing its contents in the initial substrate and the residue for each treatment. For those with significant differences, the magnitude of the difference was then compared between treatments using ordinary one-way ANOVA, except for SDF loss which was compared using an unpaired t-test as only two treatments showed significant differences.

4.3. Results

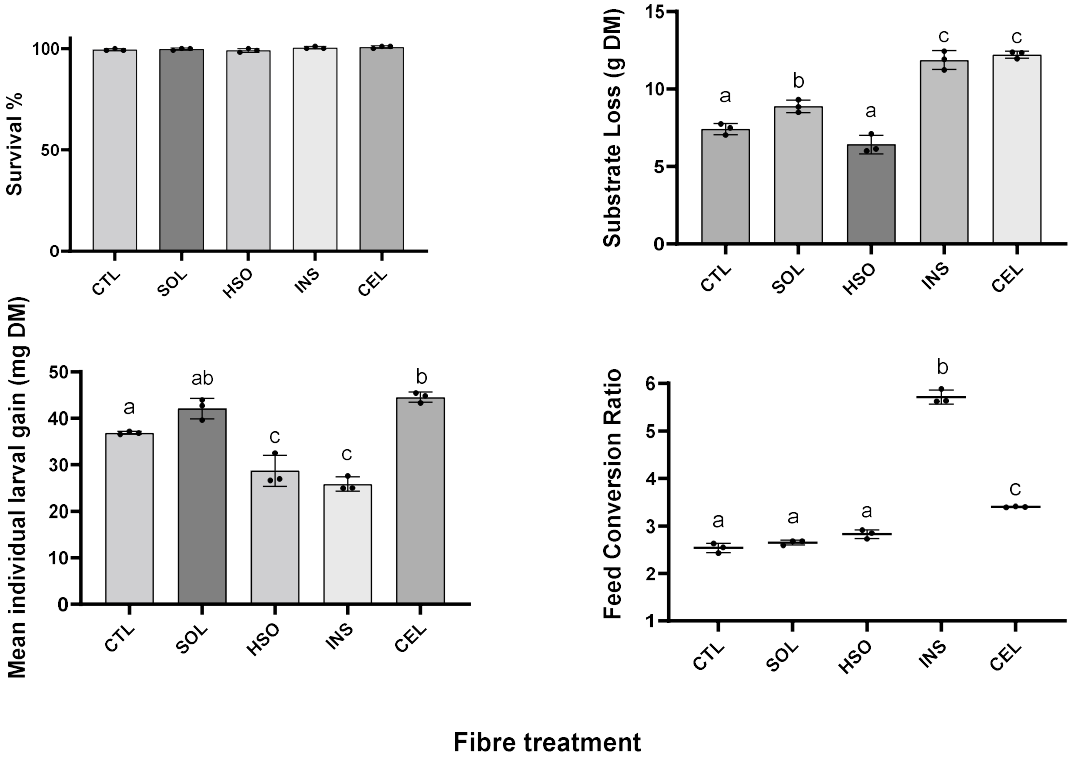

Figure 4.4 Survival, dry substrate loss (g dry matter), mean individual larval gain (mg dry matter) and FCR of each treatment. Common letters are not significantly different by ordinary one-way ANOVA test at the 5% significance level. Error bars indicate standard deviations.

There was near 100% survival in all treatments, hence fibre inclusions at the tested levels do not influence larval mortality (Figure 4.4). The addition of fibre to each substrate relative to the control resulted in greater substrate loss in all cases, except for the HSO

treatment. Insoluble fibres increased substrate loss by the largest degree (4.4 g greater than control), with the magnitude of the increase independent of the fibre's composition as either pure cellulose or the mixture of cellulose, lignin and hemicellulose as in the washed apple fibre treatment, INS. Pectin content of the HSO treatment reduced substrate loss compared to the SOL treatment. Relative to the control (37.0 ± 0.3 mg per larva), the SOL treatment did not significantly influence larval dry mass gain at the 5% significance level. However, the additional pectin of the HSO treatment significantly reduced larval gain (29.0 ± 3.3 mg, adjusted P = 0.0035) to a similar degree as the high apple fibre treatment (26.0 ± 1.5 mg, adjusted P = 0.0003). High cellulose resulted in the greatest larval dry mass (45.0 ± 1.1 mg). The FCR was similar between the control (2.5 ± 0.1) and treatments with soluble fibre additives, whereas FCR increased in the CEL (3.4 ± 0.0) and INS treatments (5.7 ± 0.2).

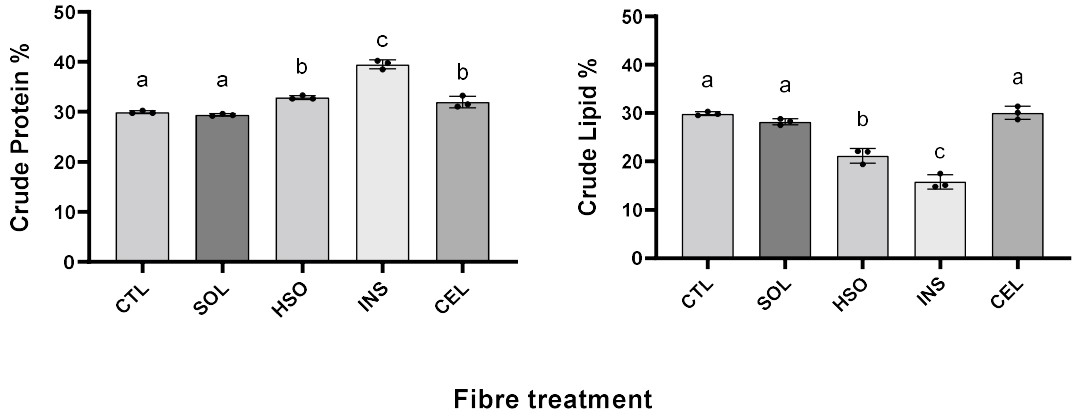

Figure 4.5 Larval protein and lipid compositions. Common letters are not significantly different by ordinary one-way ANOVA test at the 5% significance level. Error bars indicate standard deviations.

All protein contents ranged between 30 and 40% dry mass and lipid contents between 10 and 30% (Figure 4.5). The level of pectin in the SOL treatment had no influence on larval protein or lipid contents at 5% significance. HSO and INS treatments showed moderately increased larval protein contents while drastically decreasing lipids, with the largest effects observed in the INS treatment. Cellulose marginally increased protein contents, however had similar lipid contents as the control.

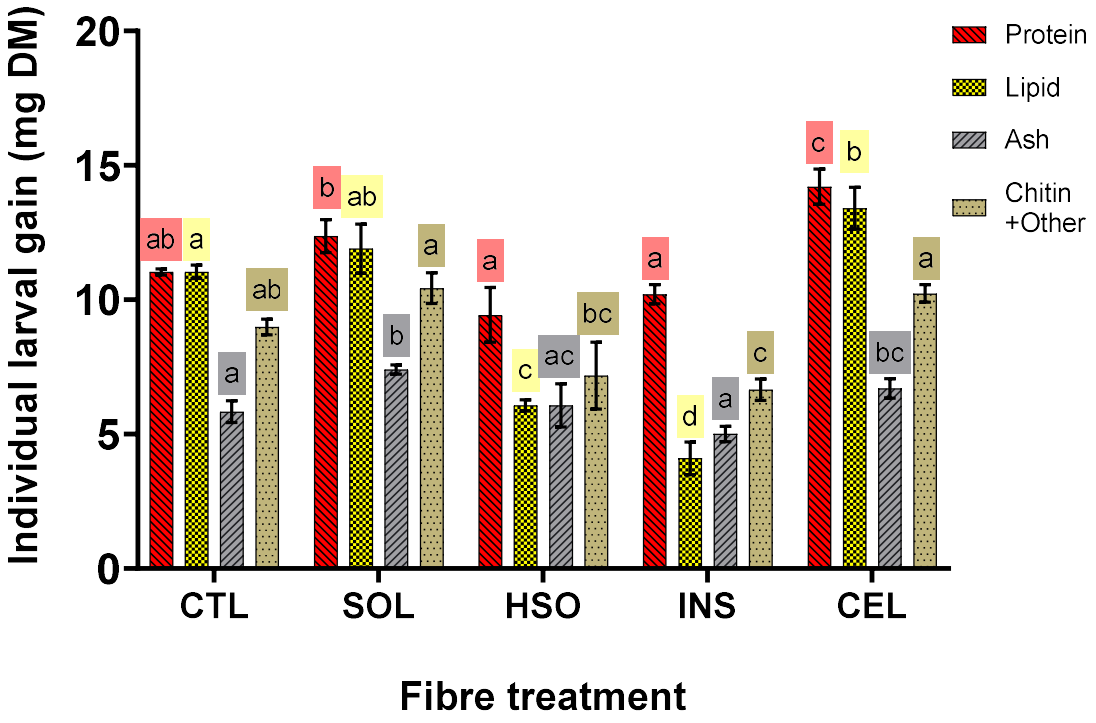

Figure 4.6 Gains in larval protein, lipid, ash and chitin per larva in mg dry matter. Common letters are not significantly different by ordinary one-way ANOVA tested at the 5% significance level. Error bars indicate standard deviations.

There were no significant differences in individual larval protein gains between treatments and the control (Figure 4.6), with the exception of the CEL treatment which increased protein gain from 11.0 ± 0.1 mg of the control to 14.0 ± 0.7 mg per larva (adjusted P = 0.0008). Lipid gains mirrored the trend of larval lipid compositions, except for CEL which significantly increased lipid gain by 2.4 mg per larva relative to the control (11.0 ± 0.3 mg, adjusted P = 0.0061). The SOL treatment resulted in greater ash gain relative to the control (5.8 ± 0.4 mg) by 1.6 mg (adjusted P = 0.0188). The residual larval gain composed largely of chitin and other carbohydrates was similar to the control in all cases except the INS treatment with a reduction to 6.7 ± 0.4 mg dry mass per larva (adjusted P = 0.0307) relative to the control (9.0 ± 0.3 mg).

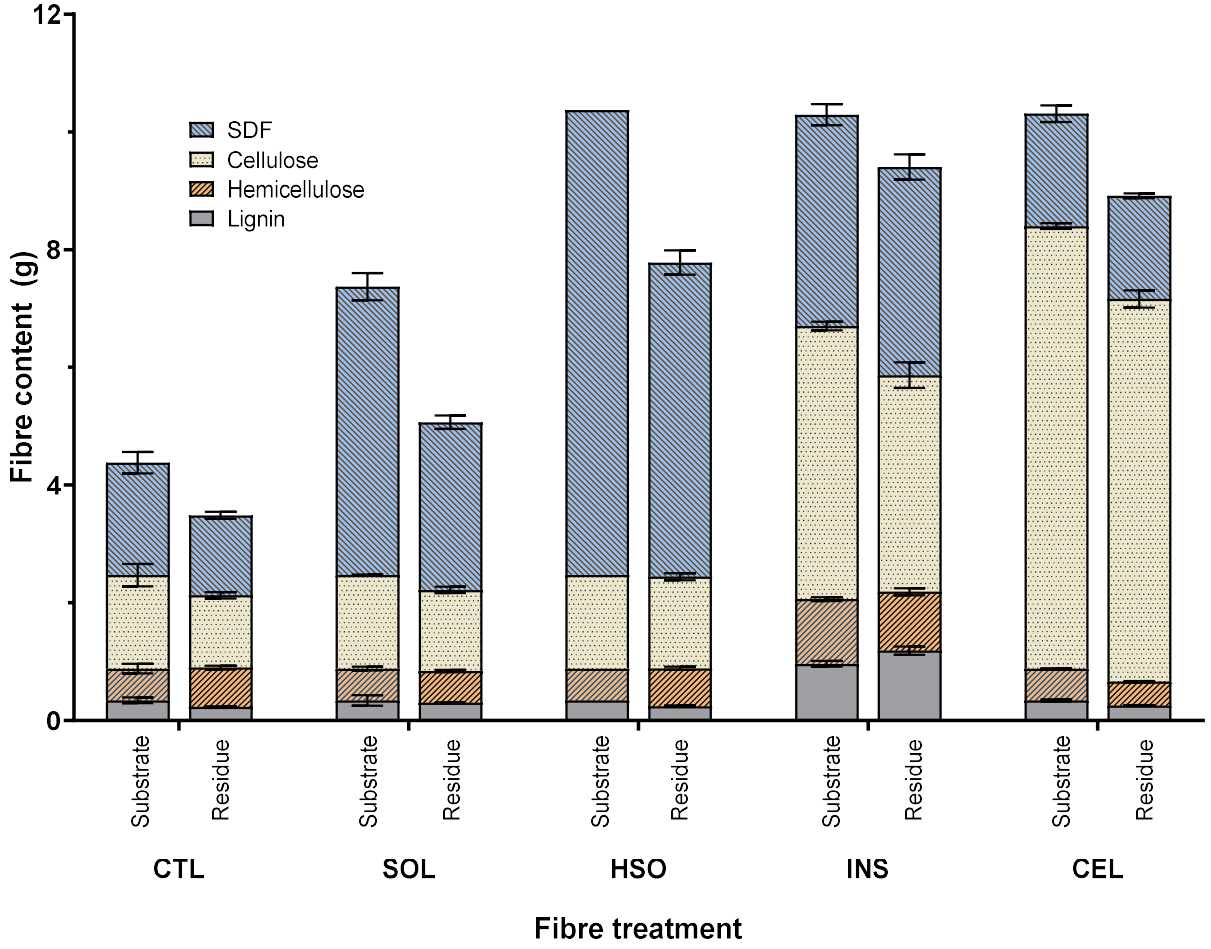

Figure 4.7 Contents of SDF, Cellulose, Hemicellulose and Lignin in starting substrates and final residues across treatments. Error bars reflect standard deviations.

Table 4.3 Loss of fibre fractions across treatments.

Fibre fraction		Treatment			
		CTL	SOL	INS	CEL
SDF	g	0.55 ± 0.19[a]	2.05 ± 0.26[b]	n.s.	n.s.
	%	29 ± 10	42 ± 5	n.s.	n.s.
Hemicellulose	g	n.s.	n.s.	n.s.	0.13 ± 0.01
	%	n.s.	n.s.	n.s.	24 ± 3
Cellulose	g	0.36 ± 0.2[a]	0.21 ± 0.06[a]	0.96 ± 0.23[b]	1.02 ± 0.16[b]
	%	23 ± 13	13 ± 4	21 ± 5	14 ± 2
TDF	g	0.89 ± 0.29[b]	2.30 ± 0.28[a]	0.88 ± 0.38[b]	1.40 ± 0.22[b]
	%	20 ± 7	31 ± 4	9 ± 4	14 ± 2

Footnote: n.s. indicates no significant difference in substrate and residue contents of the fibre fraction for that treatment. There were no significant differences (adjusted $P > 0.05$) between lignin contents of substrate and residue in any treatment. HSO was omitted as the fibre extraction on one of the substrate samples failed resulting in no replicates (N=1). Errors are standard deviations.

There was modest fibre digestion in all treatments (9 to 31% TDF loss), with all fibre fractions showing reductions in at least one treatment, except lignin. SOL lost significantly more TDF (31 ± 4%, adjusted $P = 0.0034$) than the control (20 ± 7%, adjusted $P = 0.0236$), INS (9 ± 4%, adjusted $P = 0.0433$) and CEL (14 ± 2%, adjusted $P = 0.0068$). There were no significant differences in the magnitude of TDF loss between CEL, INS and the control. Both INS (21 ± 5%, adjusted $P = 0.0316$) and CEL (14 ± 2%, adjusted $P = 0.0120$) lost significantly more cellulose than the control (23 ± 13%, adjusted $P = 0.0463$) and SOL (13 ± 4%, adjusted $P = 0.0369$). There were no significant differences in cellulose loss between INS and CEL. SOL (42 ± 5%, adjusted $P = 0.0034$) lost significantly more SDF than the control (29 ± 10%, adjusted $P = 0.0400$). Only CEL showed significant reduction in hemicellulose (24 ± 3%, adjusted $P = 0.0030$). Of all fibre types, the largest level of digestion occurred in SDF, followed by cellulose and hemicellulose.

4.4. Discussion

The inclusion level, type, and source of dietary fibre significantly influenced larval performance and composition by altering the physicochemical properties of the substrate, which in turn determined larval development. These alterations were observed in the substrate and residue textures, as well as the digestion levels of different fibre types. Much of the

discussion made here, particularly that regarding mechanisms for the influence of dietary fibres, is referenced in the literature reviewed in Chapter 2, section titled Dietary Fibre.

4.4.1. Larval Performance and Composition

Washed apple fibre in the INS treatment acted as a 'defatting agent', lowering larval gain by 30% through reduced lipid and chitin gains while increasing the FCR 2.3 times. The INS substrate viscosity appeared to increase during the trial (personal observation), possibly due to a gradual release and/or modification of intracellular pectin from the microbial and larval disruption of the apple fibre matrix. This in turn slowed larval movement and digestion as discussed in Chapter 2, accounting for the decreased larval gain. Reduced larval mobility may have provided external microorganisms greater opportunity to metabolise starches and simple carbohydrates into CO_2, accounting for the reduced larval lipid and chitin contents (Figure 4.6) and high FCR (Figure 4.4).

Pure cellulose added at 29% dry matter in the CEL treatment enhanced larval growth by 22% through increased lipid and protein gains. This was unintuitive, given the INS treatment with similar cellulose content resulted in reduced larval lipid and no change in protein. While cellulose is not a viscosity modifier, it may have similarly influenced larval mobility by providing more anchor points for the larvae to move with and access fresh substrate (see Chapter 2). This in turn could account for the increased larval gains and substrate loss. While CEL contained more cellulose, cellulose digestion was similar to INS, possibly due to reaching maximum digestion capacity. Assuming equivalent levels of cellulose digestion correspond to equivalent SCFA production, it is unlikely that cellulose fermentation contributed substantially to differences in performance and larval compositions between treatments.

Several mechanisms of concern that lead to insoluble dietary fibre's reputation as an anti-nutritional factor in animal nutrition either do not apply in BSFL or were outweighed by the benefits to larval mobility in this study. Notably, the concern of insoluble fibre increasing

the passage rate of digesta and reducing enzymatic digestion (see Chapter 2) was not supported by the increases in larval gains and low feed conversion rates of the CEL treatment. Similarly, the lack of cellulose's influence on lipid contents indicates the dilution of nutrients was not the dominant mechanism behind the lower lipid contents of the HSO and INS treatments. However, access restrictions to nutrients by intact cell walls was not a factor in this study, as the fibre additives were thoroughly homogenised and contained negligible levels of macronutrients. The fibre content endogenous to the chicken feed - which may have encapsulated nutrients - was also identical in all treatments. Hence, future studies should investigate more diverse fibre sources to identify the significance of fibrous cell walls influencing larval performance and composition.

Pectin, when included at 17% dry matter in the SOL treatment, had no significant effect on larval composition or performance besides increased substrate loss. However, larval lipid gains were significantly reduced when pectin was included in higher amounts in the HSO treatment. Larval mobility - as influenced by the increased viscosity from pectin - likely played a significant role in explaining these results, (see Chapter 2). The slight increase in viscosity of the SOL treatment relative to the waterier control (personal observation) may have improved larval mobility, enabling increased substrate consumption, while the excessive viscosity of HSO hindered it. An optimal viscosity for larval growth therefore likely lies between the levels of the control and HSO treatments. Interestingly, the reduced larval mobility did not enable increased microbial metabolism of the substrate into gaseous emissions as in the INS treatment. This may be a function of the substrate viscosity starting low in the INS treatment and increasing over time, while viscosity was consistently high in the HSO treatment. Excessive digesta viscosity of the HSO treatment may have also reduced nutrient digestibility and feed intake, contributing to the reduction in larval lipid content (see Chapter 2). Regarding fermentation products, added apple pectin was readily digested to similar extents in both SOL

and HSO treatments, indicating a possible contribution to the larvae nutrient pool and influencing adiposity. However, considering the differences in performance and compositions between these treatments, effects from SCFAs were either minimal or not present.

There may be some differences in the extent that larvae of each treatment purged their gut contents, most notably in the soluble fibre treatments considering their influence on gut residence time (Hooda et al., 2011). Such differences would influence the measured larval gain, hence larval gain should be interpreted with caution. Protein and lipid gains are likely more reliable measures of larval performance as they are less effected. The purging method outlined in Li and Bischel (2022) may be worthwhile in future.

Given the variety of possible contributory mechanisms, it is not possible to confidently distinguish between the influence of each on larval performance and composition, requiring further investigations. Quantitative measures of substrate textures such as viscosity, additional fibre sources and inclusion levels, and isolation methods to distinguish between larval and microorganism metabolism would be beneficial.

4.4.2. Fibre Digestion

Fibre digestion was variable (Figure 4.7), reflecting the variations reported in literature and confirming that fibre digestion is not simply dependent on initial fibre content. Soluble fibre showed the most significant digestion at 42% in the SOL treatment, lower than the >70% in Gold et al. (2018a). Cellulose was consistently digested across treatments ranging from 13 to 23%, similar to literature values of 27.9% in Zheng et al. (2012), 50% in Li et al. (2011), 0% in Gold et al. (2018a), 49 and 67% in Rehman et al. (2017b). Lignin is recalcitrant, showing no significant digestion in the present study and similarly low reductions in literature with 0.8% in Zheng et al. (2012), 30 and 42% in Rehman et al. (2017b). The large variation in digestion extents across studies is likely due to the lack of standardised culture conditions (larval age,

cultivation time and feeding rates), substrate properties (fibre source and inclusion levels, particle sizes and compositions) and fibre measurement methodologies.

SDF content contained within the chicken feed and washed apple fibre were mostly resistant to digestion and therefore likely did not contribute significant SCFA content. SDF endogenous to the chicken feed may differ in terms of chemical structure to the pure apple pectin, however SDF from the added apple fibre in the INS treatment could be assumed similar in structure to the pure pectin. Hence, the fibre matrix likely accounts for the lack of digestion in the INS treatment, particularly the increased extent of lignification relative to the pure pectin. This variation in digestibility of SDF may be important for studies which measure non-fibre carbohydrates by difference, which typically includes SDF. Instead, the sum of free sugars and starch may be a better indicator of non-fibre carbohydrates contents, as used in Gold et al. (2020). Greater disruption of the fibre matrix may improve accessibility of soluble fibres. Compared with soluble fibre, cellulose digestion appeared less restricted by lignification considering the notable percentage reductions across all treatments, with differences in absolute reduction appearing to be positively correlated with total cellulose content of the initial substrate.

Hemicellulose is typically considered a readily degradable insoluble fibre fraction with reported reductions of 32.6% in Zheng et al. (2012), 30% in Li et al. (2011), 49 and 59% in Rehman et al. (2017b). The lack of significant reductions in hemicellulose content in the present study is therefore surprising, with only the CEL treatment showing a slight reduction, despite the CTL and SOL treatments containing identical hemicellulose contents as supplied by the chicken feed. The chemical structure and lignification of the hemicellulose were equivalent, indicating the varied substrate structure of the CEL treatment may be responsible for enabling its fermentation. However, the magnitude of this digestion was low (0.13 ± 0.01 g), hence it is unlikely that resulting SCFAs from hemicellulose fermentation influenced the

larval performance and composition. Alternatively, the lack of hemicellulose digestion may be due to differences in fibre quantification methods between the present study and literature, since solubility of hemicellulose can vary (Williams et al., 2017). Differences may also result from variations in fly genetics and commensurate microbiota. Caution should be used when comparing percentage reductions across studies, as seemingly large percentage reductions may not translate to substantial absolute reductions, as exemplified in Table 4.3.

4.4.3. Implications for Commercial BSFL Production

The present results demonstrate that fibre contents can influence larval production in terms of larval gain, protein and lipid contents, FCRs and substrate reduction. Hence, when formulating substrates from wastes for industrial BSFL production, dietary fibre contents should be accounted for. Strategic inclusion of fibre contents may aid in achieving a facility's objectives such as maximising substrate loss or larval protein content. The generalised recommendation made in literature to ferment fibre rich substrates is therefore an oversimplification (Joly and Nikiema, 2019).

The INS fibre treatment – containing large quantities of predominantly insoluble fibre cellulose – resulted in the highest protein concentration larvae with reduced levels of fat, chitin and other contents (Figure 4.5 and Figure 4.6). Facilities whose downstream markets include processed animal feed products may find this beneficial, as chitin is considered an anti-nutritional factor for many animals (Ravi et al., 2020), and the typically high larval lipid contents can restrict potential inclusion levels of BSFL in animal feeds, requiring defatting. Therefore, while acknowledging the need for confirmatory studies using real wastes, the addition of apple fibre such as from juicing waste, or fibre sources that have similar influences on substrate properties, may be beneficial in improving product quality. Contrarily, facilities who supply live larvae may be more interested in larval gain rather than composition. In this case, addition of materials akin to cellulose to manipulate the substrate texture may be useful.

Adding insoluble fibres to BSFL substrates may also be an effective way of increasing disposal of organic wastes via gaseous emissions without compromising larval protein production (Figure 4.6). This is evidenced by the higher feed conversion rates (Figure 4.4), indicating a greater amount of substrate material was lost via gaseous emissions as opposed to accumulating in larval biomass. While disposal as gaseous emissions into the atmosphere is not a circular economy solution, it may still be a sustainable option for areas that lack the infrastructure for alternative disposal methods as may be the case in less developed regions (i.e., emitting CO_2 may be better than letting the fibre-rich food waste rot in situ and pollute waterways, attract vermin, and emit more potent greenhouse gasses). Additionally, the emissions largely compose of CO_2 given the aerobic nature of the process (Parodi et al., 2020), which has value in a variety of reuse technologies especially when available from a point source (Luu et al., 2015; Tumilar et al., 2021). Contrarily, the high FCRs and relatively low fibre digestion (Figure 4.7) of the insoluble fibre treatments shows that more of the nutrient-rich and high value chicken feed was consumed to produce the larval biomass compared to the other treatments. This may be detrimental in scenarios where securing sufficient volumes of nutritious substrate is a challenge, as is typically the case for industrial BSFL production facilities (Joly and Nikiema, 2019).

Additionally, the inclusion of chemically pure cellulose significantly increased larval gain and substrate loss (Figure 4.4). Optimisations of substrate nutritional contents using cellulose as an inert filler may therefore be suboptimal (Barragan-Fonseca et al., 2021). There may be value in investigating less expensive cellulose substitutes for similar increases in larval performance and composition.

Despite the discovered influence of fibre on BSFL digestion, it is typically not feasible to optimise all influential substrate properties without an excessively wide range of ingredients to choose from. Hence, the relative impact of fibre against other substrate properties must be

examined and the most influential substrate properties optimised, likely at the expense of others. Given the complexity of possible mechanisms involved and the interactions between them, it may be the case that fibre cannot be reasonably simplified to a few commonly measured parameters that can be generalised across multiple substrates, such as TDF, NDF and ADF alone. Nevertheless, facilities should collect and share measurements of substrate fibre-related characteristics where possible, such as SDF, NDF, ADF, water holding capacity, viscosity, fermentability, particle size, degree of lignification and the use of any pre-treatments, rather than crude fibre alone, to improve predictability of performance and composition as a function of substrate fibre. Further investigation into pre and co-treatments of fibre fractions with observation of their influence on substrate texture and fibre fermentability are warranted.

4.5. Conclusion

Substrate fibre contents were found to significantly influence both BSFL performance and composition in a manner dependent on the fibre concentration and source. Variations in performance of substrates with identical protein, carbohydrate and water contents, may therefore be partly explained by variations in fibre contents. Soluble fibre endogenous to the chicken feed and washed apple fibre was resistant to digestion likely due to inaccessibility imposed by lignin in the fibre matrices, considering added pure apple pectin was significantly digested with a total SDF reduction of 42 ± 5%. Contrarily, cellulose digestion appeared less restricted by lignification as there were notable reductions across all treatments, with differences in absolute reduction appearing positively correlated with total cellulose content of the initial substrate. The observed effects appear largely a function of substrate texture modifications induced by the fibre additives, which may be a strategic tool for BSFL production facilities to tailor their products for downstream markets. The influence on larval growth and composition of digestion products from cellulose and pectin, such as short chain fatty acids, have yet to be investigated. Further studies into the inclusion of fibrous wastes in

BSFL substrates are warranted to disentangle the physical and chemical mechanisms involved. This would enable more systematic strategies to further valorise and manage fibrous wastes using BSFL, thus enhancing the organics valorisation in the circular economy.

Chapter 5. Bioaccumulation of Perfluorooctanoic Acid in Black Soldier Fly Larvae via Substrate Exposure

5.1. Introduction

As shown through the prior chapters, BSFL substrate formulation requires compromising waste reduction, larval growth and composition. However, larval safety has little margin for compromise (Chapter 2) and there remain significant unknowns regarding larval safety as a function of substrate properties and their effect on compatible downstream use cases (Lievens et al., 2021). Various safety concerns regarding the use of organic wastes in BSFL substrates are shown in Table 5.1.

Table 5.1 Safety concerns of using food wastes, agri-residues or manures in BSFL substrates summarised from James et al. (2022) and FAO (2021).

Biological	**Chemical**
Bacteria	Heavy metals
Fungi	Mycotoxins
Viruses	Allergens
Parasites	Microplastics
Antibiotic-resistant genes	Hormones
Zoonoses	Pesticides
	Dioxins
	Polychlorinated biphenyls
	Polycyclic aromatic hydrocarbons
	Per- and polyfluoroalkyl substances
	Mineral hydrocarbons
	Biogenic amines
	Veterinary drugs

Per- and polyfluoroalkyl substances (PFASs) are a collection of widely used chemicals that have entered food and water cycles (Figure 5.1) and been found in organic wastes containing single digits to hundreds of ppb concentrations (Beecher and Brown, 2018; Li and Bischel, 2022; United States Environmental Protection Agency, 2021). Their resistance to degradation allows them to persist in the environment (Rayne and Forest, 2009), awarding them the title ‘forever chemicals’. PFAS exposure poses significant health consequences for humans, including but not limited to increased risk of kidney, prostate and testicular cancers,

risk of thyroid disease, liver damage, dysregulated lipid and fatty acid metabolism, reduced immune response to vaccines, lower birth weights and developmental effects in children (Chen et al., 2020; United States Environmental Protection Agency, 2021). Animal studies have found links to PFAS exposure and delayed growth and development, reduced weight and developmental abnormalities. Mechanisms behind these effects include disrupted hormone signalling such as a reduction in insulin-like growth factor (Lopez-Espinosa et al., 2016) and increased oxidative stress via decreased anti-oxidant capacity (Wielsøe et al., 2015). PFAS has also been shown to impair nutrient absorption via altered gene expression (Jantzen et al., 2016), such as intestinal tight junction genes responsible for regulating flow of nutrients across epithelial cells (Rashid et al., 2020). Furthermore, PFAS bioaccumulate in many plant and animal tissues, which biomagnifies up the food chain (Haukås et al., 2007; Lesmeister et al., 2021). Understanding the pathways of PFAS through the environment is therefore critical to managing its spread and mitigating human exposure (Abunada et al., 2020).

Whether BSFL may contribute to the biomagnification of PFAS is unknown. Substantial bioaccumulation over short time periods would restrict the use of larvae for food and feed when fed contaminated substrates yet may facilitate immobilisation or destruction by concentrating PFAS from lowly-contaminated organic materials. Minimal bioaccumulation during a typical BSFL digestion batch may avoid requirements for PFAS analysis of the substrate material and/or allow the inclusion of contaminated substrates that would otherwise require costly disposal. In either case, the influence of PFAS on larval growth and quality requires attention, notably fatty acid profiles, given the known dysregulation of lipid metabolism by PFAS exposure.

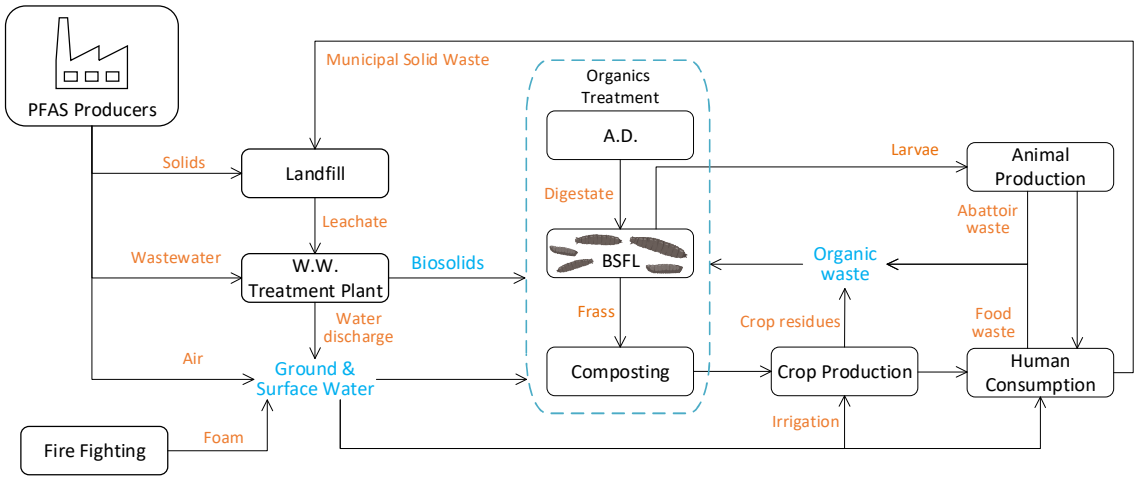

Figure 5.1 Conceptual flow of PFAS contaminants through food and water systems

This study aims to determine the time-dependent bioaccumulation potential of PFAS in BSFL and identify if the contaminant influences larval fatty acid profiles. Two larval growth trials were performed using chicken feed substrates spiked with perfluorooctanoic acid (PFOA) at concentrations representative of those found in actual organic wastes. PFOA contents in larval samples were quantified via tandem LCMS and larval fatty acid profiles of samples determined via GCMS. In the first trial, PFOA was spiked at 0.1, 10 and 1000 ppb concentrations and used for larvae cultivation for 11 days. In the second trial, PFOA was spiked at 0, 10, and 100 ppb concentrations and larvae were cultivated for 45 and 90 hours to identify if PFOA bioaccumulation was proportional to the exposure time. This second trial was necessary to supplement the findings of the first trial with shorter exposure times and an improved experimental setup.

5.2. Methods and Materials

5.2.1. Larvae Culturing and Harvest

5.2.1.1. Trial 1

Live neonates (purchased from Future Green Solutions) were fed ground chicken feed (Barastoc) until nine days old before to sieving, washing with tap water and drying with paper towels to separate the larvae from the residue.

PET cups (16 oz) with fly-mesh covered lids were used as culture containers (Lombard The Paper People, domed lids enclosed by Cyclone Miniweave fibreglass fly mesh secured over the opening with aquarium-safe silicone) (Figure 5.2). Substrates were prepared using ground chicken feed at 270 mg dry feed per larva, 60% moisture and containing either 0, 0.1, 10 or 1000 ppb PFOA with three replicates each. Once substrates were added to culture containers, 80 9-day-old larvae were introduced yielding a larval density of 2.90 larvae/cm^2. Containers were incubated in an environmental chamber (Value Scientific environmental control chamber model SHP-360 modified with several humidifiers) at 28 °C and 60–80% relative humidity. Container locations were randomly shuffled every three days to account for any local variations in incubator conditions. After 11 days, larvae were sieved from the residue, washed with tap water and returned to the environmental chamber to purge their gut contents for 24 hours. Once purged, they were washed with tap water, dried with paper towels and placed in a domestic freezer to euthanise them. Larvae and residue samples were then oven dried at 105 ^oC and frozen until further analysis.

5.2.1.2. Trial 2

Lockdowns as a result of the COVID-19 pandemic in NSW resulted in a delay of the second trial until two years after the first. Unfortunately, over this time the source of young larvae used in the previous trial became no longer available, hence an alternative source was used.

Prior to commencing the experiment, eggs (purchased from Mobius Farms) were placed on fly screen mesh and positioned above a small quantity of ground chicken feed (Organic Backyard Layer Mash Enfield Produce) at 60% moisture and placed into the incubator at 28 ^oC and 60-80% relative humidity. Over 12 days, young larvae hatched and consumed the chicken feed, prior to separation from the feed and residue for use in the growth trial.

Substrates were prepared at 240 mg dry chicken feed per larva, 60% moisture and containing either 0, 10 or 100 ppb PFOA and added to culture containers prepared akin to the first trial with three replicates each. Containers were seeded with 50 12-day old larvae of 3.4 mg average individual dry mass, yielding a larval density of 1.8 larvae/cm^2. Containers were incubated in the same conditions as the first trial. Harvests occurred at 45 and 90 hours and were undertaken as in the first trial, along with purging, euthanizing, drying and storing.

Figure 5.2 Larvae culture containers with dry substrate.

5.2.2. Process Performance

Process performance was measured in terms of larval survival and average larval gain. All calculations are in terms of dry mass. Calculations were performed as in Chapter 4.

$$Survival\ (\%) = \frac{n_f}{\mathrm{n_0}} * 100 \qquad 1$$

Where n_f refers to the number of larvae that survive to the end of the experiment and $\mathrm{n_0}$ refers to the number of larvae initially input into the experiment.

$$Mean\ Larval\ Gain\ (mg)\ = (L_f * n_f - L_0 * n_0) \quad 2$$

Where L_f refers to the mean dry mass of an individual larva at the end of the experiment (mg) and L_0 refers to the mean dry mass of an individual larva at the beginning of the experiment (mg).

5.2.3. Larvae PFOA Extraction

Larvae from were extracted using the QUECHERS method (Anastassiades et al., 2003) in duplicate using samples from two of the three biological replicates. Larval samples are high in lipids and heavily coloured, hence the choice of AOAC clean up tubes containing octadecyl silica sorbent (C18) and graphitized carbon black to remove these components from the extract.

A stock solution of 500 ppb PFOS standard (Sigma Aldrich) in water was prepared. Dry larvae samples were ground using a mortar and pestle to a fine powder prior to weighing 0.5 g into a 50 ml polypropylene centrifuge tube. Distilled water (5 ml) was added, alongside 500 µL of the prepared standard and 10 ml acetonitrile (Sigma Aldrich). Contents were briefly shaken prior to addition of extraction salts (4g $MgSO_4$, 1g Na Citrate, 0.5g Na Citrate sesquihydrate and 1g NaCl, sourced from Biotage as product Q0020-15V). Samples were rigorously shaken by hand for one minute followed by centrifugation at 4000 rpm for 5 minutes. Supernatant (6 mL) was transferred to 15 mL d-SPE tubes (sourced from Sigma Aldrich as product 55474-U) containing 400 mg C18 sorbent (sourced as DSC-18), 1200 mg $MgSO_4$, 400 mg graphitized carbon black (sourced as ENVI Carb) and 400 mg primary secondary amine sorbent (PSA). The samples were vortexed at max speed for 1 minute prior to centrifugation at 4000 rpm for 5 minutes and the resulting supernatant filtered through 0.2 um membrane syringe into autosampler vials, ready for LCMS.

5.2.4. LC-MS/MS and PFOA Quantification

A Thermo Scientific TSQ Altis Triple Quadrupole Mass Spectrometer was used to analyse the standards and sample extracts by LC-MS/MS. The mobile phase consisted of (A) 95% H2O, 5% acetonitrile, 0.1% formic acid and 20 mM ammonium acetate, (B) 95% acetonitrile, 5% H2O, 0.1% formic acid and 20 mM ammonium acetate. The flowrate was set at 0.3 mL/min. The initial mobile phase gradient composition was held at 100% A – 0% B (A and B lines were complimentary) for 2 min before gradually ramping up to 20% B over 1 min. Then from 20% to 50% over 5 min (3 to 8 min) and from 50% to 85% over 7 min (8 to 15 min). Before a 100% organic wash at 16 min for 3 min (16 to 19 min) at 0.4mL/min and equilibrating for 4 min (19.01 to 23 min) at 0.4mL/min. The first 2 min of the analysis was diverted to waste before diverting to the LC-MS/MS.

There were two columns used for the delay of coeluting compounds and the separation of the selected PFAS analytes. The isolator column used was an Agilent Zorbax Rapid Resolution High Definition (RRHD) Eclipse Plus column (1.8µm packing, 3 x 50mm) and the analytical column that was used for the separation of the PFAS analytes was an Agilent Zorbax RRHD StableBond C18 (1.8 µm packing, 2.1 x 100mm). To avoid losses due to sample filtration, samples were injected directly in the LC-MS/MS without prior filtration and analysed in triplicate.

Peaks were integrated using Thermo Scientific Xcalibur software and recoveries calculated for PFOS, ranging between 69 and 104%. PFOS was confirmed to be a reasonable internal standard for estimating PFOA recovery using spiked larval samples and blanks. Larval PFOA concentrations (ng/g) were calculated using a calibration curve and adjusted accounting for error from blanks, PFOS recoveries and initial larval mass. Bioaccumulation, referring to the total uptake of PFOA from the environment, was measured in terms of bioaccumulation

factors (BAF) as the concentration of PFOA in dry larvae (ng/g) relative to the concentration of PFOA in the substrate (ng/g) (Diener et al., 2015).

5.2.5. Fatty Acid Analysis

Lipid from was extracted and derivatised to fatty acid methyl esters (FAMEs) using the one-step method described by Sukhija and Palmquist (1988) in duplicate using samples from two of the three biological replicates, with slight modifications. This method differs from that of Chapter 3 in that it does not require pre-extraction of the lipids and is therefore quicker and generates less waste. Dry larvae samples (30 mg), 300 uL toluene and 300 uL internal standard solution (4 ppm heptadecanoic acid in toluene) were homogenised using steel beads beat for 180 seconds at max speed. Methanolic HCl was prepared by addition of 1 part acetyl chloride in 10 parts dry methanol cooled in an ice bath, prior to addition of 900 uL to the samples. Samples were vortexed at low speeds for one minute, avoiding spread of solids on the tube walls, then left to esterify over two hours in a 70 ºC water bath before cooling to room temperature. Contents were transferred to a 20 ml scintillation vial then 2.5 ml of 6% K_2CO_3 solution and 1 ml toluene added prior to 30 seconds vortex at medium speed. The top layer was transferred to a 2 ml centrifuge tube, centrifuged at 1500 RPM for 5 minutes then the top layer transferred to the autosampler vial.

GC-MS was used to analyse several dilutions of the Supelco FAME 37 mix from Sigma Aldrich to generate retention factors of each fatty acid methyl ester and confirm peak identities. The prepared FAME solutions from each unique run were then analysed with a Perkin Elmer Clarus 680 GC 30 m column (Perkin Elmer Elite 5 ms - 0.25 mm ID x 0.25 um film thickness) with SQ 8 C positive ion EI (70 eV) quadrupole mass spectrometer. Helium at a flowrate of 1.3 mL/min was used as the carrier gas. 0.5 uL sample was injected at a 50:1 split into the port preheated to 250 ºC. After 30 seconds for oven equilibration at 90 ºC, the GC oven program was held at 90 ºC for 2 mins, ramped up 10 ºC/min to 190 ºC, held for 1 minute, ramped up 3

ºC/min to 250 ºC then held for 5 minutes (total GC time 38 minutes). The GC-MS interface and MS source temperatures were 250 and 150 ºC respectively. MS scan settings included a solvent delay from 0-3 minutes before scanning from 45-550 m/z over 200 ms with 100 ms interscan delay.

TurboMass software Version 6.0.0.1811 was used for peak detection and integration for each chromatogram. Peaks that were not associated with a fatty acid methyl ester were removed manually by visual inspection of the chromatogram before quantification. Relative retention factors for each fatty acid methyl ester to the internal standard were determined from the FAME 37 mix calibration curves and concentrations of each FAME/100 g calculated using equations 3 and 4:

$$X_{RRF} = \frac{X_{RF}}{IS_{RRF}} = \frac{\frac{Xarea}{Xconcentration}}{\frac{ISarea}{ISconcentration}} \quad 3$$

$$Xconcentration = Xarea * \left(\frac{ISconcentration}{ISarea}\right) * \left(\frac{1}{X_{RRF}}\right) \quad 4$$

Where RF is the retention factor, RRF is the relative retention factor, X is the FAME of interest and IS is the internal standard.

5.2.6. Data Analysis

Graphpad prism 9.4.1 (681) was used for data analysis. Larval survival was analysed using the nonparametric Kruskal Wallace test. For Trial 1, ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare larval gains and fatty acid profiles. For Trial 2, ordinary two-way ANOVA with Tukey's multiple comparisons test was used to compare larval gains and fatty acid profiles. Assumptions of normal distribution of residuals was tested with the Shapiro-Wilk test and homoscedasticity tested with the Brown-Forsythe test.

5.3. Results

5.3.1. Trial 1

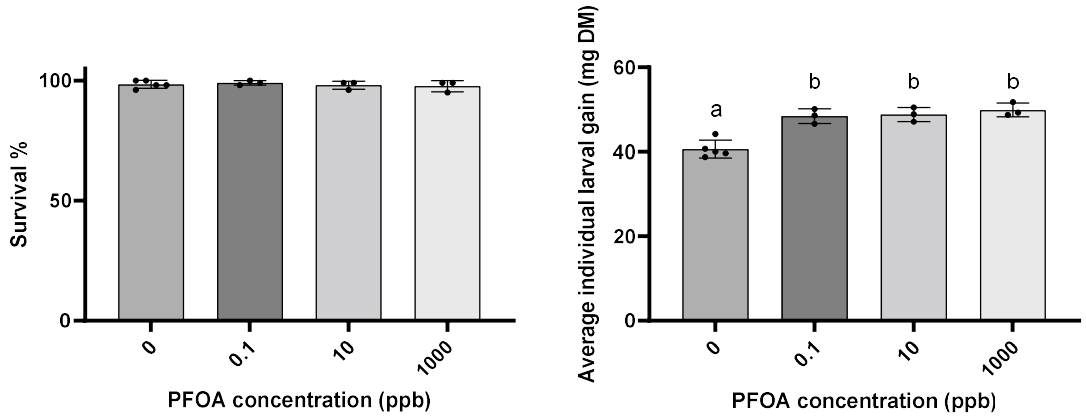

Figure 5.3 Trial 1 performance measures of each treatment in terms of survival and mean individual larval gain (mg DM). Common letters are not significantly different by ordinary one-way ANOVA test at the 5% significance level. Error bars indicate standard deviations where n=3.

There were no significant differences across survival. The larval gain of the controls (41 ± 2.1 mg DM) was significantly worse than those spiked with PFOA (mean of 49 ± 1.7 mg DM), however there were no differences between the three concentrations. Unexpectedly, conditions within the incubator were found to be heterogenous in terms of temperature and humidity, and the cultivation containers for the control were placed on a separate shelf to those of the treatment containers. It is suspected that the difference in larval gain between the controls and the PFOA spiked larvae was a function of these different environmental conditions themselves rather than the PFOA, given the lack of difference in gain between the PFOA concentrations. This confounding variable prompted the need for a second growth trial and exclusion of the control containers from the fatty acid analysis.

Table 5.2 PFOA concentrations in dry substrate and corresponding concentration in larvae of Trial 1 cultured for 11 days. Errors are standard deviations where n=3

PFOA concentration in substrate (ppb)	PFOA concentration in larvae (ppb)	Bioaccumulation factor
0	Not detected	n/a
0.1	2.55 ± 0.07	25.50
10	16.35 ± 0.64	1.64
1000	1040.90 ± 11.46	1.04

Larvae bioaccumulated PFOA in Trial 1 proportionally to the dosage in their substrate in a non-linear relationship (Table 5.2). Bioaccumulation factors decreased as substrate concentrations increased, approaching 1 at the highest substrate concentration. Control larvae did not contain detectable levels of PFOA.

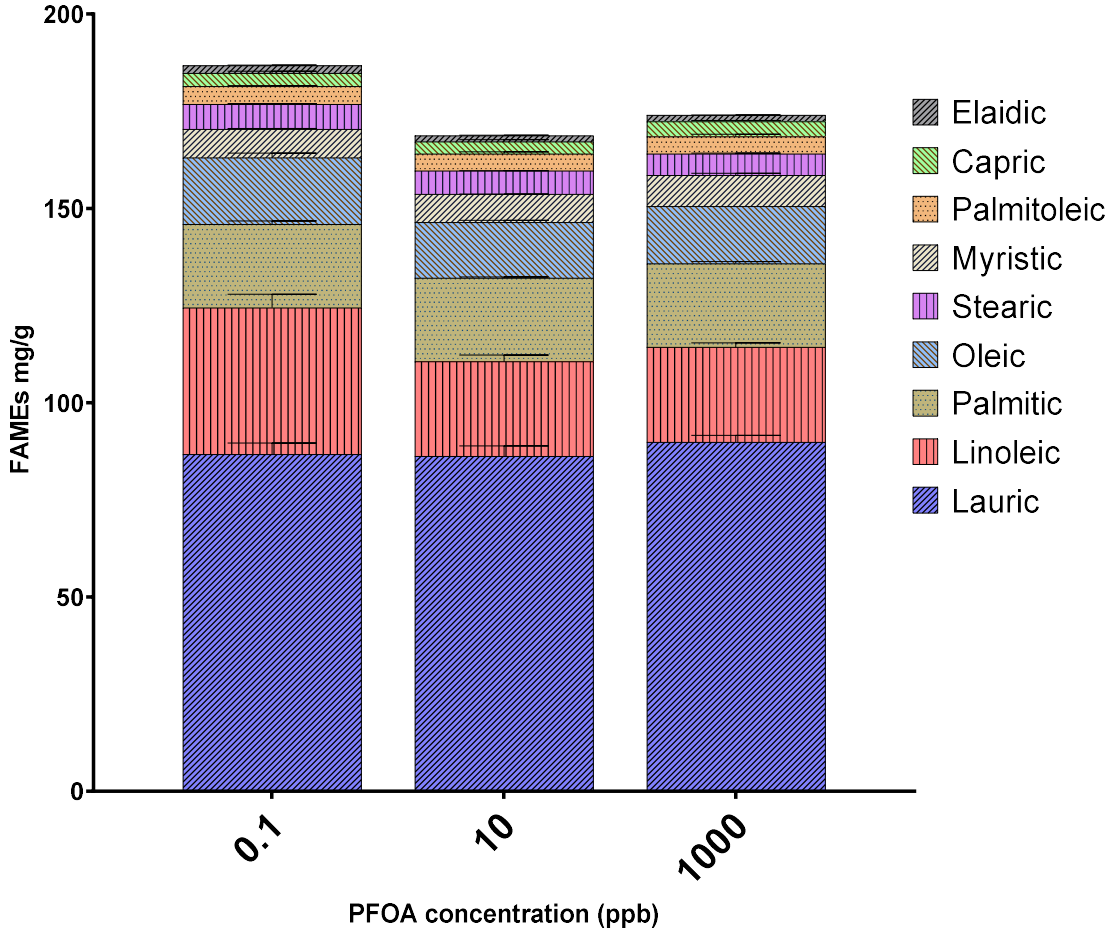

Figure 5.4 Fatty acid compositions of the larvae from each treatment group of Trial 1, excluding the controls. Error bars are standard deviations where n=3.

Table 5.3 Trial 1 treatments showing significant differences in quantities of fatty acids from Tukey's multiple comparisons tests (adjusted $P < 0.05$)

Fatty acid	Significant differences	Difference (mg fatty acid/g larvae)
Linoleic	0.1 vs 10 ppb	13.5
Linoleic	0.1 vs 1000 ppb	13.4
Total FAMEs	0.1 vs 10 ppb	17.9
Total FAMEs	0.1 vs 1000 ppb	12.9

In Trial 1, total fatty acids ranged between 168.9 and 186.8 mg/g. Lauric acid was the dominant fatty acid in all treatment groups ranging from 86.3 to 89.9 mg/g. The only significant differences were the content of linoleic acid and resulting total fatty acid contents, both of which were greater in the 0.1 ppb group (Linoleic: 37.7 ± 3.4, Total FAMEs: 186.7 ± 7.9 mg/g) than in larvae exposed to higher PFOA levels (Mean Linoleic: 24.4 ± 1.2, Total FAMEs: 171.3 ± 4.3 mg/g).

5.3.2. Trial 2

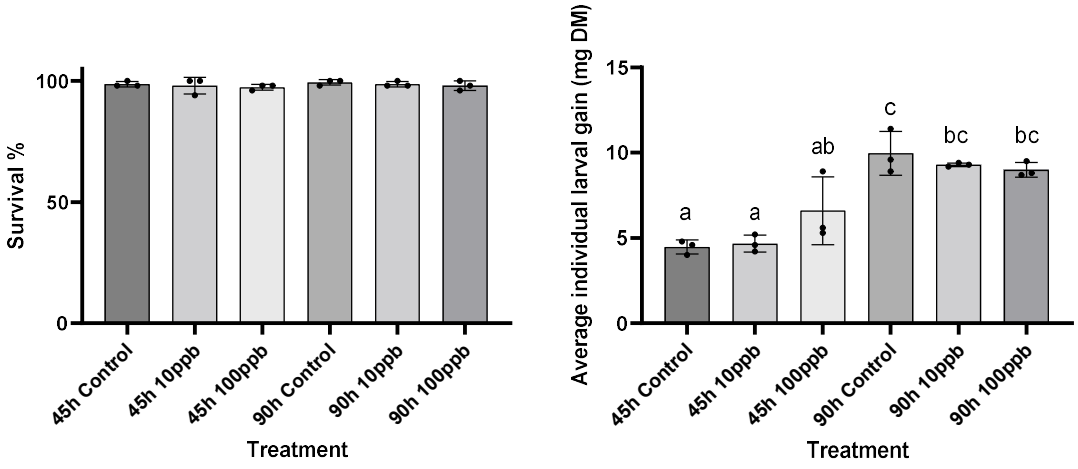

Figure 5.5 Trial 2 performance measures of each treatment in terms of survival and mean individual larval gain (mg DM). Common letters are not significantly different by ordinary one-way ANOVA test at the 5% significance level. Error bars indicate standard deviations where n=3.

As in Trial 1, PFOA content did not influence larval survival nor larval gain. There were outliers in the larval gain of 45h 100ppb and 90h Control groups, demonstrating the trial would have benefitted from further replicates. Unsurprisingly given the additional time for

consumption of substrate and larval development, larval gain of the 90h treatment groups was significantly greater at approximately double that of the 45h groups.

Table 5.4 PFOA concentrations in dry substrate and corresponding concentration in larvae of Trial 2. Errors are standard deviations where n=3.

PFOA concentration in substrate (ppb)	Exposure time (hr)	PFOA concentration in larvae (ppb)	Bioaccumulation factor
0	45	Not detected	n/a
10	45	Insufficient sample	n/a
100	45	63.44 ± 4.57	0.63
0	90	Not detected	n/a
10	90	7.24 ± 0.14	0.72
100	90	63.51 ± 2.70	0.63

Larval PFOA concentrations increase in relation to substrate concentration (Table 5.4). Exposure time does not appear to influence larval PFOA concentrations. Bioaccumulation factors are substantially lower than in Trial 1. Due to low quantities of larval sample available from the short culture times and the use of sample during method development, there was insufficient sample to determine the larval PFOA concentration in the larvae developed for 45 hours on 10 ppb substrate.

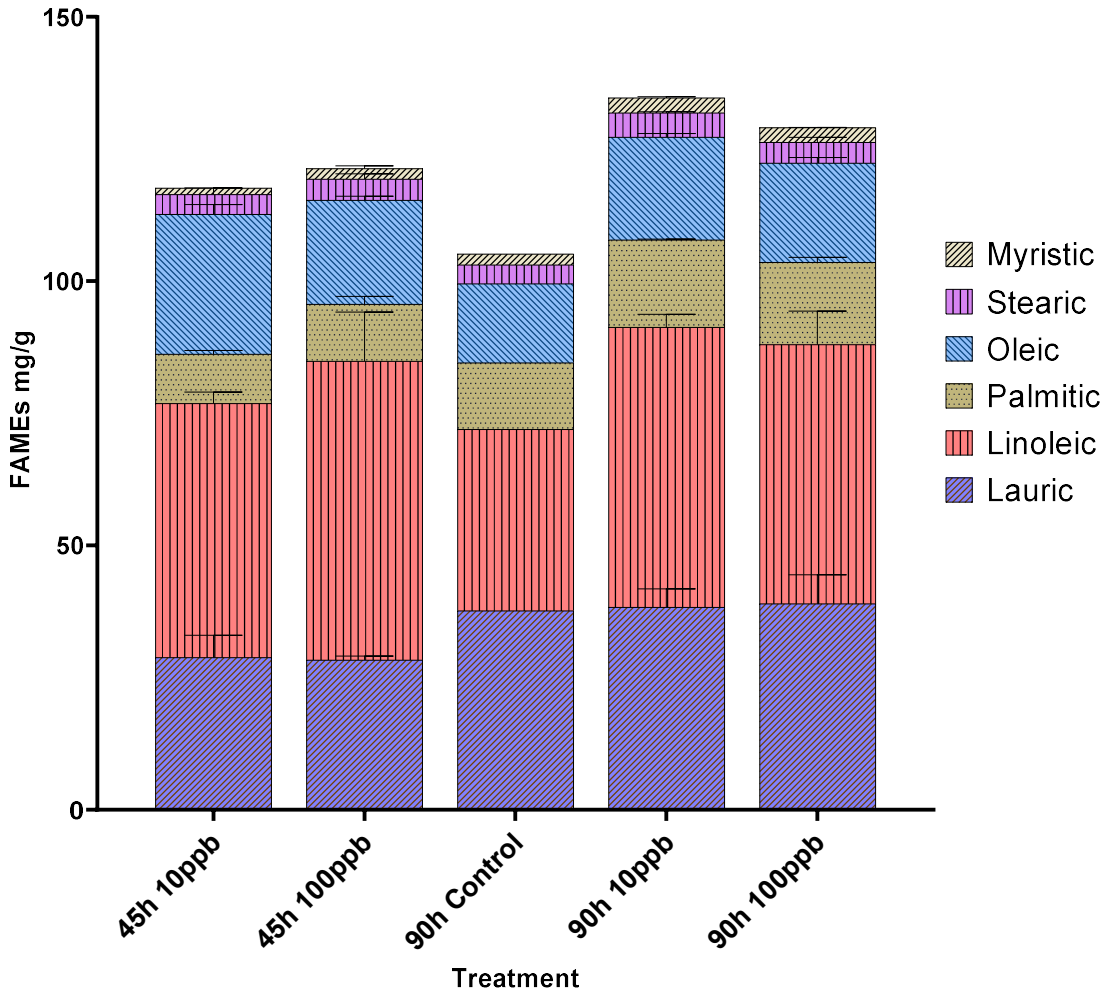

Figure 5.6 Fatty acid compositions of the larvae from each treatment group of Trial 2, excluding the controls. Error bars are standard deviations where n=3, except for the 90h Control where n=1 due to insufficient material for analysis. There was insufficient material from the 45h Control group for fatty acid analysis, hence this was not performed.

Table 5.5 Trial 2 treatments showing significant differences in quantities of fatty acids from Tukey's multiple comparisons tests (adjusted $P < 0.05$).

Fatty acid	**Significant differences**	**Difference (mg fatty acid/g larvae)**
Lauric	45h 100ppb vs. 90h 100ppb	-10.7
Total FAMEs	45h 10ppb vs. 90h 10ppb	-17.0
Total FAMEs	45h 10ppb vs. 90h 100ppb	-11.4
Total FAMEs	45h 100ppb vs. 90h 10ppb	-13.4

In Trial 2, total fatty acids ranged between 105.1 and 134.7 mg/g. Linoleic acid was the dominant fatty acid in most treatment groups ranging from 34.3 to 56.6 mg/g, with the exception of the 90h Control at 34 mg/g, for which there was marginally more lauric acid (37.7

mg/g). No differences in fatty acid contents were found between 10 and 100 ppb treatments of the same culture durations. Lauric acid was slightly increased in larvae cultured for 90 hours instead of 45 hours in the 100 ppb exposure treatment. Total fatty acid contents increased in larvae cultured for 90 hours (Mean 131.9 ± 3.9 mg/g) compared with 45 hours (Mean 119.5 ± mg/g).

5.4. Discussion

5.4.1. Larval Performance

Despite PFAS resulting in various effects on animal growth and development (Pelch et al., 2019), PFOA in this study did not influence larval survival or growth at any of the concentrations tested, in line with findings from the study by Li and Bischel (2022). Mechanisms responsible for PFAS toxicity such as altered gene expression or disrupted hormone signalling may therefore be absent or minimal in BSFL. A study on Drosophila larvae found reduced survival and growth from PFOA exposure, yet these effects were countered by nutrient supplementation of yeast and sucrose (Wang et al., 2010). This may provide an explanation for the lack of toxic effects observed in the present study given the chicken feed was supplied in excess of the larvae's requirements. Using restricted feeding regimes should therefore be studied, whereby PFAS may have greater opportunity to elicit changes in larval survival and development. However, it must be reiterated that without an unconfounded control for Trial 1, the comparative influence of PFOA exposure on larval performance over 11 days cannot be conclusively determined, requiring further validation.

5.4.2. Larval Composition

5.4.2.1. PFOA Accumulation

The concentration of PFOA in larval tissues was minimal in both trials, but higher substrate concentrations and longer exposure times led to higher larval concentrations (as shown in Table 5.2 and Table 5.4). The accumulation factors ranged from 0.63 to 1.64, with

one outlier of 25.50 in the 0.1 ppb treatment in Trial 1, which was likely due to dilution errors (Chase and Hoel, 1975). The United States Environmental Protection Agency (2022) considers these bioaccumulation factors (BAFs) to have minimal bioaccumulation potential. These findings are consistent with Li and Bischel (2022)'s results, which showed BAFs <1 for four PFAS chemicals including PFOA in BSFL spiked at 10 ppb in substrates and cultivated for 14 days. Earthworms and benthic invertebrates have also shown similarly minimal PFOA accumulation (He et al., 2016; Yun et al., 2023). However, the degree of bioaccumulation may vary for other PFAS chemicals, as it typically increases with fluoroalkyl chain length (Haukås et al., 2007).

The present study may have overestimated the BAFs due to incomplete larvae depuration of gut contents prior to extraction, but it is assumed that the extent of purging was similar across treatments due to similarities in larval growth. Though a more involved depuration system such as the one used by Li and Bischel (2022) could have mitigated this, it is unlikely that commercial BSFL producers would perform such depuration (Egnew et al., 2021). Therefore, the present depuration method is appropriate.

Given the BAFs for PFOA were close to 1, it can be suggested that if the substrate PFOA content is below the maximum regulatory limit for animal feed, then it is likely that the produced BSFL will also be below the maximum regulatory limit for substrate concentrations up to 1000 ppb. Such regulatory limits are not currently in place, despite the known health dangers of PFOA and other PFAS (Pelch et al., 2019). It is still advisable to avoid PFOA contaminated materials in BSFL substrates, since the present study only explored chicken feed while other substrates may influence accumulation and low levels may still cause negative health effects up the food chain (Schrenk et al., 2020). Post-processing of BSFL for PFOA removal such as by defatting is unlikely to be effective due to the amphiphilic nature of the compound and its tendency to bind to proteins (Haukås et al., 2007; Pérez et al., 2013).

5.4.2.2. PFOA Influence on Fatty Acid Profiles

The interference of PFAS acids with lipid metabolism in animals has been attributed to their structural similarity to fatty acids, with the substitution of Hydrogen with Fluorine atoms (Sen et al., 2022). This theory was supported in Trial 1, where the total lipid contents of 10 and 1000 ppb treated larvae were lower than those of the 0.1 ppb treatment (Table 5.3 and Figure 5.4), consistent with findings of increased lipolysis in humans exposed to PFAS (Chen et al., 2020). This effect was not replicated in the shorter durations of Trial 2 where no clear trend was observed between PFOA concentration and total larval lipid contents for larvae exposed for 45 and 90 hours (Table 5.5 and Figure 5.6). Additionally, no significant differences were found between the total lipid contents of the 10 and 1000 ppb treatments in Trial 1, despite the 100-fold increase in concentration. While this may indicate that the PFOA had maxed out its influence between 10 and 1000 ppb, further samples are required to confirm the impact of PFOA exposure on larval total lipid contents.

In Trial 1, there were significant reductions in the polyunsaturated linoleic acid of larvae exposed to 10 and 1000 ppb of PFOA, responsible for the observed reduction in total lipid contents (Table 5.3 and Figure 5.4). This finding is consistent with a study in mouse tissue, where PFOA exposure also resulted in altered polyunsaturated fatty acid composition (Lu et al., 2016). Larvae with lower linoleic acid may be less valuable as animal feed given the essential nature of this fatty acid in animal diets (Malcicka et al., 2018). This reduction may be due to the disruption of the metabolic pathways involved in the synthesis and/or consumption of linoleic acid, such as reduction in expression of fatty acid synthase (Haughom and Spydevold, 1992). However, this is in contrast to the findings in *Rana nigromaculata* and invertebrates *Caenorhabditis elegans*, where PFOA increased fatty acid synthesis and overall lipid contents, suggesting that competing enzymes in fatty acid metabolism, such as acetyl-CoA synthetase, may also be influenced by PFOA (Li et al., 2020; Zhang et al., 2019).

Additionally, PFOA may induce oxidative stress, which can cause lipid peroxidation (Li et al., 2021). PFOA exposure did not yield significant differences in fatty acid profiles between treatments in Trial 2 (Table 5.5 and Figure 5.6) possibly due to the large variance in the measures of linoleic and lauric acid, as well as the short exposure duration providing limited opportunity for PFOA to interfere with lipid metabolism.

Comparing between trials, the younger larvae of Trial 2 contained substantially higher proportions of linoleic and oleic acids and less lauric acid compared with the more mature larvae of Trial 1, consistent with literature (Liu et al., 2017). Differences in fatty acid profiles between the trials may additionally be due to the extended freezer storage of Trial 1 samples relative to those of Trial 2, as well as different fly genetics (Fan et al., 2022). Future trials are required to control for these confounding variables.

To further advance our understanding of the impact of PFAS on BSFL, it is crucial to broaden the scope of this study by analysing more samples to confirm the current findings. Additionally, investigating the bioaccumulation potential and the influence of other PFAS chemicals on larval survival and growth is necessary, as newer PFAS chemicals are being employed in place of PFOA, and transfer from feed to animals varies depending on the type of PFAS (Schrenk et al., 2020). Given the critical role that commensurate microorganisms play in BSFL production, there may be value in examining the effects of PFAS on these microorganisms in BSFL production systems, especially since PFOA has been found to alter microbial populations in biosolids (De Smet et al., 2018; Huang et al., 2022). While previous studies have found few chemical contaminants to be toxic to BSFL, with the exception of certain insecticides (Lievens et al., 2021), uncertainties about chemical safety remain, particularly in the context of using varied substrates and fly genetics.

5.5. Conclusion

In conclusion, this study found that PFOA did not influence larval survival or growth at any of the concentrations tested. The accumulation of PFOA in larval tissues was minimal ranging from 0.63 to 1.64, with one outlier of 25.50, indicating limited bioaccumulation potential. The interference of PFOA on fatty acid profiles was supported in Trial 1, but not in Trial 2. While the present study provides insight into the effects of PFOA on BSFL, the findings should be considered preliminary and further research is necessary to confirm the impact of PFOA exposure on larval performance and composition. Nonetheless, these findings suggest that if the substrate PFOA content is below the maximum regulatory limit for animal feed, then the produced BSFL is likely to also be below the maximum regulatory limit for substrate concentrations up to 1000 ppb. Therefore, it is advisable to avoid PFOA contaminated materials in BSFL substrates to prevent negative health effects up the food chain.

Chapter 6. The Implications of Substrate Diversification on BSFL Production from Organic Waste: A Mixed Integer Linear Programming Approach

6.1. Introduction

As discussed in Chapter 2, the inconsistent quality and performance of black soldier fly larvae (BSFL) production due to varying substrate properties poses significant challenges to large-scale deployment of the technology (Gold et al., 2020; Joly and Nikiema, 2019). This chapter examines these real-world consequences by identifying the optimal locations for BSFL facilities in New South Wales, Australia, considering access to diverse organic wastes for use as substrates.

Substrate diversification, in this context, refers to the practice of using a variety of waste types as substrates over a given period, as opposed to relying on a single type of waste. This approach can help to mitigate the effects of substrate variability by providing a broader range of nutrients to the BSFL. While the nutrient content of individual waste types may vary, diversification could potentially lead to more stable production rates over time by reducing the reliance on any single waste type. Using the developed model, several scenarios are analysed to determine the economic potential of substrate diversification to improve larval quality and production performance.

The following research questions are addressed:

1. In the absence of substrate diversification, what are the optimal locations of BSFL facilities and waste transfers in New South Wales, Australia, that maximise profitability?
2. How does substrate diversification, implemented to address substrate variability, impact these optimal locations and waste transfers?

The development of a mixed integer linear programming (MILP) model for locating BSFL facilities in NSW is a practical application of the theoretical and experimental findings of substrate sensitivity from the previous chapters. The resulting model allows for an assessment of the potential benefits of substrate diversification to achieve consistent substrate quality, serving as a preliminary tool in the optimization of BSFL facilities or other waste valorisation technologies.

6.1.1. The Potential of Organic Wastes as BSFL Substrates

Organic wastes are abundant. Food wastes in particular represent a considerable portion of biological nutrient waste globally, making excellent candidates for BSFL production. Around 1.3 billion tonnes of food are wasted each year, equating to approximately one third of all food produced for human consumption (Ishangulyyev et al., 2019). Waste generation varies across the supply chain, with a large proportion generated during the consumption stage in industrialised regions, and a substantial amount produced pre-consumer in the early stages of the supply chain, including primary production and manufacturing (Arcadis, 2019; Gustavsson et al., 2011). Due to safety concerns associated with post-consumer wastes (refer to the literature review of Chapter 2), pre-consumer wastes are more commonly targeted for BSFL production. Table 6.1 below presents a breakdown of the types and quantities of pre-consumer food wastes produced by different sub-sectors in Australia across the 2016-2017 financial year, highlighting the diversity and volume of potential substrates for BSFL production.

Despite their nutritional value, these waste materials are often not reused for human consumption due to poor marketability and low economic value (Carus and Dammer, 2018). They are often mixed with less valuable streams, such as sewage, and used in low-value retention options, including direct composting or waste-to-energy initiatives (Battista et al., 2019), as shown in Table 6.1.

Table 6.1 Types, quantities and dominant destinations of pre-consumer food wastes by sub-sector in Australia across the 2016/2017 financial year (Arcadis, 2019).

Sub-Sector	Representative wastes	Tonnes	Primary destination
Dairy Product Manufacturing	Cheese whey (liquid), Unmarketable milk (liquid), Sludge from wastewater treatment	629,200	Land application
Fruit and Vegetable Pack House	Apples, Oranges, Tomatoes, Bananas, Carrots, Onions, Potatoes	422,300	Compost
Wine Making	Grape marc (seeds, skins, pulp), Lees (dead yeast), Grape stems	223,920	Compost
Livestock	Animal trimmings and offal	123,000	Biochemical processing
Hazardous Liquid Food Waste	Grease trap and used oils	113,300	Other
Nut Processing	Almond pericarp, Macadamia pericarp, Peanut pericarp	82,520	Compost
Baked Product	Bread and crumbs, Dough, Other baked products	60,450	Compost
Seafood	Offal (trimmings and gut contents)	50,080	Landfill
Other Food Product		16,090	Compost
Confectionery	Chocolate, Candy, Sugar based ingredients	15,290	Compost
Beer Making	Brewer's grains, Lees (dead yeast)	14,570	Compost
Cereal, Pasta & Baking Mix	Grain and starchy products (e.g. pasta)	7,310	Compost
Soft Drink, Cordial & Syrup	Syrups	470	Landfill
TOTAL Food Waste		1,758,500	

The variety of food wastes outlined in Table 1 present a rich resource for BSFL production. However, substrate sensitivity—as explored in previous chapters—makes this a complex task due to the inherent variability in these waste sources. Diversifying the use of wastes of different types can partially mitigate this issue by providing a broader range of nutrients to the BSFL (Gold et al., 2020). However, this introduces new considerations for logistics, particularly regarding facility location selection and waste allocation. To illustrate these challenges and underscore the necessity of better understanding substrate sensitivity, this chapter employs a Mixed Integer Linear Programming (MILP) model. MILP was selected over other techno-economical modelling approaches due to its significant optimisation power relative to the computational demand. The following section introduces MILP and how it is employed in the present context.

6.1.2. Optimising BSFL Production from Food Wastes using MILP

Mixed integer linear programming is a mathematical optimization technique that enables efficient allocation of limited resources among competing activities (Artigues et al.,

2015). MILP extends the capabilities of linear programming, which can only handle continuous variables, by including integer-based variables in the model. This provides a more realistic representation of real-world situations where variables are often discrete, such as the number of BSFL facilities (Artigues et al., 2015). The objective function in a MILP model is a linear equation that represents the goal to be maximized or minimized, subject to a set of linear constraints that define the relationships among the variables.

In developing a comprehensive MILP model for the present study, the location, type, and quantity of pre-consumer food wastes are considered, as these determine the potential substrate for the BSFL facilities. The properties of the waste materials (such as nutrient contents) are crucial, as they influence the quality and quantity of BSFL production as covered in the previous chapters. In the present optimisation, the influences of substrate properties are greatly simplified to avoid over-complicating the problem and ensure relationships remain linear and compatible with MILP. Approximate conversion factors are used as discussed in the methodology.

These elements, alongside the capacity of the BSFL facilities and transportation costs, play a significant role in the model. Constraints on the capacity of each facility are included in the model to ensure that the solutions generated are feasible and realistic. Transportation costs are incorporated as part of the objective function to ensure that the solutions generated minimize these expenses and maximize overall profitability.

6.2. Methodology

6.2.1. Organic Waste Mapping

The organic waste mapping process occurred in three stages. Firstly, the locations of facilities which produce significant quantities of pre-consumer wastes (hereon termed ‘waste generators’) were identified. Estimates of each generator’s capacity were then sourced or otherwise approximated. A conversion factor approximating the waste quantity produced per

unit of capacity is then applied to each waste generator, yielding the approximate waste quantity generated at each generator per year. Several organic waste streams which are not food wastes have additionally been included due to their potential for BSFL valorisation.

Location data was largely sourced from the NSW Environmental Protection Agency (EPA) Protection of the Environment Operations (POEO) licenses register (NSW EPA, 2020). Waste generators captured from this data are those whose activities are scheduled under schedule 1 of the POEO Act 1997 No 156. Waste generators which do not significantly produce organics (e.g., cement production) were removed. Data from the National Pollutant Inventory (NPI) has been used to locate waste generators of interest missing in the EPA POEO register (DCCEEW, 2020). Location data from the EPA POEO register are reported as addresses rather than co-ordinates, preventing accurate mapping of generators. Co-ordinates have therefore been deduced through a combination of manual searching through Google maps and using Google's geocoding API, a free service which converts input addresses to co-ordinates (Google, 2020).

Individual EPA licenses (EPLs) were sourced and examined to identify waste generator capacities. Where capacities are reported as a range (between two values), the mean of the limits has been taken. Some capacities have been reported according to recent production figures and others as maximum processing capacities. The only measure of generator scale reported in the NPI data are employee counts, from which meaningful waste estimates are challenging to produce. As such, these have only been used for estimating waste quantities in the absence of capacity-based measures. Where locations and capacity data for significant waste generators are missing, these have been estimated by either directly contacting the company where possible, or from news articles and general internet searches otherwise (see appendices for further details).

Appropriate conversion factors from the reported generators' capacity to a waste estimate have been sourced, assumed, or estimated, per generator type, as described in appendices. It should be noted that these estimates include streams that are not treated solely as wastes (e.g., streams that become animal feed) and therefore may have a market value. The resulting waste estimates were plotted to demonstrate their regional locations (see 6.3.1).

During development of the MILP model, the waste generator dataset was simplified. This involved the removal of some outlier waste generators which produced 'low-quality' wastes (such as cotton gin trash) far from other generators. Additionally, several waste types such as those which were predominantly liquid such as whey from dairy manufacture and wastewater streams from aquaculture, due to the low yield of larvae that may be produced from them.

6.2.2. Modelling

The MILP model was developed in MATLAB. The details behind parameters and model logic are provided in the following sections and appendices. To ensure the models remained linear, a two-stage approach was applied. Firstly, potential facility locations (hereon termed 'candidate facilities') were placed within the bounds of the waste generators, creating a set of discrete candidate locations for facilities. This avoids the use of non-linear relationships for determining facility locations, as would occur by calculating the distances within the objective function directly. Great circle distances between each candidate facility and waste generator were then calculated using the Haversine formula and a tortuosity factor from literature. Secondly, the MILP problem is defined in terms of its decision variables and constraints before solving using the 'intlinprog' function in MATLAB. The strategies and algorithms used in intlinprog are outlined in full in the documentation MathWorks (2023). After the problem has been solved once, the program iterates the complete process, generating new candidate facilities within a defined radius of the previously selected

facilities, thereby incrementally improving on the result. If a solution is less optimal than the previous, the model backtracks, adopting the previous solution.

Assumptions and simplifications have been used to minimise the complexity of the model both for reducing computational requirements and to avoid detracting from the clarity of the findings which are intended to answer the research questions outlined in the introduction. These are outlined in the following sections and appendices.

6.2.2.1. Scenarios

The model was designed to simulate two distinct scenarios: Scenario 1, where each facility is limited to processing a single type of waste, and Scenario 2, in which facilities can process multiple types of waste. This is done by imposing a constraint on the model for Scenario 1, preventing any facility from processing more than one type of waste. For Scenario 2, this limit is increased to ten types, allowing for substrate diversification. This approach provides a direct, 'apples to apples' comparison between the scenarios of limited and diversified substrate use without necessitating any other changes to the model's sets, parameters, variables, objective function, or constraints. The model formulation is presented in the following sections in accordance with the guidelines of Teter et al. (2016). Nomenclature used in the model formulation is outlined in Table 6.2.

Table 6.2 Nomenclature used in the model formulation. See appendices for calculations and justifications.

Term	Type	Dimensions	Explanation	Value (Base case)	Units
$i \in I$	Index and set	I × 1	Index and set for candidate facilities		Unitless
$j \in J$	Index and set	J × 1	Index and set for substrates		Unitless
$k \in K$	Index and set	K × 1	Index and set for waste generators		Unitless
P_L	Parameter	Scalar	Value per unit mass of BSFL lipid produced	500	AUD/tonne
P_M	Parameter	Scalar	Value per unit mass of BSFL meal produced	1,000	AUD/tonne
P_k	Parameter	K × 1	Price per unit mass of waste from generator k	0	AUD/tonne
$D_{i,k}$	Parameter	I × K	Distance between generator k and facility i	Calculated	km
P_t	Parameter	Scalar	Cost per kilometre per tonne for transport	1.3 (Pearman, 2018)	AUD/tonne/km
P_{EAFC}	Parameter	Scalar	Equivalent annual fixed cost per facility	Calculated	AUD/facility
P_v	Parameter	Scalar	Operating cost per unit mass of waste processed	45	AUD/tonne
G_k	Parameter	K × 1	Quantity of waste generated by generator k	Calculated	tonnes/year
F	Parameter	Scalar	Minimum fraction of facility capacity used	0.7 (Suckling et al., 2021)	Unitless
Q	Parameter	Scalar	Maximum capacity of each facility	50,000	tonnes/year
U	Parameter	Scalar	Minimum fraction of total waste to be processed	0.2	Unitless
M	Parameter	Scalar	Large constant	1,000,000	Unitless
τ	Parameter	Scalar	Tortuosity factor	1.27 (Sultana and Kumar, 2014)	Unitless
C_j^M	Parameter	J × 1	Protein conversion factor for substrate j	Calculated per (Barragan-Fonseca et al., 2021)	Unitless
C_j^L	Parameter	J × 1	Lipid conversion factor for substrate j	Calculated per (Barragan-Fonseca et al., 2021)	Unitless
S_k^P	Parameter	K × 1	Protein content of waste from generator k on a wet mass basis	Calculated per (Barragan-Fonseca et al., 2021)	Unitless
S_k^C	Parameter	K × 1	Carbohydrate content of waste from generator k on a wet mass basis	Calculated per (Barragan-Fonseca et al., 2021)	Unitless
S_j^P	Parameter	J × 1	Protein content of substrate j on a wet mass basis	Known from substrate data	Unitless
S_j^C	Parameter	J × 1	Carbohydrate content of substrate j on a wet mass basis	Known from substrate data	Unitless
$y_{i,k,j}^P$	Decision variable (auxiliary)	I × K × J	Protein content of substrate j at facility i		Unitless
$y_{i,k,j}^C$	Decision variable (auxiliary)	I × K × J	Carbohydrate content of substrate j at facility i		Unitless
$b_{i,j}$	Decision variable	I × J	Binary variable indicating if substrate j is used at facility i		Unitless
$w_{i,k,j}$	Decision variable	I × K × J	Quantity of waste from generator k used in substrate j at facility i		tonnes/year
e_i	Decision variable	I × 1	Binary variable denoting the existence of a facility at location i		Unitless

6.2.2.2. Objective Function

The objective is to maximize total profit, which is calculated as revenue less cost. Revenue is created by the sale of BSFL meal and lipid, which are produced at the facilities by conversion of organic wastes. The revenue per unit substrate from selling BSFL protein meal is calculated as the mass fraction of meal produced from a substrate (C_j^M) multiplied by the value per unit mass of the meal sold (P_M). The revenue from selling BSFL lipid is calculated similarly. Additionally, revenue may be created through the provision of the waste management service to the waste generator (P_k), however such wastes are typically 'low quality' due to poor nutrient contents or contaminants, while high quality 'wastes' typically have market value and hence may represent a cost for the BSFL facilities to acquire.

Costs are incurred from waste transport, additional variable costs (P_v) such as those associated with labour, energy, materials and maintenance, and fixed costs such as those associated with infrastructure. The transportation cost is determined by the quantity of waste transported ($w_{i,k,j}$), the cost per kilometre per tonne for transport (P_t), and the distance between each facility and waste generator ($D_{i,k}$).

All revenues and costs are calculated on a one-year basis for simplicity. The fixed costs have therefore been calculated as equivalent annual fixed costs (P_{EAFC}), as described in full in following section on Parameters. The objective function is therefore formulated as follows:

$$Maximize\ \{Revenue - Cost\}$$

$$Maximize \sum_{i\in I, k\in K, j\in J} \left[\left(C_j^M P_M + C_j^L P_L - P_k - P_t D_{i,k} - P_v\right) w_{i,k,j}\right] - \sum_{i\in I} P_{EAFC}\, e_i \qquad 1$$

Where I is the set of facilities, K is the set of waste generators, J is the set of acceptable substrates, C_j^M is the mass fraction of dry defatted BSFL meal produced from processing substrate j, P_M is the value per unit mass of dry defatted BSFL meal sold

(AUD/tonne), C_j^L is the mass fraction of dry BSFL lipid produced from processing substrate *j*, P_L is the value per unit mass of dry BSFL lipid sold (AUD/tonne), $w_{i,k,j}$ is the quantity of wet waste transported from generator k used in substrate j at facility i (tonnes/year), P_k is the price per unit mass of wet waste from generator k (AUD/tonne), P_t is the cost per kilometre per tonne of wet waste transported (AUD/km/tonne), $D_{i,k}$ is the distance between facility i and waste generator k, P_v is the operating cost per unit mass of wet waste processed (AUD/tonne), P_{EAFC} is the equivalent annual fixed cost associated with each facility (AUD/facility) and e_i is the binary decision variable denoting the existence of facility *i.*

6.2.2.3. Constraints

Each constraint in the model is a representation of real-world limitations or requirements. The objective function is maximised subject to these constraints:

Maximum Waste Processing Capacity of Facility: The total waste processed at each selected facility cannot exceed the facility's maximum capacity (Q):

$$\sum_{k\in K, j\in J} w_{i,k,j} \leq Qe_i \quad \forall i \in I \qquad 2$$

Minimum Facility Capacity Usage: Each selected facility must use a minimum fraction '*F*' of the facility's maximum capacity:

$$\sum_{k\in K, j\in J} w_{i,k,j} \geq FQe_i \quad \forall i \in I \qquad 3$$

Substrates per Facility: Each facility uses at most '*S*' substrates:

$$\sum_{j\in J} b_{i,j} \leq S \; \forall i \in I \qquad 4$$

Substrate Choice and Waste Usage: Waste streams are only used in a substrate if that substrate is chosen:

$$w_{i,k,j} \leq Mb_{i,j} \; \forall i \in I, k \in K, j \in J \qquad 5$$

Waste Generation Constraint: The quantity of waste transported from each generator to be used in any substrate is constrained by the total waste generated at that location (G_k):

$$\sum_{i\in I, j\in J} w_{i,k,j} \leq G_k \quad \forall k \in K \tag{6}$$

Minimum Waste Processing Constraint: A minimum fraction '*U*' of the total waste generated must be processed:

$$\sum_{i\in I, k\in K, j\in J} w_{i,k,j} \geq U \sum_{k} G_k \tag{7}$$

Total Waste Processing Constraint: The total waste processed by all facilities should not exceed the total waste generated:

$$\sum_{i\in I, k\in K, j\in J} w_{i,k,j} \leq \sum_{k\in K} G_k \tag{8}$$

Substrate Contents Constraints: The actual protein $\left(y^P_{i,k,j}\right)$ and carbohydrate contents $\left(y^C_{i,k,j}\right)$ of the waste processed from each generator *k*, for each substrate *j* used at each facility *i*, must match the defined protein (S^P_j) and carbohydrate content (S^C_j) of the selected substrate *j*. The constraints are enforced as follows:

$$\begin{aligned} &\sum_{k\in K} y^P_{i,k,j} - S^P_j \sum_{k\in K} w_{i,k,j} = 0, \forall i \in I, j \in J \\ &y^P_{i,k,j} - S^P_k w_{i,k,j} = 0, \forall i \in I, k \in K, j \in J \end{aligned} \tag{9}$$

The same constraints apply for the carbohydrate contents with analogous variables and coefficients:

$$\begin{aligned} &\sum_{k\in K} y^C_{i,k,j} - S^C_j \sum_{k\in K} w_{i,k,j} = 0, \forall i \in I, j \in J \\ &y^C_{i,k,j} - S^C_k w_{i,k,j} = 0, \forall i \in I, k \in K, j \in J \end{aligned} \tag{10}$$

Binary Constraints: The variables e_i and $b_{i,j}$ are binary decision variables:

$$e_i \in \{0,1\}, \forall i \in I, j \in J$$

$$b_{i,j} \in \{0,1\}, \forall i \in I, j \in J$$ 11

6.2.2.4. Larval Meal and Lipid Conversion Factors

Larval meal and lipid conversion factors were determined for each substrate according to their protein and carbohydrate contents using data from (Barragan-Fonseca et al., 2021). First, the protein, carbohydrate and moisture contents of each waste material were sourced largely from the Feedipedia database (UN FAO, 2023). This data is provided in the table below.

Table 6.3 Moisture, protein and carbohydrate contents of waste materials used in BSFL substrates, as sourced from Feedipedia's database unless otherwise indicated.

Substrate material	Moisture %	Protein % (DM)	Carbohydrate (starch + sugars) % (DM)	Source
Bakery bread waste	10	12	71	Feedipedia
Spent Brewer's grains	75	26	7	Feedipedia
Oilseed processing wastes	8	34	17	Feedipedia
Cotton trash	60	7	0	(Meyer, 2007)
Wine distillery wastes	60	12	20	Feedipedia
Feedlot grain waste	12	15	62	Feedipedia
Feedmill grain waste	12	15	62	Feedipedia
Food processing wastes	60	6	59	Feedipedia
Grain mill wastes	12	15	62	Feedipedia
Grain storage wastes	12	15	62	Feedipedia
Market wastes	80	7	14	Feedipedia
Meat processing wastes	75	53	0	Feedipedia
Almond pericarps	10	6	32	Feedipedia
Paper production wastes	44	1	0	Feedipedia
Sugar milling and refining wastes	94	12	37	Feedipedia
Winery wastes	60	12	20	Feedipedia

Using the figures presented in (Barragan-Fonseca et al., 2021), development times, yields and larval lipid contents were estimated for each substrate material based on the protein and carbohydrate dry mass percentages (data in appendix).

These were then converted into conversion factors per unit dry substrate per year for both BSFL meal and lipid, considering the substrate added in (Barragan-Fonseca et al., 2021). BSFL meal was assumed the remaining high-protein material after lipid is extracted. An example calculation for the BSFL meal conversion factor is provided below:

$$C_j^M = \frac{\frac{L}{S_0}}{T} * 365 * L_M \quad (12)$$

Where L is the total dry mass of larvae produced (g), S_0 is the total input dry substrate (g), T is the development time (days) and L_M is the mass fraction of L that corresponds to BSFL meal. For example, for a substrate composed of 100% bakery bread waste, the conversion factor for BSFL meal is calculated as follows:

$$\frac{\frac{3}{80}}{28} * 365 * 0.73 = 0.36\ g\ BSFL\ meal/g\ dry\ substrate/year$$

This is calculated on a dry mass basis for substrates at 60% moisture content, so it is adjusted to a wet mass basis by multiplying by 0.4 before use in the model. Note that it is assumed that all substrates are adjusted to 60% moisture content at each BSFL facility. Additionally, the protein and carbohydrate contents of each substrate material were adjusted to a wet matter basis before use in the model. The resulting data is provided below:

Table 6.4 Substrate data table showing wet mass nutrient contents and estimated conversion factors for each substrate material.

Substrate material	Protein % (WM)	Carbohydrate % (WM)	Meal conversion factor (g meal / g WM / year)	Lipid conversion factor (g lipid / g WM / year)
Bakery bread waste	11.2	64.2	0.143	0.053
Spent Brewers grain	6.5	1.7	0.124	0.039
Oilseed processing waste	31.0	15.3	0.112	0.048
Cotton trash	2.8	0.0	0.085	0.006
Wine distillery waste	4.7	8.0	0.168	0.037
Feedlot grain waste	13.2	54.6	0.155	0.064
Feedmill grain waste	13.2	54.6	0.155	0.064
Food processing waste	2.4	23.6	0.166	0.053
Grain mill waste	13.2	54.6	0.155	0.064
Grain storage waste	13.2	54.6	0.155	0.064
Market waste	1.4	2.8	0.147	0.020
Meat processing waste	13.3	0.0	0.000	0.000
Almond pericarps	5.1	29.1	0.171	0.033
Paper production waste	0.4	0.0	0.000	0.000
Sugar milling and refining waste	0.7	2.2	0.184	0.052
Winery waste	4.7	8.0	0.168	0.037

6.2.2.5. Product Prices

Bulk BSFL product prices are poorly defined and shown to vary greatly across locations (Joly and Nikiema, 2019). For the purposes of the present model, larval meal and lipid prices were estimated from prices over the past five years of 65% protein fishmeal and soybean oil. Per IndexMundi.com, these ranged from 1.91 – 2.62 k AUD/tonne for fishmeal and 1 – 2.8 k AUD/tonne for soybean oil over the 2018 – 2023 period. For conservatism, values approximately half of the minimums were used in the base case of the model as AUD 1,000 and AUD 500 per tonne of larval meal and lipid respectively.

6.2.2.6. Transport Costs

Transport costs were calculated as the approximated distance between facilities and waste generators multiplied by an approximate cost in AUD per tonne per km of 1.3

(Pearman, 2018). Distances were calculated as great circle distances using the haversine formula that yields the shortest distance between two points on the surface of a sphere, given their longitudes and latitudes. The resulting distance was multiplied by a tortuosity factor of 1.27 (Sultana and Kumar, 2014) to account for the fact that roads do not travel in straight lines between each facility and generator. The haversine formula is as follows:

$$d = 2R \arcsin\left(\sqrt{\sin^2\left(\frac{lat2 - lat1}{2}\right) + \cos(lat1)\cos(lat2)\sin^2\left(\frac{lon2 - lon1}{2}\right)}\right) \qquad 13$$

Where d is the distance between the two points in km, R is the radius of the earth in km, and *lat1, lat2, lon1, lon2,* are the respective latitudes and longitudes of each point.

6.2.2.7. Variable Costs

Operating costs for BSFL facilities were estimated based on data reported in (Joly and Nikiema, 2019) for several case study locations. The most suitable case study for the current Australian context was identified as Enterra Feed in Canada, due to its close similarity in terms of median income. At the time of writing, according to World Population Review, the median income for Australia is 17,076 USD and for Canada, it's 18,652 USD.

Enterra Feed processes 100 tonnes of waste per day, equating to approximately 36,500 tonnes per year. The facility operates with 32 full-time equivalent employees, which is assumed to represent labour costs. The ratio of operators to daily tonnes of waste processed is 0.3.

Based on Joly's study, labour costs as a percentage of total operating costs can range from 30 - 65%, hence for the present model, a proportion of 50% has been selected.

Given that 32 employees are paid a median income of 17,076 USD per year to process 36,500 tonnes of waste, the labour cost per tonne of waste processed comes out to approximately 15 USD. With the assumption that labour costs represent 50% of total operating costs, the

overall variable cost is approximately 30 USD per tonne of waste processed. In Australian dollars at the time of writing, this equates to about 45 AUD per tonne of waste processed.

6.2.2.8. Fixed Facility Costs

Fixed facility costs were calculated prior to each run using the 6/10ths rule (Moore, 1959), which is encompassed as follows:

$$Cost\ 2\ =\ Cost\ 1\left(\frac{Size\ 2}{Size\ 1}\right)^{0.6} \quad 14$$

The values of Cost 1 and Size 1 were adopted from Enterra Feed, reported in (Joly and Nikiema, 2019) as USD 7,500,000 infrastructure costs and 100 tonnes capacity per day, respectively. The price of 1 USD in AUD at the time of writing is 1.5 AUD, and the capacities input in the model are per year. Hence, the calculation became:

$$Cost\ 2\ =\ 7500000 * 1.5\left(\frac{Size\ 2}{36500}\right)^{0.6} \quad 15$$

All other revenue and cost streams are calculated on a yearly basis; therefore this fixed cost is converted to an equivalent annual fixed cost (EAFC) based on a projected lifespan of the facility. The equation for calculating the equivalent annual cost is:

$$P_{EAFC} = Cost\ 2 \times \left[\frac{r \times (1+r)^n}{(1+r)^n - 1}\right] \quad 16$$

Where r is the discount rate and n is the number of years of projected lifespan. Assuming a lifespan of 20 years and a discount rate of 9% (Synergies Economic Consulting, 2022), an equivalent annual fixed cost per facility can be calculated as follows:

$$P_{EAFC} = Cost\ 2 \times \left[\frac{0.09 \times (1+0.09)^{20}}{(1+0.09)^{20} - 1}\right] \quad 17$$

6.2.2.9. Sensitivity Analysis

The complete developed waste generator dataset excluding meat and paper processing was run six times for Scenario 2, with the solution which yielded the highest profit presented in Figure 6.5. Scenario 1 was significantly more time intensive to run and hence, due to time

constraints, a subsample of 50 waste generators (hereon termed ‘the subsample’) was selected and used for comparing Scenarios 1 and 2. Nine equivalent runs were performed for both Scenarios 1 and 2 on this subsample, with the solution which yielded the highest profit in both scenarios presented in Figure 6.6 and Figure 6.7. Additionally, this same subsample was used for sensitivity analysis of several parameters including the maximum facility capacity, minimum total waste processed constraint, waste acquisition price, transport price, and prices of larval meal and lipid. Each parameter setting was run at least twice.

6.3. Results and Discussion

6.3.1. Organic Wastes in New South Wales

The mapping results from this section are provided in Figure 6.1, with summaries of waste estimates and numbers of generators broken down per generator type in Figure 6.2 and Figure 6.4.

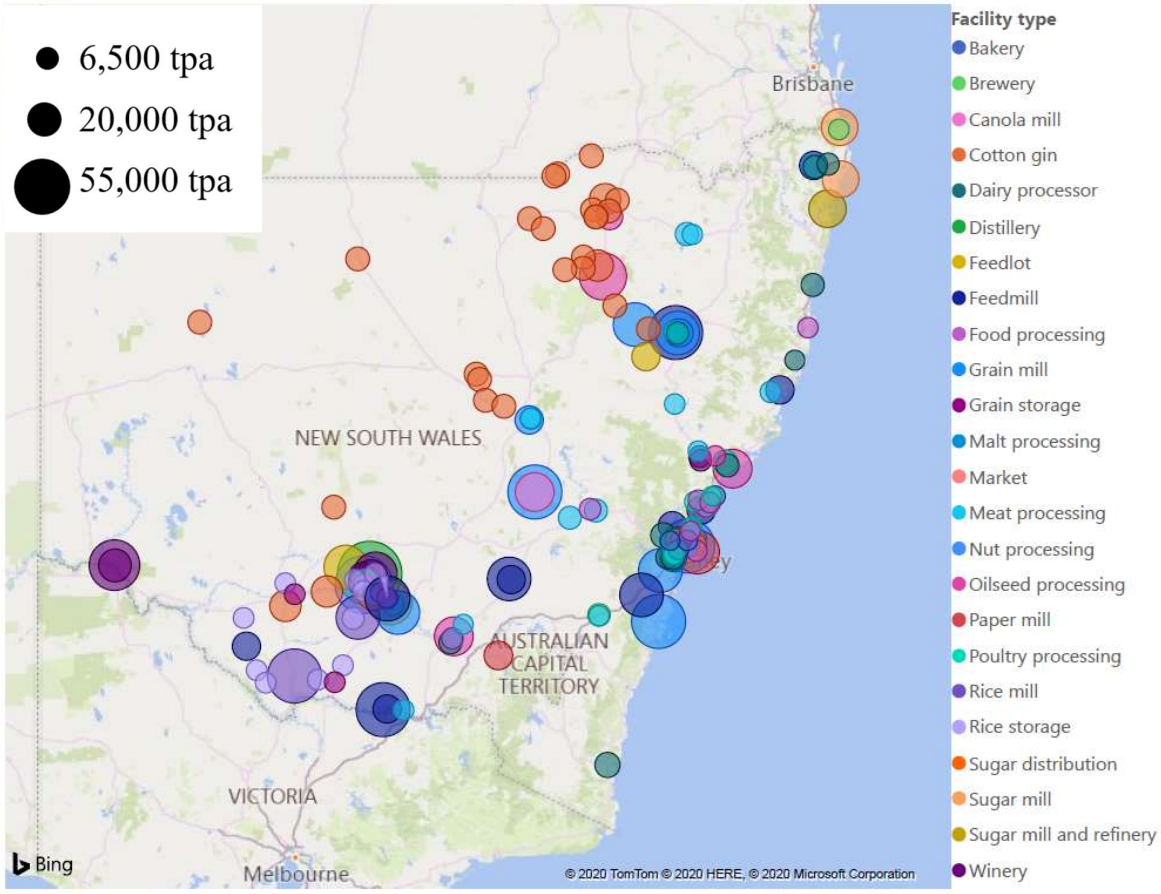

Figure 6.1 Overview of significant pre-consumer organics estimates for NSW.

Figure 6.1 shows pre consumer organics are generated across the state particularly in the east, with significant urban clusters around the Sydney Metropolitan, Central Coast and Hunter regions. Notable clusters in more rural areas include the Riverina and New England regions. The types of wastes produced vary across the state.

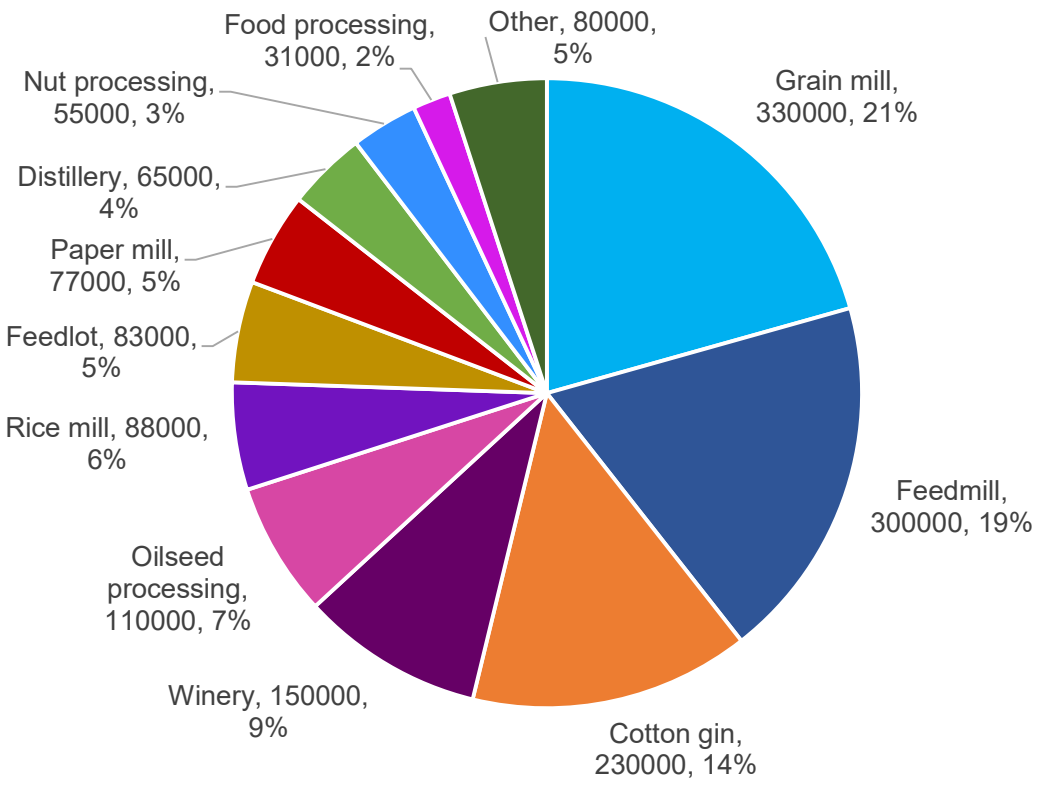

Figure 6.2 Summary of the total NSW solid pre-consumer waste estimates per generator type.

Figure 6.2 presents the combined waste estimates across the state for pre-consumer generators (excluding supermarkets) whose streams are predominantly solid. Cereals and grain by products from milling dominate, followed by cotton gin trash and winery grape marc produced largely in regional NSW.

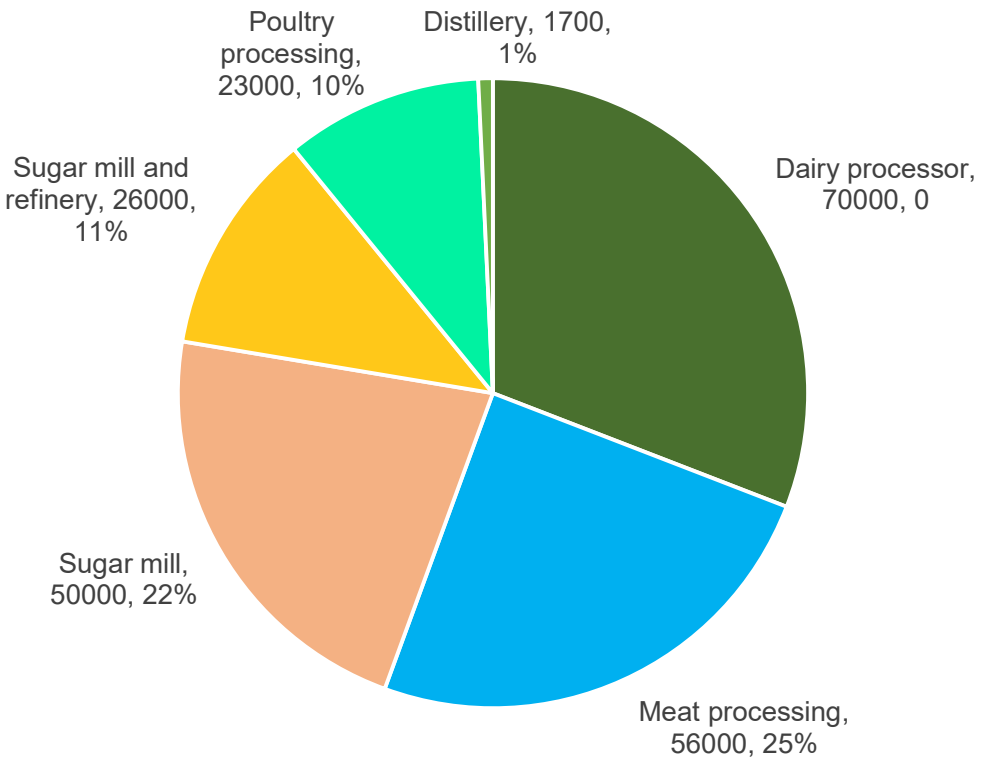

Figure 6.3 Summary of the total NSW liquid pre-consumer waste estimates per generator type.

Figure 6.3 presents the combined waste estimates from pre-consumer generators which are predominantly liquid. Dairy processing whey, meat processing organics and molasses from sugar processing form the bulk of these wastes, generated across the state. These wastes are likely less amenable to BSFL production due to their dilute nutrient content and the need for dewatering.

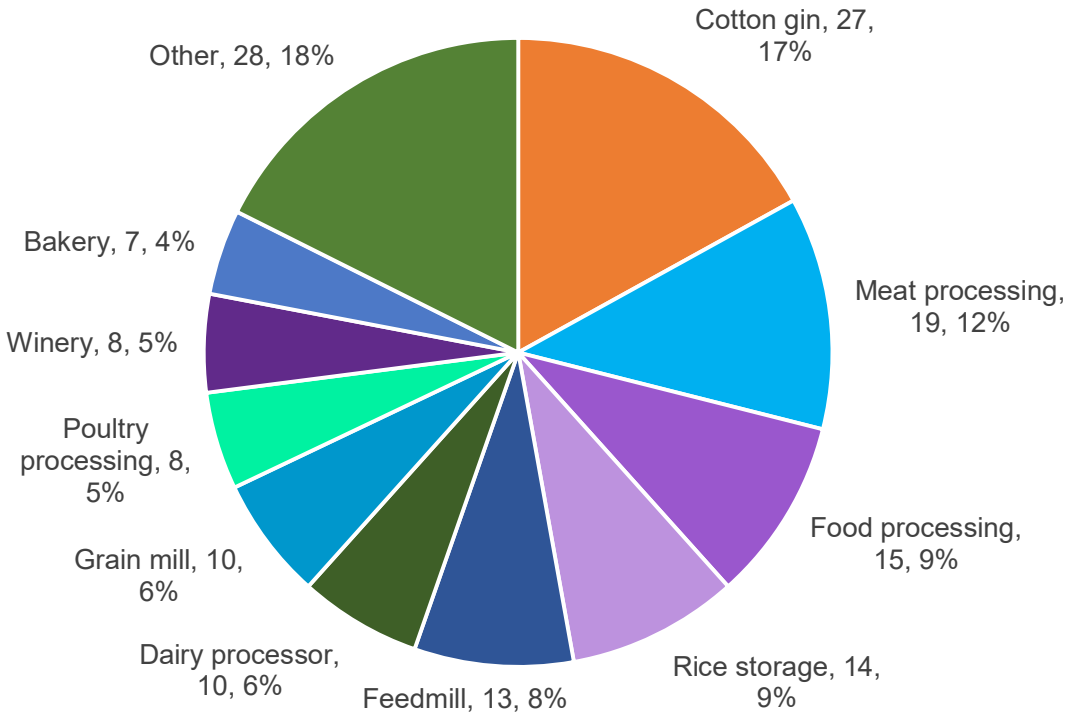

Figure 6.4 Summary of the number of NSW facilities generating pre-consumer organic wastes (both solid and liquid) per generator type.

Figure 6.4 presents the count of significant generators identified per category. Viewing figures Figure 6.2, Figure 6.3 and Figure 6.4 together provides an overview of the concentration of these waste streams, with lower quantities distributed across many generators yielding a low concentration as in the case of rice storage compared with grain mills and dairy processors, for which large quantities are produced from fewer generators. Targeting streams of higher concentration will facilitate easier logistics. Generator types in the 'Other' segment have four generators or less and include oilseed processing, paper mills, feedlots, rice mills, distilleries, grain storage, malt processing, sugar mills and refineries, breweries, markets, and nut processing.

6.3.2. Model Outputs

The model outputs provide insights into the impact of substrate diversification on the optimal locations and profitability of BSFL facilities. For the full NSW dataset allowing

waste diversification, the model identified 27 locations for placement of BSFL production facilities when using the base-case constant processing capacity of 50,000 tonnes per year. Locations with multiple facilities indicate the placement of a single, larger capacity facility in that location may be beneficial (Figure 6.5). A breakdown of the overall results from this run is provided in Table 6.5.

Table 6.5 Results from the model after 10 iterations for Scenario 2 for all generators across NSW excluding meat and paper processing for the base-case settings of parameters. Facility processing capacities are set to 50,000 tpa.

Result	**Value**
Facilities selected	27
	Million AUD/year
Total Profit	114
Total Revenue	233
BSFL Meal Revenue	198
BSFL Lipid Revenue	35
Total Costs	119
Total Transport Costs	21
Fixed Facility Costs	40
Variable Facility Costs	57
	Tonnes/year
Total Waste Processed	1,275,200
Fraction of total waste available that was processed	0.82
BSFL Meal Produced	197,516
BSFL Lipid Produced	70,605

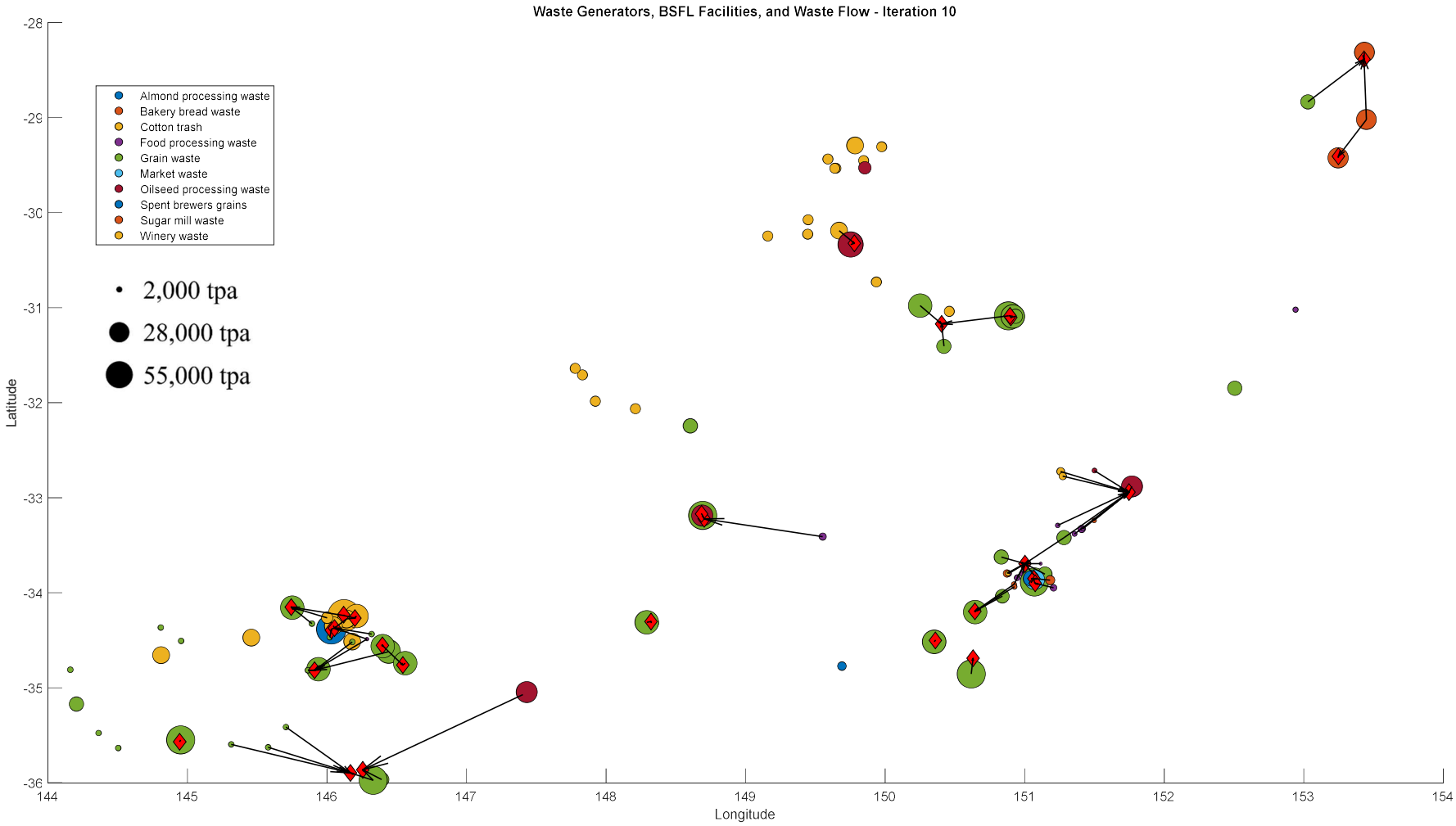

Figure 6.5 Output from the model after 10 iterations for Scenario 2 for all generators across NSW excluding meat and paper processing for the base-case settings of parameters. Facility processing capacities are set to 50,000 tpa. The sizes of generator markers are proportional to the waste generated at that location.

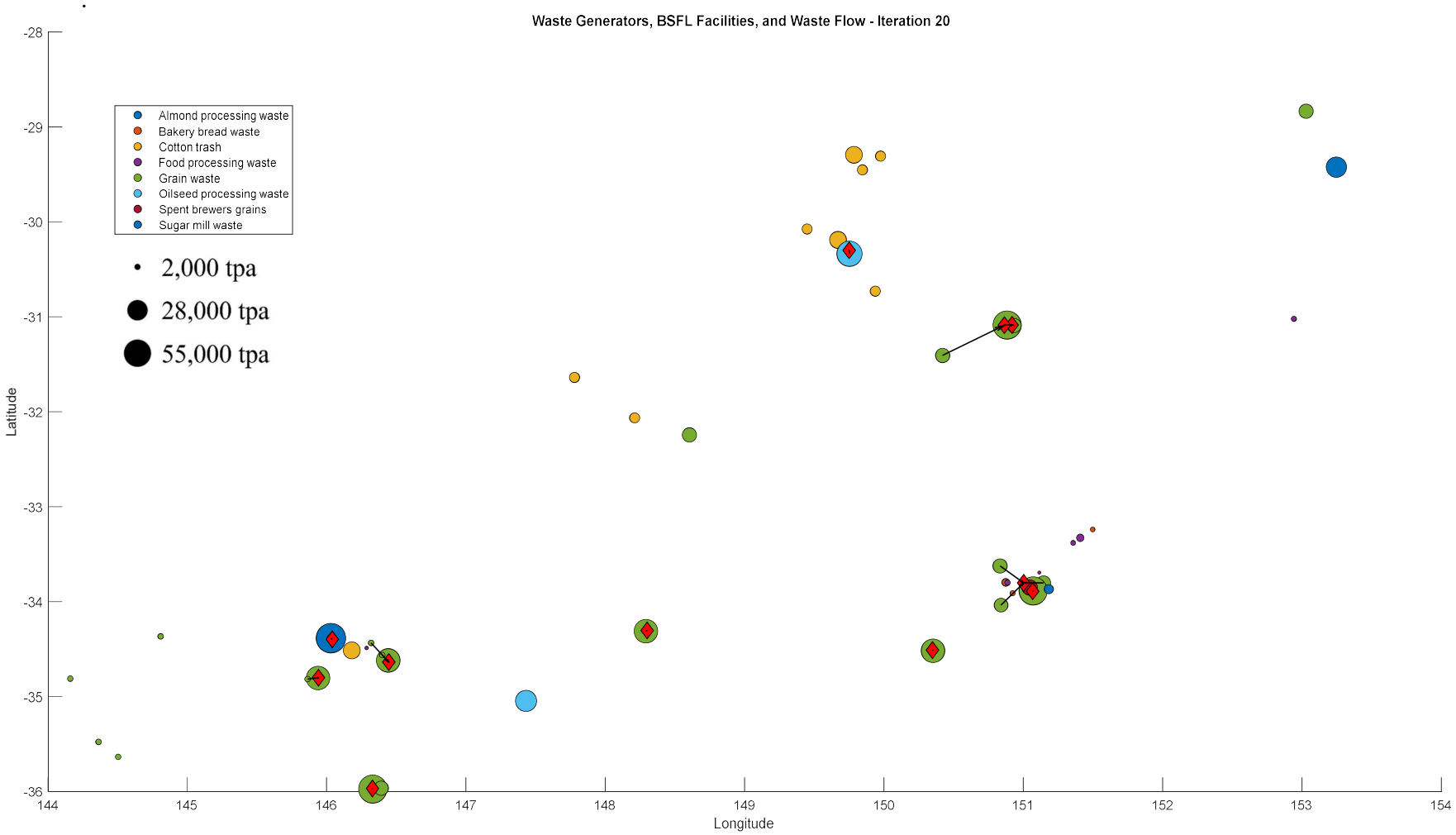

Figure 6.6 Output from the model after 20 iterations for Scenario 1 for a subsample of 50 generators across NSW excluding meat and paper processing for the base-case settings of parameters. Facility processing capacities are set to 50,000 tpa. The sizes of generator markers are proportional to the waste generated at that location.

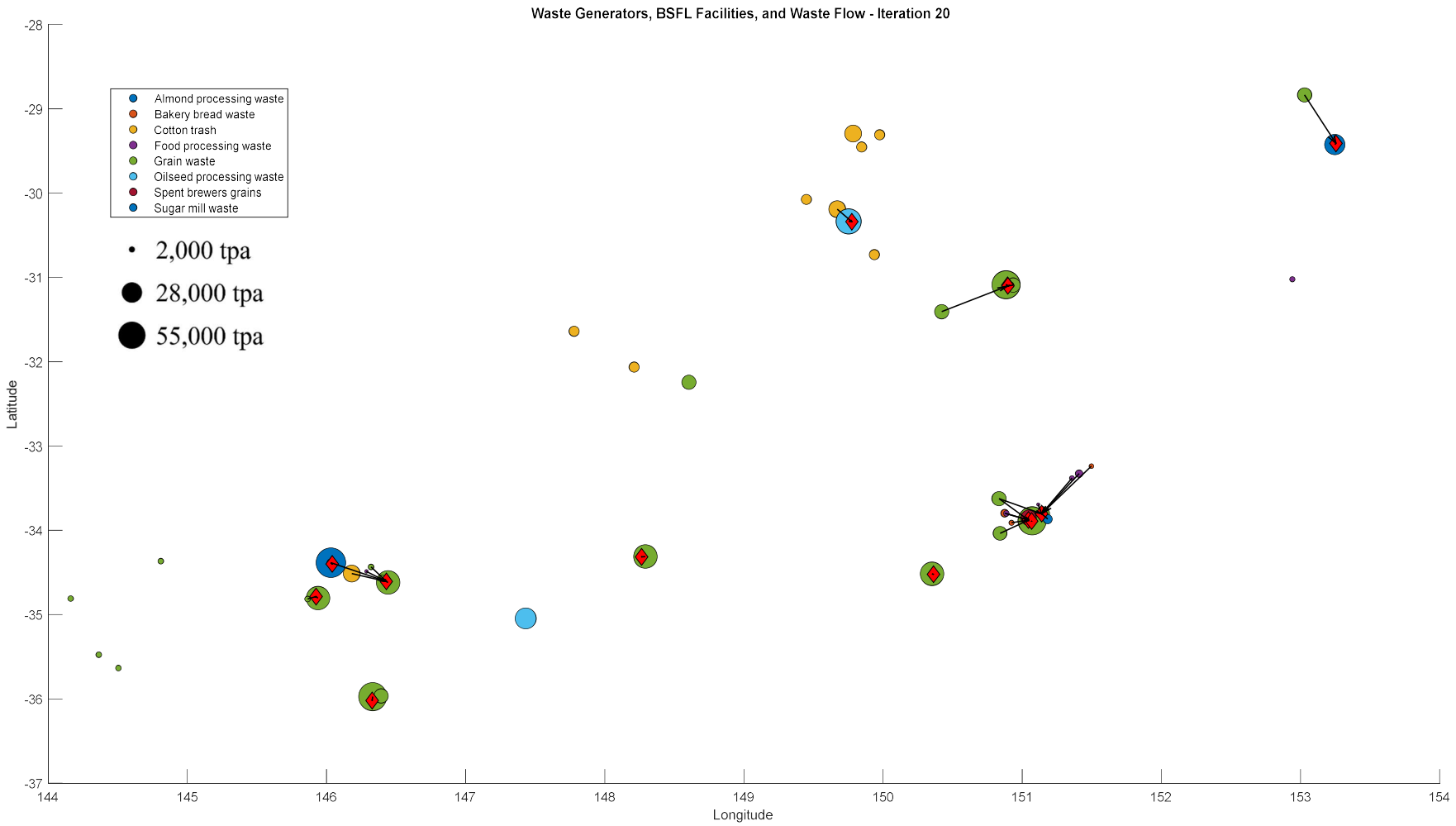

Figure 6.7 Output from the model after 20 iterations for Scenario 2 for a subsample of 50 generators across NSW excluding meat and paper processing for the base-case settings of parameters. Facility processing capacities are set to 50,000 tpa. The sizes of generator markers are proportional to the waste generated at that location.

For the full NSW dataset allowing waste diversification, grain waste was the most commonly selected (Figure 6.5). This reflects the large volumes available (Figure 6.2), its spread across the state (Figure 6.1), and its favourable conversion factors (Table 6.4). Other wastes, including those from wineries, oilseed processing, almond processing, markets, sugar milling, food processing, baking, and brewing, were similarly selected when within the vicinity of several other waste generators. The exception was cotton trash, which was less frequently selected due to its less favourable conversion factors.

Comparing between Scenarios 1 and 2, the locations of optimal facilities were similar for the subsample base-case (Figure 6.6 and Figure 6.7). However, Scenario 2, which allowed for substrate diversification, enabled the placement of three additional facilities. These locations were not profitable without waste diversification, indicating that diversification enables the valorisation of waste sources with either lower conversion factors or lower quantities.

The selection of waste types also differed between the two scenarios. In areas where there are clusters of various types of waste, diversifying wastes proved profitable for many of the facilities. This yielded benefits primarily in terms of BSFL meal production with minimal additional costs from transport (Figure 6.8). In some locations, a single waste type was insufficient to enable the placement of a facility, whereas when even just one additional waste type was available, a profitable facility could be placed.

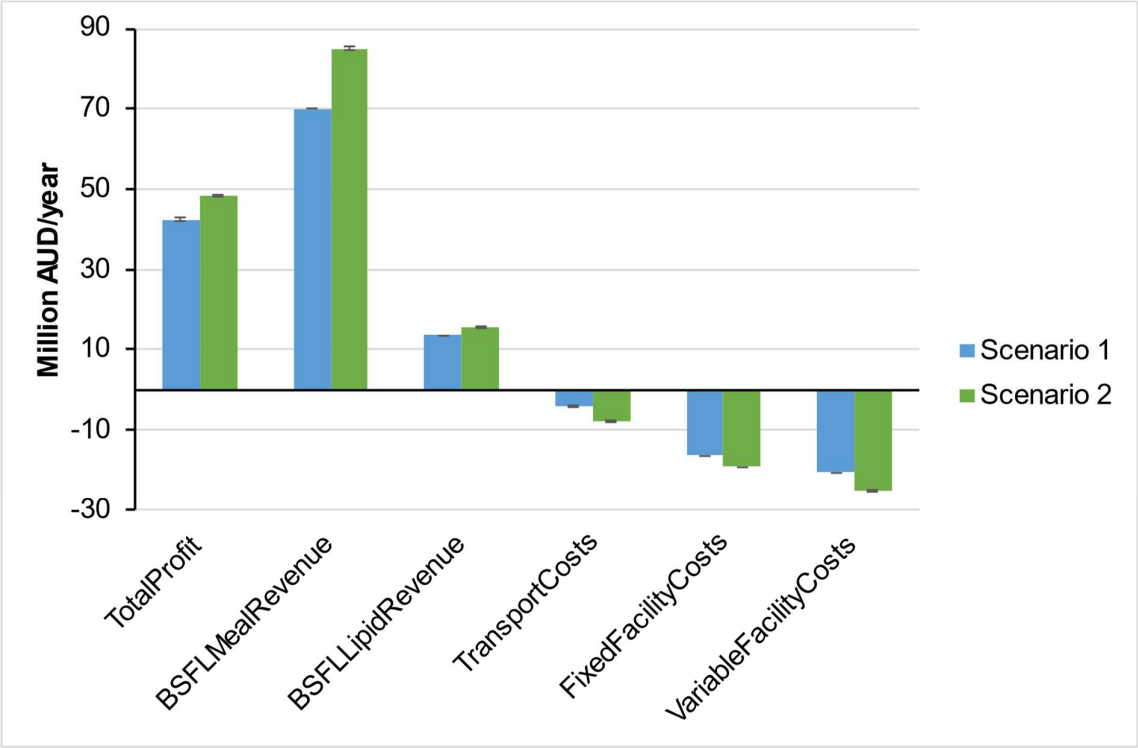

Figure 6.8 Breakdown of profit, revenues and costs for the base-case scenarios using a subsample of 50 generators from the waste data set.

All components of both revenue and cost increased when multiple waste types were allowed to be processed at each facility (Scenario 2). The increase in revenue, particularly from the sale of BSFL meal, was greater than the increased costs, yielding a significant increase in the total profit of 14% relative to Scenario 1 for the selected subsample. In both scenarios, revenue was dominated by BSFL meal sales, while costs were dominated by near equal parts variable and fixed facility costs. Transport contributed the least to overall costs, as is often the case in production industries (Figure 6.8).

6.3.3. Sensitivity Analysis

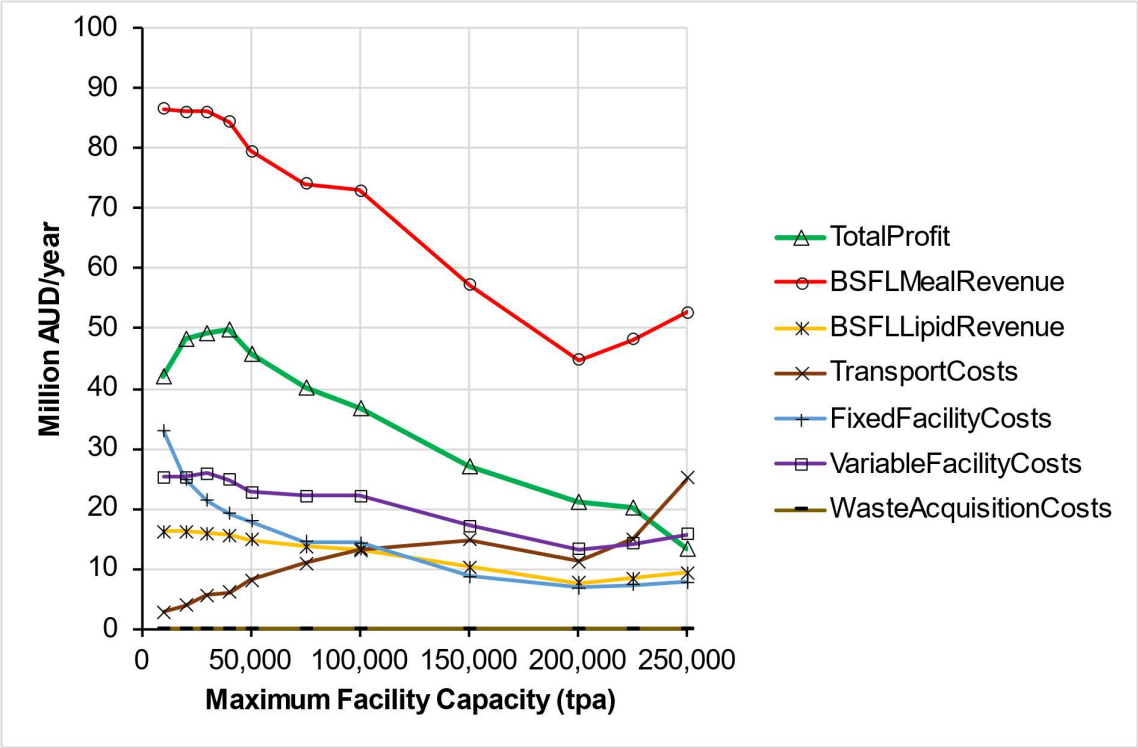

Figure 6.9 Sensitivity analysis of profit and its components to changes in the maximum facility capacity Q. Analysis was performed for Scenario 2 on the subsample of 50 waste generators.

With the exception of BSFL prices, profit and its components were most sensitive to variations in the maximum facility capacity *Q*. Maximum profit was achieved at a maximum waste processing capacity of approximately 40,000 tpa, slightly below that of 50,000 tpa as selected for the base-case. This stems largely from the balancing of fixed facility costs and transport costs (Figure 6.9). Larger facilities benefit from economies of scale which can lower the average cost per unit through increased production because fixed costs are spread out over more units of output, as accounted for in the calculation of equivalent annual fixed cost using the 6/10ths factor. On the other hand, smaller facilities can access lower transport costs as they can be located closer to waste sources, which offsets some of the benefits of economies of scale. Comparison with capacities of traditional organic waste treatment facilities such as windrow composting, mechanical-biological treatment and anaerobic digestion provides additional confirmation that the optimal facility capacity is appropriate. At

a commercial scale, such facilities in NSW have processing capacities between approximately 10,000 and 300,000 tpa organic waste, with a median of 50,000 tpa (personal communication with undisclosed waste management company).

However, the optimal facility capacity might be influenced by additional substrate properties not accounted for in the current model. For instance, the conversion factors used in the model are estimated from the protein and carbohydrate contents of the wastes alone, which do not truly reflect the real conversion factors. If the actual conversion factors are lower for certain waste types or vary significantly among different waste types, then a wider variety of waste sources might be necessary to achieve high meal production. In such a case, being close to a singular waste type might be less beneficial in terms of reducing transport costs, which is a significant way in which smaller facilities can increase profit.

Moreover, the seasonality of waste supply could also influence the optimal facility capacity. If the availability of certain waste types fluctuates significantly throughout the year, then a facility might need to process different types of waste in different seasons to maintain high meal production. This could necessitate a larger facility that can handle a wider variety of waste types, which would benefit from economies of scale with respect to fixed facility costs.

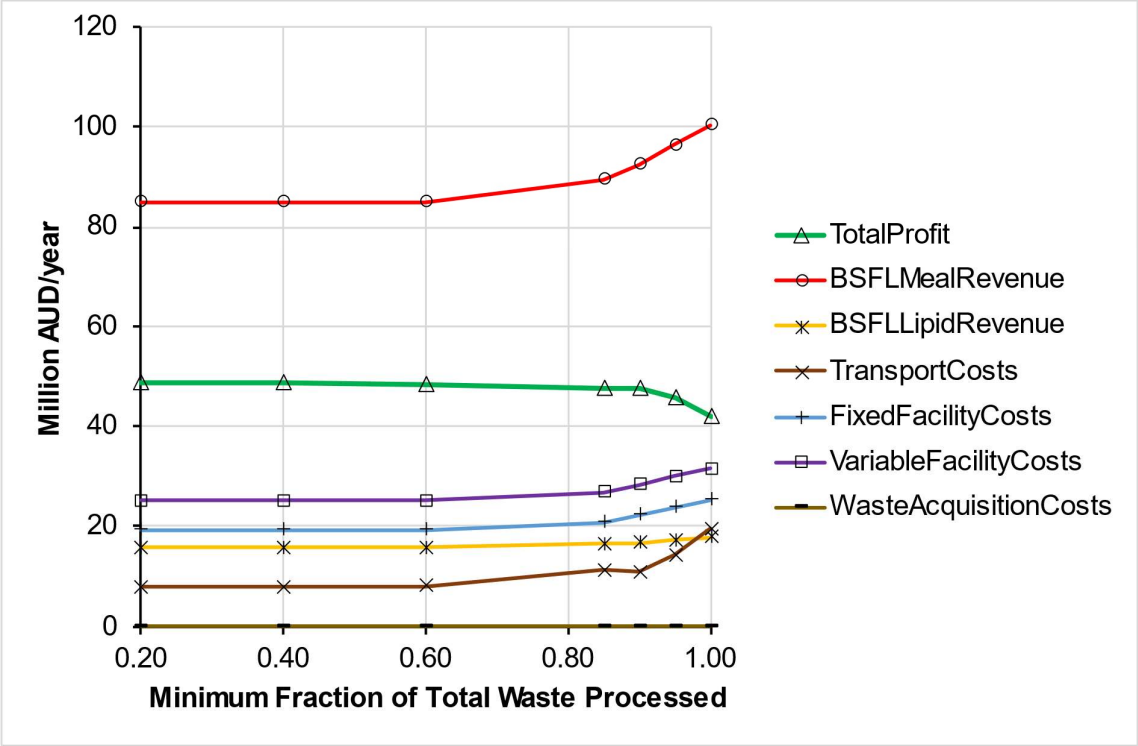

Figure 6.10 Sensitivity analysis of profit and its components to changes in the minimum fraction of total waste processed (*U*). Analysis was performed for Scenario 2 on the subsample of 50 waste generators.

Without the 'minimum waste processed' constraint ($U = 0$), the model identifies the point of maximum profit as processing approximately 80% of the total waste available ($U = 0.8$) for Scenario 2. Hence, any settings of '*U*' below this value does not influence the solution, as confirmed in Figure 6.10. Forcing larger quantities of waste disposal above these optima results in transport costs rising faster than revenues, as shown when constraining the model to process all available wastes.

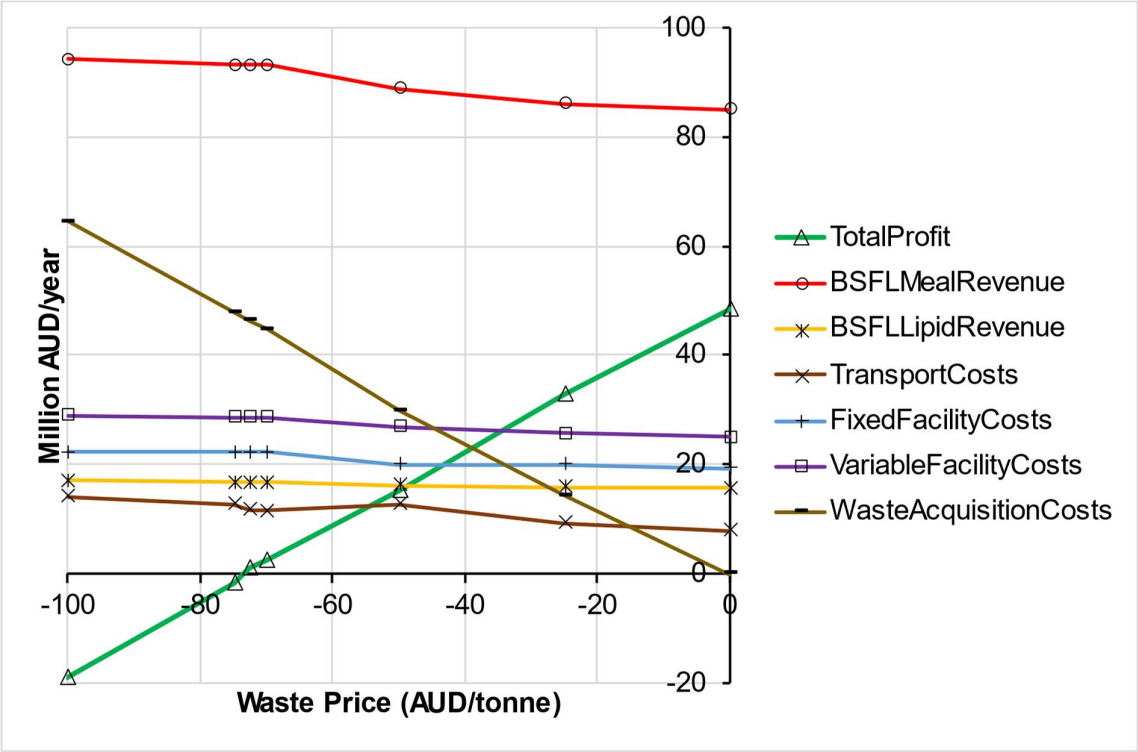

Figure 6.11 Sensitivity analysis of profit and its components to changes in waste acquisition prices (P_k). Analysis was performed for Scenario 2 on the subsample of 50 waste generators.

Figure 6.11 shows that for the base-case, due to the significant revenue BSFL meal may produce, facilities may pay up to approximately 75 AUD per tonne of wet waste prior to losing money (the breakeven point). This is using the blanket assumption that all wastes cost the same, which is a substantial simplification. In reality, costs will depend on market competition for the resource such as animal feed (something about opportunity cost here?), as may be influenced by the materials proximate composition including water content. Similarly, some materials may generate income through gate fees, which could offset the purchase of higher cost and potentially higher performing materials. Future research should address this gap by estimating prices for such materials. Additionally, varied waste prices are likely to alter the optimal facility locations and waste transfers, thereby influencing the other profit components which are currently unperturbed in Figure 6.11.

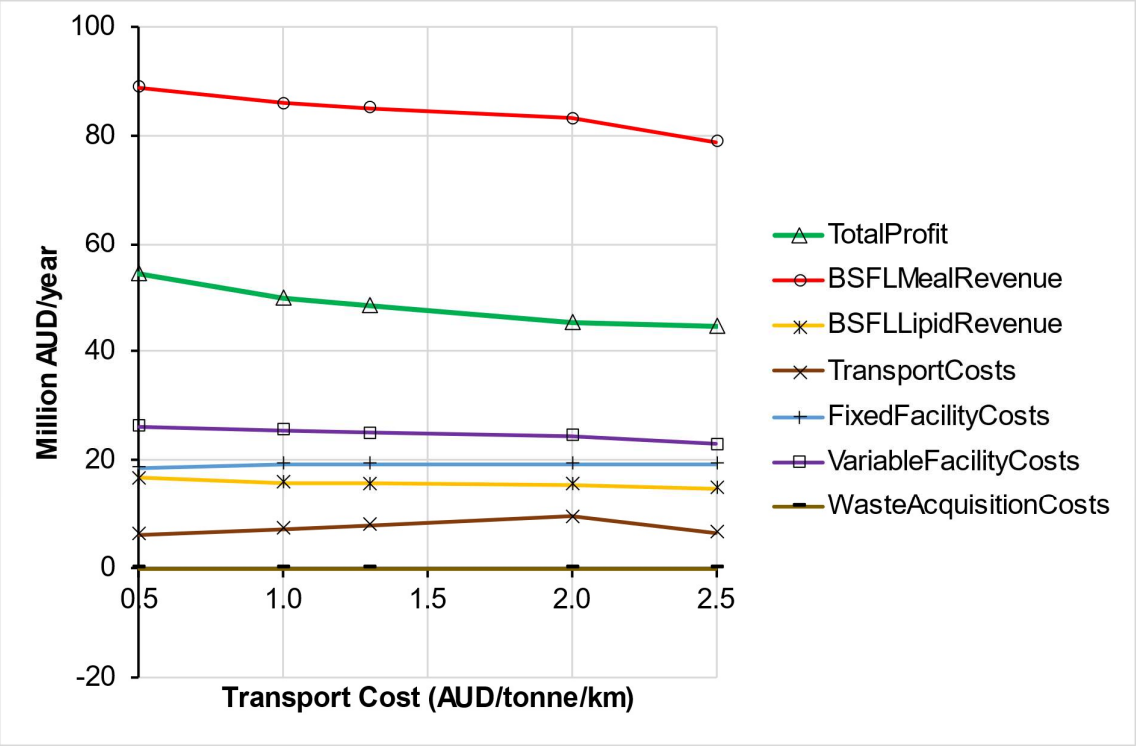

Figure 6.12 Sensitivity analysis of profit and its components to changes in transport costs per tonne per km (P_t). Analysis was performed for Scenario 2 on the subsample of 50 waste generators.

Changes in transport costs per tonne per km had little effect on profit or its components (Figure 6.13). This is due to the low contribution of transport costs to the overall costs in the optimised base-case (Figure 6.8). The reduction in profit at the price of 2.5 AUD/tonne/km compared to 0.5 reflects the model sacrificing BSFL meal production in favour of minimising transport distances and resulting costs, limiting waste transfers from previously profitable waste generators.

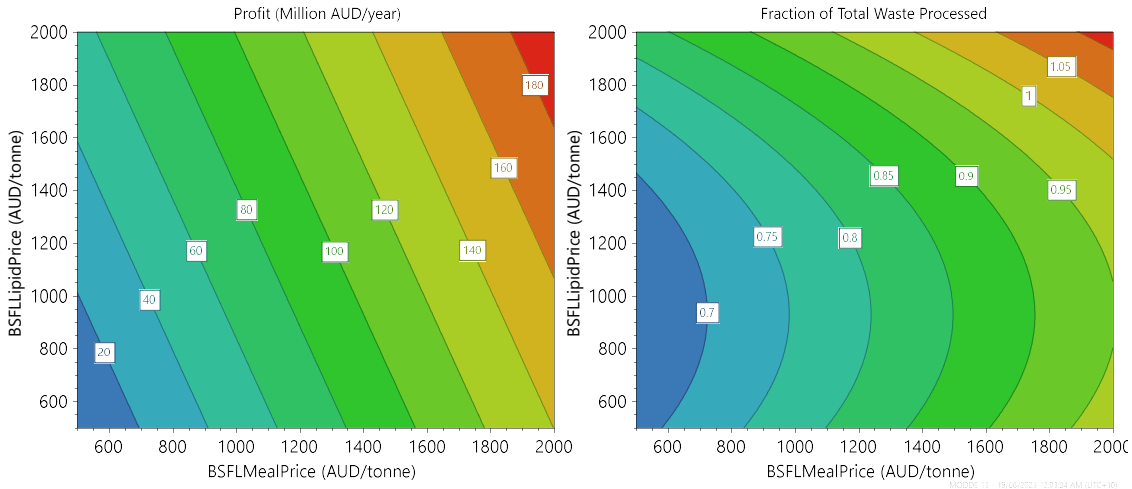

Figure 6.13 Variations in total profit and fraction of total waste processed in response to changes in BSFL meal (P_m) and lipid prices (P_L). Analysis was performed for Scenario 2 on the subsample of 50 waste generators.

Prices of larval products, most significantly the BSFL meal, have the largest influence on profitability (Figure 6.13). Hence, research into development of high value products from BSFL meal and lipids holds significant promise of ensuring the viability of the technology and enabling the valorisation of wider varieties of organic wastes. This is demonstrated in Figure 6.13, showing the strong relation between BSFL meal price and the optimal quantity of total waste processed. However, as noted in Chapter 2, such high value products also have potential to alter Figure 6.11, tipping the balance away from using waste materials in substrates in favour of paying to acquire reliable, non-waste materials, such as manufactured poultry feeds.

In any case, while larval product prices are the most influential parameters on profitability, they are also some of the most uncertain due to the lack of an established/mature demand for the products in bulk quantities. Additionally, even in established markets such as those for fishmeal and soybean, commodity prices fluctuate drastically overtime. Hence, a conservative estimate of the prices is used for the base case of the model, despite sources that present significantly higher price points for BSFL meal and lipid (Joly and Nikiema, 2019; Suckling et al., 2021).

6.3.4. Circular Economy Benefits

Regarding the circular economy benefits of BSFL production in the present context of NSW, a careful assessment is required. Often, the circular economy benefits associated with BSFL production are significantly tied to their ability to divert waste from landfill. However, as shown in Table 6.1, landfill is not the dominant destination for pre-consumer organic wastes across Australia. Composting, which sits low on the waste-management hierarchy, is an often-employed solution given the availability of suitable land across the country (Table 6.1). It can be assumed that net composting capacity across the state can meet the management demands of the mapped pre-consumer organics.

While diverting wastes destined for composting towards BSFL production could be seen as a beneficial alternative from a nutrient-retention or circular economy perspective, the situation is more complex. The waste mapping process uncovered that many of these pre-consumer organics in NSW are already reused at higher levels of value retention. Examples include direct use in animal feed and biochemical processing (see appendices). Given that animal feed is the dominant target market for BSFL products (Bessa et al., 2020), the benefits of diverting such streams to BSFL production are not straightforward.

Nevertheless, there may still be scenarios where it is beneficial to divert streams from direct inclusion in animal feeds, to BSFL production instead. These may include concentration of nutrients in the BSFL meal relative to the initial waste, or removal of contaminants and anti-nutritional factors such as aflatoxins from microbially compromised wastes. Additionally, there are logistical benefits such as weight reduction through moisture losses during the BSFL production process, as well as microbial stabilization. Both aspects may enable economical transport of nutrients further distances than may be achieved by shipping the raw waste itself. Furthermore, substrate diversification and blending of low-quality wastes with more amenable wastes may enable the valorisation of wastes that are

currently underutilised or less suitable for reuse, such as fibrous materials or manures. By using these in BSFL substrates, their contained nutrients may be efficiently introduced into the food system.

In the presented model, grain waste was the most often selected when optimising across the full dataset of wastes in NSW (Figure 6.5). These are broadly regarded as destined for animal feed (see appendices), however, this varies according to the specific generator and the particular batch according to various factors. Such factors include proximity of the waste generator to local farms, environmental conditions such as droughts increasing costs of alternative animal feeds, and cleanliness of the waste batch. Batches deemed unfit for animal feed may be more accessible for BSFL production. However, it is unknown whether such wastes would be fit for BSFL, both in terms of palatability for the larvae, and in terms of safety. This would require assessment on a case-by-case for particular waste generators and waste batches.

Several additional scenarios exist where BSFL production from the identified pre-consumer wastes is beneficial from a circular economy perspective. For example, the diversion of waste from lower-value retention options, such as biogas generation, could be beneficial. Moreover, if BSFL products become widely accepted as a form of direct human nutrition in place of various animal products, diverting wastes from inclusion in animal feed may be considered beneficial from a circular economy perspective. However, these potential benefits require a detailed life cycle assessment. This assessment should be built on reliable data and account for the opportunity costs in relation to existing and alternative use cases for NSW. It is important to note that such assessments are heavily context-dependent (Wenzel, 1998). For instance, while it may appear beneficial from a circular economy perspective to divert wastes from biogas generation towards BSFL production, this does not guarantee

benefits from an economic, social, or environmental perspective for that locality (Corvellec et al., 2022).

6.3.5. Limitations and Future Work

6.3.5.1. Improvement of Data Quality and Addressing Simplifications

The model presented in this study, although beneficial for providing initial insights, relies on empirical data and simplifications that might not always be perfectly applicable. These aspects likely affect the understanding of how waste diversification impacts optimal facility locations, since the details of revenue and cost structures of BSFL production can alter the profitability and hence the attractiveness of different locations, especially when considering substrates of varied waste types.

In particular, the data generated through the waste mapping regarding waste quantities and their composition carry a significant degree of uncertainty. Considering the output of the model is only as good as the input data, significant effort was placed on the data mapping stage with companies and peak bodies representing waste generators contacted directly to provide data. However, many were hesitant to provide even limited information due to commercial confidentiality, resulting in the need for estimating waste quantities at each generator location (see appendices). As such, while data for some generators is reliable, others are highly uncertain. This is particularly pertinent to the grain waste estimates considering their heavy selection in the model and high uncertainty. The actual profitability may in fact be significantly lower than what has been presented in the current model in Table 6.5. Hence, the presented model outputs should not be relied upon for decision making, and it is critical that more reliable data regarding waste generators is acquired.

In addition, the model simplifies or otherwise does not account for several aspects that could influence BSFL production. These include prices associated with selling substrate residue (also referred to as frass in the literature), using a fixed variable facility cost factor

irrespective of facility size, up and downstream processing such as dewatering and lipid extraction, and variations in prices of larval products based on quality. For example, a singular larval meal price has been assumed, despite the protein content of meal varying according to the substrate used, ranging from 43 to 63% estimated for the substrates of the present study. In real markets, meal of low-protein content may be graded as low in quality and thereby have a lower sale price (Drewery et al., 2022), as is the case for commodities such as fishmeal (Tridge, 2023). Furthermore, the use of the tortuosity factor from literature rather than data from actual road networks simplifies the calculation of transport costs and possibly obscuring influential real-world complexities of waste transportation. Additionally, varied waste streams require pre-treatments such as de-watering and particle size reduction which incur associated costs and which have not been accounted for in the current model. These disregarded factors could influence how waste diversification affect the determination of optimal locations for BSFL facilities.

6.3.5.2. Implementation of Waste Blending

The model was formulated to enable not only waste diversification, but also waste blending to yield more optimal substrates with higher conversion factors than individual wastes alone. Waste blending is proposed as a potential method for mitigating sensitivity of BSFL production to varied substrate properties (Gold et al., 2020), yet the consequences of such blending on BSFL production logistics remain unexplored. The present formulation was developed such that a set of discretised substrate blends with increased conversion factors may be selected by the model, in an effort to address this knowledge gap. However, time and computing resources were insufficient to implement this into MATLAB. Future research should implement waste blending to explore whether it may be an economically feasible strategy for addressing substrate sensitivity of BSFL production from organic wastes.

6.3.5.3. Application in Other Contexts

Other regions like those with less land, less waste management infrastructure, and less alternative cascading reuse options, may yield more circular economy benefits compared to NSW, where much of the pre-consumer wastes for BSFL production are already in use. Alternatively, more circular economy benefits may result from focusing on post-consumer wastes such as may be produced in point sources from shopping centres, universities, schools, hospitals or supermarkets, or even more contaminated materials such as wastewater biosolids and municipal solid wastes. However, as noted in Chapter 2, this requires significantly further research on safety confirmations and larval tolerance to anti-nutritional factors.

6.3.5.4. Exploration of Economies of Scale

A key limitation to the model is that all facilities are forced to be the same size, i.e. facility capacities are not decision variables. While larger numbers of small facilities can be placed right next to each other to still make use of large point sources, they remain penalised relative to a single large facility since they do not benefit as much from the economies of scale built into the equivalent annual fixed facility cost calculation.

There are also unknowns regarding the extent to which economies of scale should be applied in the present model. For instance, fixed and variable facility cost assumptions based on data from Enterra may not hold universally, as suggested by (Joly and Nikiema, 2019), who observed in several case studies that the cost per unit waste processed tended to be larger for larger facilities, countering the concept of economies of scale. This could imply either confounding factors in the data used for determination of costs per unit waste processed (such as differences in labour costs across jurisdictions) or additional infrastructure costs that outweigh the benefits of economies of scale.

Furthermore, there is uncertainty regarding the calculation of equivalent annual fixed facility costs using the 6/10ths rule. The 6/10ths rule, which is based on the geometric

properties of three-dimensional shapes, often applies to equipment or facilities where cost is related to physical size, typically a function of volume or mass (Moore, 1959). However, for BSFL production facilities, this rule may not apply in the same way due to the unique nature of the BSFL production process. BSFL are often developed in shallow plastic trays, with a limit to the depth of substrate (Lalander et al., 2020). This limit is imposed by several factors. Larvae tend to consume nutrients more readily from the upper levels of the substrate, possibly due to easier access and lower metabolic costs associated with burrowing. If substrate layers are too deep, larvae may not reach the lower levels before those levels are depleted of oxygen, leading to anaerobic conditions that can be detrimental to larval development (Palma et al., 2018). Furthermore, substrate at lower levels may be wasted as microbes consume the readily available nutrients while larvae focus on the upper levels.

While methods for mixing the substrate could potentially enable deeper substrate layers, there is currently no evidence that such methods are cost-effective. Therefore, to increase the capacity of a BSFL production facility, the number of trays or the area of each tray would need to be increased, rather than their depth. This suggests that the cost of the trays, and hence the overall cost of the facility, might scale more linearly with capacity, rather than following the 6/10ths rule. Therefore, the overall cost scaling exponent for BSFL production facilities might be higher than 0.6. However, more detailed cost analyses would be needed to determine the exact value.

Moreover, costs associated with the trays are unlikely to be the dominant source of cost for a BSFL production facility. Other significant costs, such as variable facility costs associated with labour, utilities, waste acquisition and pre-processing, and the cost of processing and selling the BSFL products, might scale differently with capacity, requiring further investigation.

6.3.5.5. Supplementary Modelling Techniques to Incorporate Additional Substrate Properties

For future work, exploring non-linear programming approaches such as genetic algorithms or gradient-based methods could be beneficial. Development of further mechanistic and empirical relationships between substrate properties (e.g. carbohydrate and fibre profiles from Chapters 3 and 4) and conversion factors are also critically needed, considering the low confidence in those estimated from substrate protein and carbohydrate contents alone. Expanding the model to include stochastic or dynamic elements could provide a more accurate representation of real-world complexities, and considering the timeframe beyond a single year could give a more comprehensive understanding of the long-term viability and implications of different facility location strategies.

Additionally, in the current model, substrate diversification is averaged over a time frame of one year. This means that the model assumes a consistent supply of different waste types throughout the year, which may not reflect real-world conditions where the availability of certain waste types likely varies seasonally. This impacts the usefulness of the model outputs for real-world decision making. For example, if certain waste types are only available at certain times of the year, this could affect the profitability and optimal locations of BSFL facilities. To address this, future research could explore shorter time scales in the model.

There is also some room for improvement in the local search ability of the implemented model. As can be seen in Figure 5, the facility locations would have benefitted from additional local search iterations to further reduce the combined transport distances. The choice of the number of iterations with which to run a particular scenario is a compromise between computation time and quality of the solution, with diminishing returns with each iteration. A high number of iterations therefore limits the number of scenarios that may be analysed in a given time. However, further scenario analyses where multiple parameters are

adjusted systematically may yield novel insights. Hence, future efforts could be put towards optimising the implementation and running the model with additional computational resources.

6.4. Conclusions

This chapter aimed to examine the implications of substrate sensitivity on black soldier fly larvae (BSFL) production from food waste, with a specific focus on identifying optimal locations for BSFL facilities in New South Wales, Australia, while considering access to diverse organic wastes as substrates. The main objective was to assess the impact of substrate diversification on the optimal locations and waste transfers, considering the sensitivity of BSFL production to differences in organic waste protein and carbohydrate contents. Achieving this objective was first facilitated by mapping quantities and locations of point-sources of pre-consumer organics wastes. The objective was then achieved by developing and implementing a MILP model, and by comparing profits, optimal facility locations, and waste transfers between two scenarios: one with substrate diversification and the other without. Substrate diversification was shown to significantly increase the overall profit by 14% when using a subsample of 50 waste generators from the developed NSW dataset. Additionally, the sensitivity of the model to changes in parameters was assessed, including facility capacities, waste processing constraints, waste acquisition costs, transport costs and product prices. Product prices and maximum facility capacities yielded the most significant variations in the model output. Circular economy benefits from the diversion of the selected organics streams to BSFL production in NSW are discussed and determined to be variable, dependent on local contexts.

This is the first known model to explore BSFL production logistics optimisation using multiple waste types. The model provides a preliminary tool for optimising BSFL production in a manner compatible with the circular economy, facilitating large-scale deployment of

BSFL production using pre-consumer food wastes as substrate components. However, the heavy reliance on assumptions and empirical data limitations calls for further research to refine and validate the model. Future studies should explore more comprehensive models that incorporate additional substrate properties to match real BSFL production scenarios more closely.

Chapter 7. Conclusions and Recommendations

7.1. Conclusions

The need for a circular economy is apparent, and Black Soldier Fly Larvae (BSFL) are increasingly recognized as a potential solution for cycling organic waste. However, securing a supply of 'high quality' waste materials from which to create a successful substrate is challenging. This requires large-scale producers to use multiple waste streams as substrate materials, however BSFL quality and process performance are highly sensitive to substrate properties in ways which are not understood. To mitigate this issue, some producers are turning to non-waste materials traditionally used as animal feed, sacrificing the circular economy promise of BSFL. A deeper understanding of the influence of substrate properties on BSFL production is needed. With this knowledge, reliable waste-based substrates could be formulated, and the circular economy potential of BSFL could be realised. This thesis develops our understanding of how substrate properties influence BSFL quality and process performance, such that BSFL producers may better optimise production using waste-based substrates.

The approach taken to achieve this was to comprehensively review the literature and identify specific substrate properties that would bring the most benefit in understanding further. Experiments were designed and performed to fill these knowledge gaps, incorporating learnings from each sequential experiment. Finally, mathematical modelling was employed to explore how substrate properties influence BSFL production at a regional scale to demonstrate the influence on the circular economy, thereby demonstrating the need for continued research in this area. By enhancing our understanding of how substrate properties influence BSFL quality and process performance, this thesis assists in the

optimisation of waste-based substrates for BSFL valorisation, contributing to the broader goal of advancing the circular economy.

7.1.1. Key Findings and their Contributions to the Field

Each chapter of this thesis contributes significant findings to the field of BSFL production research. Chapter 2, the literature review, presents the potential of BSFL production as a tool of the circular bioeconomy and identifies a key barrier to realising this potential: the unexplained variability in BSFL quality and process performance. This variability is attributed to a limited understanding of the role of substrate properties, including macronutrient contents, physical features and safety considerations. The review emphasizes the need for appropriately controlled experiments that explore these properties, considering their interplay with the larvae and microorganisms present in the production system. It also highlights the lack of mathematical models that account for the influence of substrate properties on BSFL production, a gap that, if filled, could maximise the technology's potential for the circular economy.

In Chapter 3, the first investigation into the importance of specific dietary carbohydrates on black soldier fly larvae is presented. The study examines the hypothesis that the types of carbohydrates within the substrate can significantly influence larval survival, waste reduction, bioconversion, and waste conversion efficiency, as well as the crude lipid content and fatty acid profiles of the produced larvae. The study finds that certain carbohydrate types, particularly galactose and arabinose, significantly influence BSFL treatment performance, with bioconversion reductions of -14.7 ± 6.8% and -63.0 ± 2.6% relative to the control, respectively. Additionally, larvae crude lipid contents are significantly increased by wheat starch (12.6 ± 3.0%) and decreased by galactose (-15.0 ± 1.4%) and xylan additives (-27.5 ± 3.4%), however fatty acid profiles are largely unaffected and remain

dominated by lauric acid. There are minimal significant effects from mono- and di-saccharides or starch additives.

These are valuable insights as lipid contents and fatty acid profiles are highly variable quality factors of BSFL, and this variability is largely believed to be a function of substrate carbohydrate contents. As such, this study provides confirmation that while dietary carbohydrates are influential on larval quality to an extent, there remain other substrate properties responsible for variations in these quality factors. It was also discovered that the hemicellulose xylan and hemicellulose-sourced galactose and arabinose – components of dietary fibres – were detrimental to larval performance, supporting the decision to explore dietary fibres in the following study.

Dietary fibres, identified in the literature review as significantly understudied macronutrients in BSFL substrates, are examined in Chapter 4. This study reveals a more significant role for dietary fibres in BSFL substrates than previously recognised. Specifically, fibre additives are found to significantly influence BSFL performance, including growth and conversion efficiency, and BSFL quality in terms of proximate composition. These effects are dependent on the concentration and source of the fibre. Most notably, substrates with pure cellulose as an additive are found to enhance larval growth by 22.6 ± 2.2% through both protein and lipid gains. In contrast, the addition of apple fibre containing a complex of cellulose, hemicellulose and lignin, decreases overall growth by -29.7 ± 3.2% through reductions in lipid and chitin contents, without affecting protein gain.

The dominant mechanism for these effects appears to be physical modifications to the substrate, such as texture improvements. Additionally, the study introduces the potential influence of fibre fermentation products, such as short chain fatty acids, on BSFL production. The extent of fibre fermentation is assessed by measuring fibre digestibility, considering not just crude fibre, but also fibre fractions. This reveals accessibility differences due to

lignification, with cellulose less impeded by lignification than hemicellulose and soluble fibre. These findings challenge the widespread belief that fibre is a mono-dimensional, inert or anti-nutritional component in BSFL substrates.

Together, non-fibre carbohydrates and dietary fibre provide the vast majority of carbon for larval and microbial metabolism. Additionally, they form the largest components by mass of all feasible BSFL substrates, varying greatly in composition across organic wastes. Hence, the studies in Chapters 3 and 4 fill a significant and fundamental gap in understanding the influence of substrate properties – specifically macronutrients – on BSFL production. Having explored the influence of macronutrients on BSFL production, it is crucial to address another key aspect identified in the literature review: the need for safety confirmations. This is particularly important when considering the use of waste materials as substrates, as a lack of safety confirmations currently presents a barrier to the wider adoption of these materials in BSFL production.

In Chapter 5, the focus shifts to per- and polyfluoroalkyl substances (PFASs), specifically perfluorooctanoic acid (PFOA), due to their widespread presence in various waste materials, their persistence in the environment, and the potential health risks associated with their bioaccumulation and biomagnification in food chains. The study finds that PFOA content up to 1000 ppb in chicken feed substrates does not impact larval survival or growth, and results in minimal bioaccumulation in larval tissues, with bioaccumulation factors ranging from 0.63 to 1.64. However, there is some evidence of interference in fatty acid metabolism as a result of PFOA exposure in the 11-day trial. These are significant findings as it provides reassurance about the safety of BSFL production when using waste substrates that may have been exposed to PFOA, thereby helping to alleviate concerns about potential health risks of BSFL production and promoting its acceptance and adoption.

Finally, Chapter 6 diverges from the previous studies in both methodology and scale by employing mathematical modelling to create an optimisation model of locations and waste transfers between theoretical BSFL producers and waste sources across the state of New South Wales, Australia. This approach is chosen to strengthen the connection to the circular economy potential of BSFL production, which is the underlying purpose of this thesis. It also demonstrates the significance of the findings on substrate sensitivity from the previous chapters, illustrating how a varied substrates can influence BSFL production logistics. Furthermore, this chapter addresses the gap identified in the literature review regarding the lack of modelling tools that account for substrate sensitivity in BSFL production. Such a tool provides valuable insights to industry stakeholders and policymakers in developing circular economy solutions to organic wastes at a state-wide scale.

7.1.2. Implications for BSFL Production and the Circular Economy

The findings of this thesis have significant implications for the future of BSFL production research and practice. Firstly, the literature review demonstrates our lack of understanding of the interplay between larvae, microorganisms and substrate properties in BSFL production systems, and the need for experimental studies and mathematical models to assist in filling these gaps. This suggests that the research conducted in this thesis could help to push the current limits of BSFL production in terms of growth and substrate conversion.

The studies on carbohydrate and fibre profiles in substrates reveal that these properties can explain some of the variations in larval performance and quality across substrates with identical protein, carbohydrate, and water contents. This indicates that these properties could influence the selection, blending and/or pre-treatment of waste-based substrates for BSFL production. The finding that dietary fibres can enhance BSFL production suggests that some waste materials, which were previously considered low quality due to their high fibre contents, may actually be more valuable than previously thought. In addition,

this finding implies that strategic addition of fibrous materials may be another method for tailoring larval products to downstream markets or use cases.

While further investigations are warranted, the findings on PFOA bioaccumulation in BSFL suggest that if the PFOA content in the substrate is below the maximum regulatory limit for animal feed, the produced BSFL is also likely to be below the maximum regulatory limit for substrate concentrations up to 1000 ppb. This finding could provide reassurance to BSFL producers and regulatory bodies about the safety of using waste substrates exposed to PFOA, potentially leading to an expansion in the range of waste materials that can be safely used in BSFL production.

The findings of Chapter 6 highlight the potential of substrate diversification in enhancing the profitability of BSFL production, with a 14% increase in overall profit observed when using a diverse range of organic waste sources. The study also underscores the importance of considering local contexts when assessing the circular economy benefits of diverting organic waste streams to BSFL production.

Lastly, the deeper understanding of BSFL substrate properties developed in this thesis can lead to more consistent and efficient BSFL production, potentially altering the design and operation of BSFL production systems.

7.2. Recommendations

7.2.1. BSFL Production Research

Future research should aim to understand the importance of substrate nutrient contents, physical features and safety considerations at a deeper mechanistic level. Disentangling the interplay between these properties, the larvae and microorganisms involved is valuable in this regard. Similarly, the standardization of BSFL production studies, such as those recommended in Bosch et al 2020, is essential. This includes the introduction of

measures that enable comparisons to be made 'apples with apples', allowing for quantitative explorations of the literature such as through meta-analyses.

Exploring at a deeper mechanistic level would also benefit from methods sourced from other disciplines which are beyond the scope of this thesis, such as next generation sequencing to identify contributing microorganisms. Specialised and dedicated laboratories which bring together interdisciplinary expertise and techniques will be beneficial. Collaboration between such labs and industry producers is recommended to ensure labs are applying methods and data applicable to industry, and industry has access to emerging discoveries in BSFL production research.

7.2.2. Recommendations Emerging from Thesis Chapters

Based on the literature review, several overarching recommendations can be made. Firstly, there is a need to delve into the potential of high-value products like chitin-based materials and antimicrobial compounds. These products could expand the market of BSFL-based commodities and facilitate the establishment of the BSFL industry. However, it's crucial to consider the risk of shifting towards non-waste substrates for improved reliability and quality control. This shift could detract from the valorisation, a key aspect of the circular bioeconomy. Thus, strategies should simultaneously be developed to motivate producers to predominantly use waste substrates, thereby maintaining the system's circularity.

Further investigation into anti-nutritional factors, such as tannins, caffeine, enzyme inhibitors, limonene, lectins, polyphenols, and volatile fatty acids is also necessary. These factors, frequently present in many proposed organic wastes for BSFL substrates, have exhibited adverse effects on different insect species and earthworms, underscoring their relevance for BSFL studies. The potential accumulation of these factors from the substrate or their production by the larvae, which may limit the choice of suitable substrates, should be explored.

In addition, other substrate properties warrant investigation, including amino acid profiles, lipid profiles, texture, and micronutrient contents. Techniques for modifying these properties, such as through intentional microorganism treatments, need further exploration. While microorganism treatments have shown promise in improving BSFL production in terms of product quality and performance measures, identifying suitable species and conditions remains a challenge.

Findings from Chapter 3 suggest future research should verify the results can be reproduced with waste-based substrates of equivalents carbohydrate profiles, considering the chemically pure carbohydrates used in this study are more readily available than those in real wastes. Once verified, the following recommendations may be pursued:

- Explore methods to offset the anti-nutritional effects of hemicellulose-sourced carbohydrates.
- Identify optimal inclusion rates of specific carbohydrates such as galactose and arabinose to balance between performance loss and larval lipid reduction, as may be beneficial in cases where low-lipid larvae are desired.

From Chapter 4, more research into the inclusion of fibrous wastes in BSFL substrates is needed to unravel the involved physical and chemical mechanisms. In this regard, the following recommendations are made:

- Investigate the role of intact cell walls in limiting nutrient access using diverse fibre sources.
- Quantify the impact of viscosity, texture, and structure on larval performance and composition.
- Investigate the fermentation products of cellulose, hemicellulose, and pectin, which could be facilitated by novel methods of larval faecal harvesting.

From Chapter 5, additional research is required to validate the impact of PFOA exposure on larval total lipid contents due to the limited sample size used in this study. Additional recommendations include:

- Investigate other PFAS chemicals, especially given the recent replacement of PFOA in manufacturing with newer generations of organofluorine compounds.
- Investigate additional contaminants such as pesticides, dioxins, hormones and antibiotics, as contaminants such as these restrict the range of acceptable substrate materials to those which are often already diverted from landfills, such as pre-consumer food wastes, while preventing valorisation of materials like municipal solid wastes.

Recommendations from Chapter 6 encourage the development of more advanced models for BSFL production logistics, accommodating a greater range of substrate properties. Additional recommendations are to:

- Expand the model's application beyond pre-consumer wastes and the New South Wales context.
- Enhance waste quantity and bioconversion estimates using updated industry data.
- Incorporate supplementary modelling techniques such as non-linear programming to aid in selecting, pre-treating and formulating substrates from organic wastes.

7.2.3. Improving Compatibility of BSFL with the Circular Economy

Once the interplay between larvae, microorganisms and substrate properties are better understood, translating this understanding into practical value may be achieved by developing methods to manage the substrate sensitivity of BSFL production, aiming to make waste-based substrates the default feedstock. Additional benefits may be realised by recognizing complementary industries and processes with which industrial symbioses may be established. Engaging with government entities and stakeholders from such industries may enable a top-down approach to optimise the deployment of BSFL production facilities in relation to waste sources, thereby creating region-wide circular economies.

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Appendices

Chapter 3

Performance measures of each treatment as absolute values are presented below.

Appendix Table 1 Performance measures of the different treatments of Chapter 3. Values from the 80% chicken feed benchmark are underlined. Letters within each column indicate significant differences to the benchmark (a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$).

Treatment	Survival %	Dry Waste Reduction %	Dry Bioconversion %	Dry Waste Conversion Efficiency %
Chicken feed 80% (Benchmark)	98.3 ± 1.9	58.5 ± 2.3	28.1 ± 0.5	48.0 ± 1.0
Chicken feed 100%	98.8 ± 2.2	54.7 ± 6.0	26.6 ± 1.5	48.8 ± 2.7
Sunflower Oil	89.2 ± 0.7	48.9[a] ± 2.2	28.0 ± 0.5	57.4[c] ± 2.1
D Glucose	89.6 ± 2.6	64.4 ± 3.8	28.0 ± 1.2	43.5 ± 1.1
Sucrose	87.1[a] ± 8.3	62.3 ± 4.7	28.7 ± 1.3	46.2 ± 3.1
D (-) Fructose	86.7[a] ± 3.1	62.1 ± 2.7	27.6 ± 0.9	44.4 ± 0.6
Corn starch	91.7 ± 5.6	55.0 ± 2.7	25.8 ± 0.9	47.0 ± 1.3
Wheat starch	84.6[b] ± 4.0	57.4 ± 1.6	26.1 ± 0.2	45.4 ± 1.5
D (+) Galactose	81.7 ± 7.1	48.2[a] ± 5.6	23.9[b] ± 1.9	49.6 ± 2.4
D (+) Mannose	86.7[a] ± 2.6	58.8 ± 5.1	26.5 ± 1.0	45.3 ± 2.8
D (+) Xylose	87.9[a] ± 2.9	51.0 ± 3.0	25.7 ± 1.2	50.3 ± 0.5
D (-) Arabinose	81.3[c] ± 2.5	28.8[c] ± 0.4	10.3[c] ± 0.9	35.9[c] ± 2.7
Xylan	98.3 ± 0.7	61.3 ± 2.7	23.9[b] ± 1.1	39.1[c] ± 2.1

Chapter 4

4.1. Sample calculations

Below are the formulas used for calculating the performance measures, larval compositions and fibre contents of substrates and residues, in terms of the recorded data. Example calculations using the recorded data for the control sample 19 are provided.

Appendix Table 2 Terms used in the calculations of performance measures, larval compositions, and fibre contents of Chapter 4.

Term	Symbol	Used in equation
Larvae count at harvest	n_f	1, 2
Initial larvae count	n_0	1
Total dried larvae mass at harvest	$TDLar_f$	2, 4
Initial dried larvae mass	$TDLar_0$	2, 4
Initial dry substrate	$TDSub$	2, 4
Total residue dry mass	$TDRes$	2, 4
Filled culture container mass at harvest	FC	3
Empty culture container mass	EC	3
Total fresh larvae mass at harvest	$TFLar_f$	3
Residue sample fresh mass	$SFRes$	3
Residue sample dry mass	$SDRes$	3, 9, 10, 12, 14, 16
Nitrogen content	$N\ \%$	5
Larvae sample dry mass	$SDLar$	6, 7
Mass of recovered lipid and tube	$Lip\&T$	6
Lipid tube mass	T	6
Mean total lipid mass of blanks	$TLip_b$	6
Volume of recovered lipid extract	RLE	6
Volume of Matyash solution added	MS	6
MTBE content of Matyash solution	$MTBE\ \%$	6
Mass of crucible and larval ash	$C\&ALar$	7, 9, 10, 12, 14, 16
Initial crucible and celite mass	C_0	7, 9, 10, 12, 14, 16
Initial crucible and celite mass of blank	C_{b0}	7, 9, 10, 12, 14, 16
Final crucible and celite mass of blank	C_{bf}	7, 9, 10, 12, 14, 16
Mass of crucible, celite and TDF	$C\&TDF$	9
Mass of crucible, celite and NDF	$C\&NDF$	10
Mass of crucible, celite and ADF	$C\&ADF$	12
Mass of crucible, celite and ADL	$C\&ADL$	14
Mass of crucible, celite and residue ash	$C\&Ash$	16

4.1.1. Performance measures

$$Survival(\%) = \frac{n_f}{n_0} * 100 \quad (1)$$

$$Survival\ (\%) = \frac{80}{80} * 100 = 100\ \%$$

$$Mean\ Individual\ Larval\ Gain\ (mg) = (TDLar_f - TDLar_0)/n_f \quad (2)$$

$$Mean\ Individual\ Larval\ Gain\ (mg) = \frac{3.068\ g - 0.122\ g}{80} * 1000\frac{mg}{g} = 36.83\ mg$$

$$SubstrateLoss(g) = TDSub - TDRes = TDSub - \left(FC - EC - TFLar_f\right) * \left(1 - \left(\frac{SFRes - SDRes}{SFRes}\right)\right) \quad 3$$

$$Substrate\ Loss\ (g) = 15.81 - (46.79 - 18.10 - 10.23) * \left(1 - \left(\frac{10.34 - 4.52}{10.34}\right)\right) = 7.74\ g$$

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{Substrate\ loss}{larval\ gain} = \frac{TDSub - TDRes}{TDLar_f - TDLar_0} \quad 4$$

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{7.74}{3.07 - 0.12} = 2.63$$

4.1.2. Larval composition

$$Protein\ \% = N\ \% * 4.76 \quad 5$$

$$Protein\ \% = 6.36 * 4.76 = 30.27\ \%$$

$$Lipid\ \% = \frac{\frac{(Lip\&T) - T}{RLE} - TLip_b}{\left(\frac{}{MS * MTBE\ \%}\right)} * 100 \quad 6$$

$$Lipid\ \% = \frac{\frac{(1.1168\ g) - 1.0900\ g}{\left(\frac{2000\ uL}{8995\ uL * \frac{10}{13}}\right)} - 0.0033\ g}{0.2998\ g} * 100 = \frac{0.0894}{0.2998} * 100 = 29.8\ \%$$

$$Ash\ \% = \frac{C\&ALar - C_0 - (C_{bf} - C_{b0})}{SDLar} * 100 \quad 7$$

$$Ash\ \% = \frac{15.5935g - 15.5288g - (15.5038g - 15.5033g)}{0.4380\ g} * 100 = \frac{0.0642}{0.4380} * 100 = 14.7\ \%$$

$$Chitin + Other\ \% = 100\% - Protein\ \% - Lipid\ \% - Ash\ \% \quad 8$$

$$Chitin + Other\ \% = 100 - 30.3 - 29.8 - 14.7 = 25.2\ \%$$

4.1.3. Residue fibre contents

$$TDF\ \% = \frac{C\&TDF - C_0 - \left(C_{bf} - C_{b0}\right)}{SDRes} * 100 - Ash\ \% \quad 9$$

$$TDF\ \% = \frac{(32.4332) - 32.0185 - (32.0887 - 32.0853)}{0.9943} * 100 - 1.8 = 39.6\ \%$$

$$NDF\ \% = \frac{C\&NDF - C_0 - \left(C_{bf} - C_{b0}\right)}{SDRes} * 100 - Ash\ \% \quad (10)$$

$$NDF\ \% = \frac{(32.2704) - 32.0185 - (32.0817 - 32.0887)}{0.9943} * 100 - 1.8 = 24.3\ \%$$

$$SDF\ \% = TDF\ \% - NDF\ \% \quad (11)$$

$$SDF\ \% = 39.6 - 24.3 = 15.3\ \%$$

$$ADF\ \% = \frac{C\&ADF - C_0 - \left(C_{bf} - C_{b0}\right)}{SDRes} * 100 - Ash\ \% \quad (12)$$

$$ADF\ \% = \frac{(32.2131) - 32.0185 - (32.0730 - 32.0817)}{0.9943} * 100 - 1.8 = 18.7\ \%$$

$$Hemicellulose\ \% = NDF\ \% - ADF\ \% \quad (13)$$

$$Hemicellulose\ \% = 24.3 - 18.7 = 5.6\ \%$$

$$ADL\ \% = \frac{C\&ADL - C_0 - \left(C_{bf} - C_{b0}\right)}{SDRes} * 100 - Ash\ \% \quad (14)$$

$$ADL\ \% = \frac{(32.0642) - 32.0185 - (32.0725 - 32.0730)}{0.9943} * 100 - 1.8 = 2.9\ \%$$

$$Cellulose\ \% = ADF\ \% - ADL\ \% \quad (15)$$

$$Cellulose\ \% = 18.7 - 2.9 = 15.8\ \%$$

$$Residue\ Ash\ \% = \frac{C\&Ash - C_0 - (C_{bf} - C_{b0})}{SDRes} * 100 \quad (16)$$

$$Residue\ Ash\ \% = \frac{(32.0344) - 32.0185 - (32.0710 - 32.0725)}{0.9943} * 100 = \frac{0.0174}{0.9943} * 100 = 1.8\ \%$$

Chapter 6

6.1. Organic Waste Mapping

6.1.1. Waste Estimate Conversion Factors

This section contains details of the waste mapping conversion factors, including estimating production capacities of each generator site and conversion factors from these capacities to waste quantities generated. In some cases, individual companies have supplied data regarding these figures. Due to a non-disclosure agreement, the identity of these companies has been excluded from this chapter.

6.1.1.1 Almond Processing

As of December 2019, there was one significant nut processing facility identified in NSW from the EPA POEO register, located in the Riverina region. Capacity of this facility was sourced from the EPA license and reported as annual processing capacity. Resulting data quality of this capacity has been deemed medium to low as it is reported as a range. The kernel as the main product from almond processing has been reported as only 14.5% of the mass of a fresh almond, resulting in 85.5% waste in the form of the hull and shell (pericarp) (Godini, 1984). Therefore, a waste estimate of 85% of the processing capacity has been used. This waste is typically composted, used as animal feed or incinerated (Almond Board of Australia, 2020).

6.1.1.2 Bakery Bread Waste

Location and capacity data for plant bakeries are comparatively difficult to source as these facilities are not included in the EPA POEO register. As such, the National Pollutant Inventory (NPI) is the main data source used to identify these facilities. As of December 2019, seven such facilities have been identified in NSW. Bakery wastes include unused dough, damaged and rejected products, breads, and unused ingredients (Heuzé V. et al., 2018). The conversion factor from employee counts to waste estimates has been sourced from

(Dusoruth et al., 2018) which reports an estimate of 6.75 tonnes of waste per employee per year. The resulting waste estimate qualities have been deemed very low. Production information from a bakery site in Sydney was sourced from the respective company as just over 1 million loaves produced per week. Assuming 330 days on stream time, this equates to approximately 47 million loaves per year. Further assuming that all loaves are 700 g as per the local supermarket, 33,000 tonnes of bread is produced per year. In the absence of waste estimate information, a crude assumption was used of 10% of production mass becoming waste, as was assumed in the National Food Waste Baseline for all farm production (Arcadis, 2019). The resulting waste estimate quality is therefore very low. One of the large bread manufacturers contacted reportedly processes dough and bread waste into stockfeed, selected bread lines into breadcrumbs, organic trade waste sludges to composting or soil conditioning and other suitable organic wastes to bioreactors (assumed to refer to anaerobic digestion).

6.1.1.3 Brewery Wastes

As of December 2019, three breweries have been identified in NSW operating at a significant enough capacity to be included in this study, as estimated from publicly available EPA Protection of the Environment Operations (POEO) licenses register. These are located in Cumberland, Goulburn and Tweed local government areas (LGAs). Production capacities have been sourced and/or estimated in litres from internet sources, with medium to high confidence in their quality.

Waste from brewing is typically brewers' grains (86 mass %), yeast (11%), trub (2%, precipitate from heating wort) and diatomaceous earth (1%, referred to as Kieselguhr and used as a fining agent) (Thiago et al., 2014). A waste generation assumption of 16% production capacity has been used (Thiago et al., 2014). The quality ratings of the resulting estimates have been determined medium.

According to the National Food Waste Baseline (NFWB) report of early 2019, most beer making wastes in Australia are composted with a smaller fraction used as animal feed, as has been assumed for these facilities (Arcadis, 2019). In times of drought and high stockfeed prices, more of these wastes may be used as animal feed (direct communication).

6.1.1.4 Cotton Ginning

From the EPA POEO license register, 27 significant cotton gins can be identified in NSW as of December 2019. The largest are located in Narrabri, Moree Plains, Hay, Murrumbidgee and Leeton LGAs. The averages of the EPA ranges for processing capacity have been used to estimate the capacities of the facilities and the data quality of these estimates have been deemed medium to low given the wide ranges.

Material processed in cotton gins are typically 55 mass % seed, 35% lint and 10% 'trash' referring to sticks, leaves, twigs, dirt and other organic debris (Heuzé V. et al., 2015). Cotton seed is typically used for oil production and animal feed (Meyer, 2007). It is therefore assumed that 10% of processing capacity 'cotton trash' forms the majority target waste and resulting estimates are deemed low quality due to lack of direct data from representative facilities. Cotton Australia claims the trash is used for oil spills, animal feed, ethanol production and compost (Cotton Australia, 2020).

6.1.1.5 Dairy

As at December 2019, 16 dairy facilities have been identified as significant in NSW, as estimated from publicly available EPA POEO licenses register, composed of four milk producers and 12 processors. The largest are located in the Bega, Cumberland and Lismore LGAs. Production or processing capacities data quality are mostly medium to low, with two deemed high.

Wastes from milk production and animal accommodation include manure, wastewater, grain and animal losses, which have not been quantified due to data sourcing and

estimation difficulties. Reportedly, around 4% of on-farm milk production is wasted and 5% of input milk to manufacturing is considered waste largely due to quality control rejects (Arcadis, 2019). Wastes from manufacturing are predominantly liquid, composed of whey from cheese and yoghurt production and wastewater (Arcadis, 2019). Whey is the remaining liquid after curd separation and is 95% water and is typically termed acid or sweet according to the process pH it is generated from. Greater quantities of whey are generated from hard cheeses than soft. Large dairy processors can justify the investment in drying and marketing whey, while smaller facilities rely largely on land application (Arcadis, 2019). The NFWB highlights whey as a significant underused pre-consumer food waste in Australia (Arcadis, 2019).

Quantities of wastes from primary dairy production (animal accommodation) have not been estimated due to lack of information and accessibility. Waste production from processors has been estimated using Bega Cheese' publicly available information. Bega processes 700 million litres of milk, which equates to 700,000 tonnes when a density of 1 kg/L is used. This is used to produce 200,000 tonnes of dairy products (cheese, whey, butter and others), resulting in a 500,000 tonne difference which is assumed waste (Bega, 2020). Therefore, an assumption of 2.5 tonnes of waste per tonne of dairy produced has been used. Where information on dairy production is unavailable, a rough estimate of 500 L of wastewater (mostly whey) per tonne of raw milk processed has been used (Enviro Concepts, 2016). Resulting estimates have been deemed low quality bar the Leppington Pastoral Company wastewater data which is publicly available. Land application and wastewater treatment form the main waste disposal options for dairy facilities in Australia as have been assumed here per the NFWB (Arcadis, 2019).

6.1.1.6 Feedlot Wastes

As of December 2019, three feedlots of significant scale were identified from the EPA POEO license register. These are located in Yanco, Tabbita and Caroona. Capacities have been estimated as the average of the EPA documented capacities and have been deemed medium to low quality.

Feedlots are intensive feeding zones with wastes including manure, wastewater and wasted feeds (grains) (ALFA, 2023). A blanket assumption of 20% of processing capacity becomes waste has been made in the absence of any direct information, hence of a very low data quality. Information on the treatment of these wastes was not available and have since been assumed wastewater treatment, compost and/or animal feed.

6.1.1.7 Feedmill Wastes

As of December 2019, 14 feed mills of significant scale have been identified from the EPA POEO register, the largest located in Tamworth Regional, Federation, Wingecarribee, and Lake Macquarie LGAs. Capacities have been estimated as averages from EPA capacity ranges, aside from those labelled as greater than 250,000 which have been treated as simply having 250,000 tonnes annual processing capacity. Quality of these capacity estimates have been deemed medium to low.

Feed mills are facilities that process general agricultural products such as grains and animal parts into stockfeeds. They typically include blending and extruding sections. Wastes are generated through poor inventory management, spills and off-spec batches (Format Solutions, 2020). Grains are typically the bulk processed material (Satake, 2020).

It will be assumed that wastes from grain milling are similarly produced in feed mills and form the bulk of this stream, hence an estimate of 20% of processing capacity has been assumed waste accounting for bran and germ. This is likely an over-estimate as these grain milling by-products are typically used in formulating stockfeeds and hence are unlikely to be

considered as wastes in a feed mill. It is unclear what happens to feed mill wastes unfit for conventional sale as stockfeed.

6.1.1.8 Food Processing Wastes

As of December 2019, 14 significant food processing facilities have been identified in NSW, with capacities reported as a mixture of processing and production capacities (from the EPA, deemed medium to low data quality) and employee counts (from NPI, deemed high data quality) when better data is unavailable. Most are located in the Sydney Metropolitan Area and Riverina region.

This is a diverse category, including producers of grain products such as breakfast cereals and pasta, chilled and frozen foods, sauces, canned goods such as beans and soups, dressings and snackfoods such as potato and corn chips. Due to the variety of products these facilities produce, it is difficult to estimate waste production without direct data from each facility. As such, a very low-quality estimate of 5% of processing and production capacity has been assumed waste as estimated from data provided by direct contact with a major NSW food processor. For employee counts, the waste conversion factor used is 10.13 tonnes per employee per year (largely for baked products) excluding the Wrigley's facility for which the factor used is 3.38 tonnes per employee per year (Dusoruth et al., 2018). The NFWB estimates most wastes from food manufacturing in Australia are used for animal feed or compost (Arcadis, 2019). It can be assumed that some streams are ineligible for either treatment, such as organics contaminated by cleaning chemicals, and inevitably end up in landfill.

6.1.1.9 Grain Milling including Rice

As of December 2019, 10 significant grain mills have been identified from the EPA POEO register, reported with annual processing capacity and deemed medium to low data

quality. These are located around the Sydney Metropolitan Area, North West, Central West and Riverina regions.

Grain milling includes the processing of grains into flours, bakery mixes and ingredients such as starch. Most grains processed in NSW are wheat, with other significant grains including oats, barley, rice, and maize (ABARES, 2020). The endosperm accounts for approximately 80% mass of the wheat grain, hence 20% of processing capacity has been assumed waste as estimated from the non-endosperm portion of wheat grain (NSW DPI, 2008). Bran and germ form most of this non-endosperm portion and are included in various products such as wholegrain wheat and breakfast cereals; hence this conversion factor overestimates waste production. The NFWB reports wastes from grain milling under its 'Manufacturing' sector as destined for animal feed, compost and energy recovery (Arcadis, 2019).

6.1.1.10 Grain Storage

As of December 2019, 16 significant grain storage facilities are listed in the EPA POEO register with capacities all between 30 and 100,000 tonnes of annual processing capacity (medium to low data quality). Most are in the Riverina and Murray regions. Wastes from grain storage facilities are avoidable, stemming from poor inventory management. It is assumed therefore that waste generation is very low, with a crude assumption of 3% of processing capacity becoming waste (very low data quality). The NFWB reports disposal options from transport and storage in the food system as animal feed, composting and landfill (Arcadis, 2019).

6.1.1.11 Malt Processing

As of December 2019, two significant malting facilities are listed in the EPA POEO register, with processing capacities reported as a range (medium to low quality). These are located in the New England and Sydney Metropolitan Areas.

Malting of barley refers to the controlled germination and drying of barley, changing the grains character for uses in beer, beverage, and food production (Cargill, 2018). The rootlet, referred to as the culm, is removed and typically forms 3-4% of the waste from malting, while barley dust and screenings form an additional 3-4% (personal communication with malting company), hence a conversion factor of 7% processing capacity has been used for estimating waste generation. All organic co-products are reportedly sold for animal feed (personal communication).

6.1.1.12 Market Wastes

Waste from the Sydney Markets in Flemington is a significant point source in NSW. The estimate of fruit and vegetable waste produced from Sydney Markets is 25,000 tonnes per year (Cormack, 2016), and assumed to be most of the organic waste produced. This waste is used in Earthpower's anaerobic digester. Additional sources of similar fruit and vegetable waste will be from packhouses which have not been included due to their absence from both EPA and NPI datasets. The NFWB reportedly sourced data on such facilities from the Australian Agricultural Production Statistics (Arcadis, 2019).

6.1.1.13 Meat Processing Including Poultry

As of December 2019, 28 significant meat processing facilities including poultry processing have been identified in NSW from the EPA POEO register and NPI data. Production capacity ranges (medium to low data quality) and employee counts (high data quality) have been used to estimate waste generation. They are distributed across NSW including urban and regional areas.

Aside from wastewater generation, meat processing facilities reportedly generate little waste (Arcadis, 2019). According to the National Food Waste Baseline, 123,000 tonnes of organic waste was produced in Australia from livestock processing and 4,364,866 tonnes of meat produced in 14/15, hence a waste generation of approximately 3% of production which

has been used as the conversion factor (low data quality). A conversion factor of 6.75 tonnes per employee per year (Dusoruth et al., 2018) has been used when only an employee count is known (very low data quality). Non-primary products such as entrails, bones, feathers, skins and other typically uneaten animal parts are used in bio-based processing with a small portion sent to anaerobic digestion (Arcadis, 2019).

6.1.1.14 Oilseed Processing

Six facilities process oilseeds at a significant scale in NSW as of December 2019, as identified from the EPA POEO licenses register and NPI data. The largest are located in Narrabri, Newcastle, Wagga Wagga and Cabonne LGAs. The averages of the EPA ranges for processing capacity have been used to quantify scale of the facilities, bar the Narrabri facility for which only a lower limit of 250,000 tpa processing capacity is known therefore a value 10% above this limit has been used. These estimates have been deemed medium to low quality. Atlantic Pacific Foods was not found in the EPA register and therefore its scale is reported as an employee count and deemed high quality (per NPI data).

Canola (rapeseed) and cottonseed accounts for 90% of Australia's oilseed production (AOF, 2020), hence the waste conversion factor for these facilities has been estimated per canola processing. The main products from canola milling are canola oil (40-42 mass % of seed) and meal (40 mass % of seed). As the meal is used sold as a primary product (particularly in animal feed formulations), it is not considered a waste. Wastes of interest are largely made up of hulls and unrecovered kernel fragments (12-20% of seed weight) (UN FAO, 2019). In the absence of direct data, an average waste production of 16% processing capacity has been used, largely from hulls. Resulting estimate qualities have been deemed very low. For facilities where scale is reported as an employee count, a conversion factor of 17.01 tonnes per employee per year has been used (Dusoruth et al., 2018) with resulting estimate quality deemed very low. The processing and disposal options for these wastes are

unclear without a deeper investigation, however two of the facilities use biomass for energy production so it could be assumed these wastes are used in this application. The NFWB indicates most nut processing wastes (also largely hulls) are composted, as could occur for these wastes (Arcadis, 2019).

6.1.1.15 Paper Production

As of December 2019, four significant paper production facilities have been identified in NSW from the EPA POEO register with each facility's annual production capacity reported as a range (medium to low data quality). They are located in the Murray, Sydney Metropolitan and Riverina regions.

Wastes from paper production are a combination of paper, cardboard, pulp and other residues including sludge recovered after wastewater treatment. The conversion factor has been determined from data of a significant paper mill in Sydney generating a 9% waste conversion factor. Australasian Pulp and Paper Technical Association indicates most paper and cardboard waste is recycled in across Australia (AFPA, 2016), while a report from the USDA forestry service suggests sludge from paper production is largely landfilled or incinerated (Scott and Smith, 1995).

6.1.1.16 Sugar Milling and Refining

As of December 2019, four significant sugar processing facilities have been identified in NSW from the EPA POEO register, with reported processing capacities extracted and deemed medium to low data quality, except for the Harwood facility for which the processing capacity was sourced directly and deemed high data quality. They are located in Richmond/Tweed, Grafton and Sydney Metropolitan regions.

Excluding sugar cane bagasse, much of the waste from sugar production is in the filter press mud (mill mud) and molasses. According to the Australian Sugar Milling Council, the generally accepted calculation quantity of mill mud produced is 7% on cane tonnage and the

quantity for molasses is 3% on cane tonnage (personal communication), yielding a conversion factor of 10 kg waste / 100 kg cane processed. Both by-products are used in stockfeed, and molasses undergoes further refining into ethanol. Excesses are assumed composted or landfilled. Across Australia in a typical drought-free year and a harvest of 33 million tonnes of cane crop, molasses demand for stockfeed accounts to approximately 365,000 tonnes and demand for refining into ethanol is approximately 305,000 tonnes (yielding 60 million litres of ethanol).

From sugar refining, additional by-products such as clarifier scum are generated at an assumed 2% on raw sugar processed (Sugarmill.co.in, 2019). As the portion of raw sugar processed vs cane processed is unclear in the processing capacities reported in the EPA licenses, this will be assumed negligible and not contribute to the conversion factor used.

In the case that molasses is fermented and refined for ethanol production, a by-product termed dunder is produced proportional to the amount of water used during distillation (typically around 5 litres of water used per litre of ethanol produced). As it is unclear if molasses refining occurs at the identified facilities, this information has not contributed to the conversion factor used.

6.1.1.17 Winery Waste

As of December 2019, eight grape processing/winery facilities have been identified as significant in NSW, as estimated from the publicly available EPA POEO licenses register. These are located in the Griffith and Wentworth LGAs. Grape processing capacities have been sourced from direct contact with facility operators and internet sources (AV, 2020). Where better capacity information was unavailable, an average between the EPA reported capacity ranges has been used resulting in an estimate of medium to low quality.

The bulk of winery organic waste is derived from grape pressing and crushing, generating grape marc (aka pomace) made up of seeds (17 fresh mass %), skins and pulp

from the grapes (Heuzé V. and Tran G., 2017). Other wastes of lees and grape stems are also generated.

It has been assumed that the significant waste generation of grape marc is equal to the difference between grapes processed and wine produced. The NSW average yield per tonne of grapes is 716 L wine (Wine Australia, 2019). Assuming a wine density of 980 kg/m^3, this equates to 700 kg of wine produced per tonne of grapes processed. Hence waste marc production is 300 kg per tonne of grapes processed and 430 kg per tonne of wine produced. These have been used to estimate waste production per winery in NSW where direct data is unavailable and have been deemed medium to low in quality. According to the NFWB, most wine making wastes in Australia are composted with smaller fractions used in bio-based processing, as has been assumed for these facilities where direct data is unavailable. It is anticipated that a portion is also used as animal feed, though this is not noted in the NFWB (Arcadis, 2019).

Direct data was supplied for a large winery facility in NSW. With a production capacity of 120,000 tpa, the facility reportedly generates 35,000 tpa of organic waste composed of 25,000 tpa grape marc (seeds and skins after pressing, sent to a third party for alcohol spirit extraction), 5,000 tpa grape stalk (from crushing, sent to on-site composting and blended with manure, grape marc, gypsum or lime as needed for vineyard application) and 5,000 tpa brewers grain (used for animal feed during drought and compost for vineyards otherwise).

6.1.1.18 Wine Distillery Wastes

As identified from the EPA POEO licenses register in December 2019, there is a single significant wine distillery in NSW located in Griffith alongside the significant wineries and grape processing facilities of the state. In the NPI data, an additional facility near Sydney is also identified however it is understood this is only a bottling plant and no distilling takes

place (waste then largely liquid from inventory management faults). The upper limit of the EPA range has been used for processing capacity quantity, treated as low-quality data. Wine distillery wastes are like winery wastes, consisting largely of grape marc. Significant quantities of wastewater are also generated in wine distilleries; however this has not been included in this estimate due to its variability and logistical difficulties in its reuse. The wastewater of this facility is used as class A irrigation water (personal communication).

In the absence of direct data, grape ethanol has been treated as the primary product (ignoring additional products such as tannins and tartaric acid), with 40 tonnes of grapes generating approximately 3,800 litres of ethanol (Minnaar and Jolly, 2010). As with winery waste, distillery waste has been estimated as the difference between raw input grapes and output ethanol, assuming lees and other waste production as negligible. Using an ethanol density of 0.8 kg/L, this equates to 3040 kg of ethanol per 40 tonnes of grapes processed. Assuming an optimistic 95% recovery results in 2888 kg ethanol / 40 tonnes grapes or 72 kg ethanol / tonne grapes. Therefore, 14 tonnes of grapes need processing to produce 1 tonne of ethanol, generating 13 tonnes of waste grape marc. The resulting waste data quality estimate has been deemed very low, likely over-estimating the true waste generation given grape ethanol is not the sole product. Wastes are used partly for animal feed, composting and land application after wastewater treatment (Tarac, 2020). It is unclear if and how much material becomes landfill without further information.

6.2. Modelling

6.2.1. Selection of a Hybrid Optimisation Approach: Shotgun with Local Search and Backtracking

The hybrid approach, comprising a single large shotgun followed by local search iterations, was selected due to its balance between execution time and solution quality (profit). The local search step involves placing two new candidate facilities randomly within

a small radius of a previously selected facility location, and then selecting the most favourable location out of the three options.

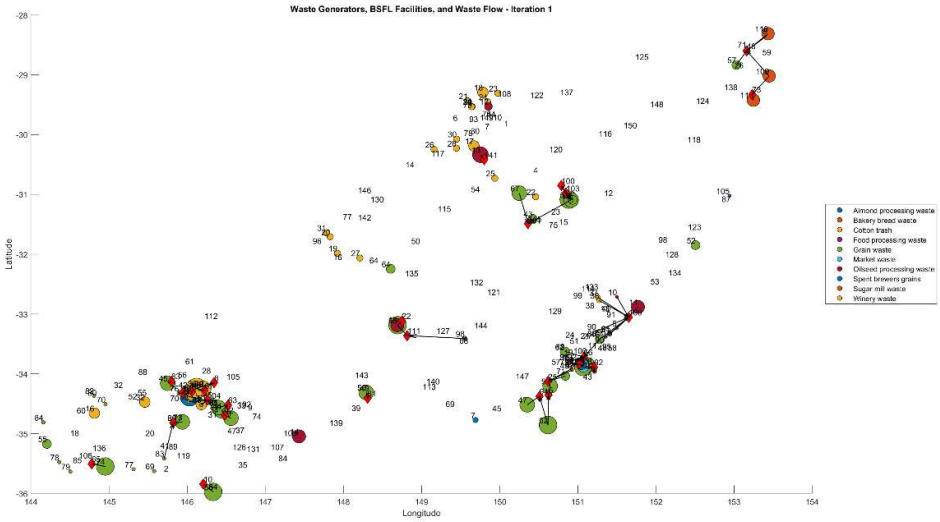
Integer programming problems, such as this model that seeks to optimise waste transfers and candidate facility locations, are inherently complex and often classified as NP-hard problems (Artigues et al., 2015). These issues present a computational challenge due to the vast number of potential solutions, particularly as the problem size increases. The optimisation solver must navigate this extensive solution space, making computational efficiency a primary concern.

While the solver is equipped to find a good solution, it does not guarantee the absolute optimal solution for complex problems. The solver employs underlying heuristics and strategies, such as branching methods and cutting planes, to guide its path through the solution space. As a result, the solver may reach a local optimum and fail to explore other areas of the solution space that might harbour a superior solution.

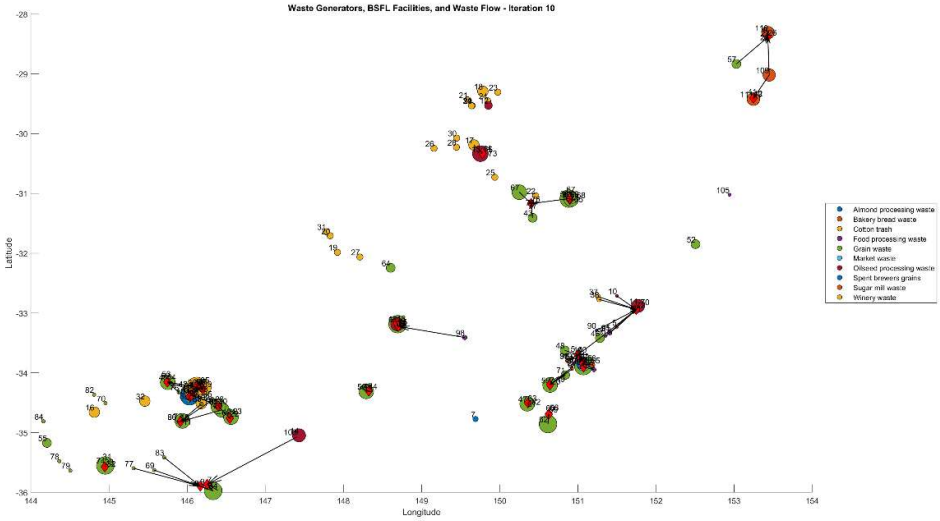
The iterative nature of this model, which generates new candidate locations based on the selections of previous iterations, introduces additional complexity. While the best locations from a previous iteration are retained as candidates, the inclusion of new candidates can subtly alter the constraints and feasible solution space of the problem. Consequently, an optimal or feasible solution in one iteration may not maintain these qualities in the subsequent iteration.

The backtracking feature integrated into this model serves as a safeguard against these inherent complexities. By retaining the best solution discovered up to that point, the model ensures that it does not discard a potentially favourable solution due to the limitations of the solver or changes in the feasible solution space. Thus, while the model operates under these restrictions, it is designed to function efficiently and effectively within them.

Appendix Appendix Figure 1 and Appendix Figure 2 demonstrate the improvements generated by the incorporation of the hybrid approach.



Appendix Figure 1 Locations of selected facility locations (red diamonds) and waste generators (coloured circles) at the first iteration for the full NSW dataset. Distances between facilities and generators have not been completely minimized. Floating numbers indicate locations of candidate facilities, including those which were not selected by the solver.



Appendix Figure 2 Locations of selected facility locations (red diamonds) and waste generators (coloured circles) at the 10th iteration for the full NSW dataset. Distances between facilities and generators have been further minimized.

6.2.2. Locating Initial Candidate Facilities

The choice of location of initial candidate facilities is restricted by the need to avoid non-linear relationships, necessitating the exploration of different modelling options. Several strategies were attempted, including grid-based, randomly generated, generated within a hull, or generated within a radius of waste generators. The approach of randomly locating initial candidate facilities within a radius of waste generators, bounded by a hull formed by the generators, yielded the most optimal results in preliminary testing and hence was chosen for this study.

While it might initially appear more efficient to position a processing facility adjacent to a waste generator with high conversion efficiency, this doesn't always result in the most optimal arrangement when considering the system as a whole. A simple scenario can illustrate this: Suppose there are three waste generators, where the first one produces a small quantity of high-efficiency waste, while the other two generate larger quantities of lower-efficiency waste. If a processing facility is positioned directly beside the first generator, although the conversion efficiency is maximized, high transportation costs would be incurred in transporting significant volumes of waste from the other two generators.

In contrast, by strategically positioning the processing facility in a location that balances proximity among all generators, overall transportation costs can be reduced while still processing the high-efficiency waste. Even though this might mean not being located directly next to the high-efficiency generator, this approach can potentially optimize overall system efficiency and profitability.

Therefore, the selected approach of generating initial candidate facilities randomly within a radius of generators, bound by a hull, offers a flexible starting point. It incorporates the location of multiple waste generators and ensures a more comprehensive evaluation of potential facility locations.

6.2.3. BSFL Meal and Lipid Conversion Factor Data

Data used for estimating the meal and lipid conversion factors are provided below.

Appendix Table 3 Data estimated from Barragan-Fonseca et al. (2021) used in estimating conversion factors.

Waste	Protein content (% DM)	Carbohydrate content (% DM)	Est. Dev time (D)	Est. Yield (g DM)	Est. Larval Protein content (%)	Est. Larval Lipid content (%)
Bakery bread waste	12	71	28	3	43	27
Spent Brewer's grains	26	7	28	2.5	43	24
Oilseed processing wastes	34	17	28.5	2.5	43	30
Cotton trash	7	0	30	1.5	43	7
Wine distillery wastes	12	20	24	2.7	43	18
Feedlot grain waste	15	62	25	3	43	29
Feedmill grain waste	15	62	25	3	43	29
Food processing wastes	6	59	25	3	43	24
Grain mill wastes	15	62	25	3	43	29
Grain storage wastes	15	62	25	3	43	29
Market wastes	7	14	24	2.2	43	12
Meat processing wastes	53	0	0	0	0	0
Almond pericarps	6	32	26	2.9	43	16
Paper production wastes	1	0	0	0	0	0
Sugar milling and refining wastes	12	37	24	3.1	43	22
Winery wastes	12	20	24	2.7	43	18
Blend High P High C	24	55	24	2.9	43	32
Blend Balanced	17	45	24	3.1	43	26
Blend High P Low C	24	35	26	2.8	43	28
Blend Low P High C	10	55	26	3.2	43	25